UNIVERSIDADE
BEIRA INTERIOR

Strategies to improve added value of aromatic and medicinal plants

Versão final após defesa

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Dissertação para obtenção do Grau de Mestre em
Bioquímica
(2° ciclo de estudos)

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julho de 2022

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Para a minha maior inspiração e motivação, a minha mãe.

Agradecimentos

Para que a realização desta dissertação corresse da melhor forma possível, foi necessário o apoio e incentivo de várias pessoas, às quais quero desde já exprimir todo o meu agradecimento.

Em primeiro lugar, gostaria de agradecer à Professora Lúcia Silva, por me aceitar neste projeto e não só me oferecer toda a orientação necessária, sempre com muita simpatia, como me orientar durante a realização de toda a parte laboratorial nos laboratórios da UBI. Gostaria também de agradecer, por me proporcionar um novo desafio, de experienciar uma parceria com um departamento e faculdade diferentes, o Departamento de Ciências e Tecnologia da Biomassa, da FCT-UNL. Posto isto, gostaria também de agradecer à Professora Ana Luísa Fernando, por aceitar este projeto e me integrar nos seus laboratórios e me apoiar na descoberta de uma nova área. Agradeço também às minhas colegas de laboratório, Laura, Raquel e Filipa, por toda a companhia e ajuda durante este processo e, acima de tudo, ao João e à Carolina, os meus braços direitos, por se disponibilizarem para me ensinar e apoiar durante todo o processo laboratorial decorrido. Agradeço à Engenheira Violeta Lopes, do Banco Português de Germoplasma Vegetal, por fornecer as amostras de *Daucus carota* para a realização da dissertação.

Agradeço também aos meus amigos e à minha família, por tornarem esta jornada um pouco mais leve, proporcionando-me momentos para relaxar, me divertir, ouvirem todos os desabafos e estarem sempre lá para me apoiar durante todo o processo.

Muito obrigada a todos os envolvidos ao longo desta etapa, não seria igual sem a vossa presença! Agora, só resta seguir rumo a uma nova aventura!

“Pedras no caminho? Guardo todas. Um dia vou construir um castelo.”

Nemo Nox

Resumo

Devido à necessidade de utilizar mais produtos naturais em vez de sintéticos, é importante caracterizar diferentes óleos essenciais e suas possíveis aplicações. Um exemplo do seu potencial de utilização reside em aplicações associadas à indústria alimentar. Mais especificamente, na área das embalagens, onde é importante encontrar compostos ativos a incorporar em embalagens, para que estas se tornem ativas, a fim de melhorar o tempo de prateleira e proporcionar opções mais seguras e com menos produtos químicos sintéticos na conservação de alimentos.

Desta forma, o objetivo desta dissertação consistiu em caracterizar e estudar diferentes óleos essenciais (*Daucus carota* subespécies *maritimus, hispidus, maximus, carota* e *sativus; Aloysia citrodora* e *Cymbopogon citratus*) e a sua possível aplicação em filmes edíveis à base de quitosano. Neste trabalho, os óleos essenciais foram previamente analisados por GC-MS, a fim de compreender a sua composição. Foram identificados os seguintes compostos maioritários: acetato de geranil, *trans*-metil-isoeugenol e α-pineno, para *Daucus carota* subsp. *maritimus*; acetato de geranil, óxido de cariofileno e *trans*-metil-isoeugenol para *Daucus carota* subsp. *hispidus*; *cis*-asarona, acetato de geranil e elemicina para *Daucus carota* subsp. *maximus*; acetato de geranil, *cis*-asarona e *trans*-metil-isoeugenol para *Daucus carota* subsp. *carota* e por fim carotol e daucol para *Daucus carota* subsp. *sativus*; para *Aloysia citrodora* os compostos maioritários apresentados foram geranial, neral e limoneno, e por fim, *Cymbopogon citratus* apresentou como compostos maioritários geranial, neral e β-mirceno.

Os óleos essenciais de *Aloysia citrodora* e *Cymbopogon citratus* foram ainda aplicados em filmes de quitosano tendo sido testada a sua funcionalidade através de ensaios de migração e a sua aplicabilidade na conservação de carne de frango. O processo de migração de compostos ativos foi monitorizado por ensaios de migração em diferentes simuladores de alimentos (etanol 95%, etanol 50%, etanol 10% e água destilada), e os teores de compostos fenólicos totais e a atividade antioxidante foi medida no simulador ao longo do tempo (10 dias). Para o óleo de *Cymbopogon citratus*, a maior migração de fenóis observou-se com etanol 50%. Já para o óleo de *Aloysia citrodora* a maior libertação de fenóis ocorreu com etanol 95%.

Na aplicação em embalagem de carne de frango, as amostras de carne foram envolvidas nos biofilmes durante 13 dias em ambiente refrigerado. Ao longo deste tempo foram

feitos testes físico-químicos (humidade, pH, acidez titulável, cor, azoto básico volátil e índice de substâncias reativas ao ácido tiobarbitúrico) e foi medido o seu grau de contaminação microbiana. Comparando com a amostra não embalada, estas embalagens permitiram aumentar o prazo de vida útil da carne uma vez que se verificou menor produção de azoto básico volátil, um decréscimo de pH, humidade e oxidação lipídica, e um menor crescimento de microrganismos. Em comparação com o controlo de quitosano, a incorporação de óleos atrasou a oxidação lipídica da carne até ao 9º dia, sem se terem verificado diferenças entre óleos. Mas em termos de atividade antimicrobiana, estes óleos não melhoram a atividade do quitosano. Verifica-se, igualmente, que estes óleos conferem uma tonalidade amarela à carne, pela sua migração. Embora sem diferenças significativas, o óleo essencial de *Cymbopogon citratus* apresentou resultados um pouco mais eficazes no decréscimo da oxidação lipídica e o óleo de *Aloysia citrodora* apresentou um menor crescimento a nível de contaminação microbiana.

Palavras-chave

Daucus carota; *Aloysia citrodora*; *Cymbopogon citratus*; Óleos essenciais; Bioplásticos

Abstract

Due to the public need to use more natural rather than synthetic products, it is important to look at different essential oils and their possible applications. An example of this search is in the food industry, more specifically, in the packaging area, that where it is important to find active compounds to incorporate into the packages, in order for these to become active and improve the shelf-life time and to provide safer options with less synthetic chemicals in the food conservation.

In this way, the aim of this dissertation consisted into characterizing and studying different essential oils (*Daucus carota* subspecies *maritimus, hispidus, maximus, carota* e *sativus; Aloysia citrodora* e *Cymbopogon citratus*) and their possible applications into chitosan based edible films. In this work the essential oils were previously analyzed by GC-MS, in order to understand their composition. The major compounds found for *Daucus carota* subsp. *maritimus* were geranyl acetate, *trans*-methyl-isoeugenol and α-pinene; geranyl acetate, caryophyllene oxide and *trans*-methyl-isoeugenol for *Daucus carota* subsp. *hispidus*; *cis*-asarone, geranyl acetate and elemicin for *Daucus carota* subsp. *maximus*; geranyl acetate, *cis*-asarone and *trans*-methyl-isoeugenol for *Daucus carota* subsp. *carota* and finally carotol e daucol for *Daucus carota* subsp. *sativus*; for *Aloysia citrodora* the major compounds presented were geranial, neral and limonene, and for *Cymbopogon citratus* geranial, neral e β-myrcene.

The *Aloysia citrodora* and *Cymbopogon citratus* essential oils were further applied in chitosan films, being tested their functionality by migration assays and their applicability in the conservation of poultry meat. The migration of the active compounds was monitored by migration assays in different food simulant mediums (ethanol 95%, ethanol 50%, ethanol 10% and distilled water), as well as the total phenolic content and antioxidant activity in the simulant medium, throughout the time of ten days. For the *Cymbopogon citratus* the higher migration occurred at ethanol 50%, on the other hand, for *Aloysia citrodora* the higher release of phenols occurred with ethanol 95%.

When applied to the poultry meat packaging, the meat samples were wrapped in biofilms for 13 days in a refrigerated ambient. Throughout the time, were realizes physical-chemical trials (moisture content, pH, titratable acidity, color, basic volatile

nitrogen and thiobatbituric acid reactive substances index) but also the microbial contamination level. Compared to the non-wrapped sample, the packaging allowed an increase to the shelf-life of the meat, since it as shown lower basic volatile nitrogen production, decreased pH level, moisture and lipidic oxidation, as well as lower microbial growth. In comparison with the chitosan control, the incorporation of the essential oils provided a decrease in the lipidic oxidation until the 9th day, without difference between the oils. In terms of antimicrobial activity, the oils did not improve the chitosan activity. As also been verified a yellowish tone to the meat provided by both oils, due to migration. *Cymbopogon citratus* presented results a little more effective in the oxidation decrease and *Aloysia citrodora* presented a lower increase of the microbial contamination.

Keywords

Daucus carota; *Aloysia citrodora*; *Cymbopogon citratus*; Essential Oils; Bioplastics

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List of Acronyms

AAI	Antioxidant Activity
ACEO	Aloysia citrodora Essential Oil
ATP	Adenosine Triphosphate
BVN	Basic Volatile Nitrogen
CCEO	Cymbopogon citratus Essential Oil
CFU	Colony Forming Unit
Ch	Chitosan
CNC	Commercial Cellulose Nanoparticles
DPPH	2,2-diphenyl-1-picrylhydrazyl
EO	Essential Oil
FRAP	Ferric-reducing Antioxidant Power
GA	Gallic Acid
GC-MS	Gas Chromatography coupled with Mass Spectrometry
HD	Hydrodistillation
I%	Inhibition percentage
IC50	Concentration that Inhibits 50 percent
IPP	Isopentenyl Diphosphate
LPS	Lipopolysaccharides
MDA	Malonaldehyde
ROS	Reactive Oxygen Species
Subsp.	Subspecies
TBARS	Thiobarbituric Acid Reactive Substances
TEP	1,1,3,3-tetraethoxypropane
TFC	Total Flavonoid Content
TPC	Total Phenolic Content
UV	Ultraviolet

Chapter 1
Introduction

Chapter 1 - Introduction

1. Introduction

For a long period of time, manufactures and consumers have been using synthetic preservatives in food industry [1], however, we are entering an era where it is very important for the consumer to have the healthiest options in terms of food preservation, exhibiting food products that are safe, free from synthetic chemical preservatives and that present a long shelf life [2].

Preservatives are important to increase the shelf life and maintain the quality and safety of the product, by retarding reactions like fermentation, acidification, microbial contamination and decomposition[3], nonetheless, since synthetic preservatives and their consumption have been reported to be associated with potentially life-threatening effects [3], such as allergic effects, intoxication and even cancer and other degenerative diseases [4], it is raising preoccupation among the consumers and researchers.

In other to provide more natural options of preservation, researchers are moving towards plants and herbs. On account of flavors, aroma and natural properties, the essential oils have been used in food industry for food preservation[1], but also in cosmetics, agriculture industry and sanitary products [5].

Essential oils seem to be a promising substitute to the synthetic ones, since they are rich in bioactive chemical components, like terpenes, terpenoids and phenolics, that are worldwide known for their biological activities[5]. It is also important to refer that researchers have been finding that these oils present antimicrobial activity, for instance anti-fungal, anti-bacterial and antioxidant activities, that can be used to inhibit the pathogenic microorganisms growth, ensuring the microbial safety of the products [2].

Since the composition of these oils' variates accordingly with many factors, mainly among species, it is important to analyze different species from plants, in order to provide an extensive data of compounds and properties of each. With this work we aim to analyze three different species oils and see if they have the necessary qualities to be introduced into the preservative industry.

1.1. Essential oils: properties and applications

1.1.1. What are they? How are they obtained?

Accordingly with European Parmacopoeia 7th edition, an Essential Oil (EO) is an "Odorant product, generally, of a complex composition, obtained from a botanically defined plant raw material, either by driving by steam of water, either by dry distillation or by a suitable mechanical method without heating. An essential oil is usually separated from the aqueous phase by a physical method that does not lead to significant change in its chemical composition" [6].

Essential oils are colorless liquids at ambient temperature with a complex mixture of volatile compounds, the secondary metabolites, synthesized and extracted from many medicinal plants. They are a chemically rich mixture of many bioactive chemical components, like terpenes, terpenoids and phenolics, however, their chemical composition can variate both quantitatively and qualitatively, depending on many factors, such as the harvesting season, time of the they that they are picked, geographical sources, part of the plant, their stage of maturation, among others [1,5–7].

In terms of solubility, they are insoluble in water, but have solubility in alcohol, ether, organic solvents and fixed oils[1,5]. In nature, EOs characterize less than 5% of the vegetal dry matter, and represent important functions to the plant, like attracting beneficial insects and pollinators; protecting the plant against environmental stress, as heat, cold and others; and protecting the plant against pests and microorganisms [8–10]. These functions are possible due to the antimicrobial, antifungal, antioxidant, antiviral, antimycotic, antiparasitic and insecticidal properties provided by the aromatic and chemical characteristics of the EOs [1,5]. The most associated properties to these oils are antimicrobial activity against the food-borne pathogens, by destabilization of the phospholipid bilayer of the cell membrane, enzyme system and genetic material of the bacteria, but also the antioxidant activity, by the stabilization of the free radicals and the reactive oxygen species (ROS) and also by blocking the UV-light and chelating metals [11].

The EOs contain a variety of polar and non-polar compounds, in different quantities, their representation consists in two or three predominant compounds that are present at high concentrations (between 20% and 90%), while the other compounds represent significantly lower concentrations. These compounds are divided in two fractions (Figure 1), depending on whether they result from the biosynthetic chain, the terpenes group and the terpenoids group [10,12]:

The terpenes group is with no doubt the major constituent of the EOs and can achieve up to 90% of the composition of the EO. They are characterized by their basic structural element, the isoprene, and are formed by combination of isoprene units $(C_5H_8)_n$, being n the

number of linked isoprene units. Depending on how many isoprene units the terpenes contain, they can be subcategorized, being the most common subcategories the monoterpenes (2 isoprene units, C_{10}) and sesquiterpenes (3 isoprene units, C_{15}) [5,6]. The synthesis of terpenes in medicinal plants consists in three steps: Isopentenyl diphosphate (IPP) precursor synthesis; number of IPPs to form the prenyl-diphosphate precursor of different terpenes; and finally, synthetase of different types of terpene precursor, allylic prenyl-diphosphate, that will go through a small modification to form the terpene skeleton. In the final step, it will go through redox reactions for secondary enzymatic modifications of the skeleton, in other to achieve the functional properties of the different terpenes [1].

The terpenoids group, the oxygenated derivates of the hydrocarbon terpenes, like alcohols, aldehydes, ketones, acids, phenols, ethers, esters, hetero-cycles and methoxy derivates are the less abundant in the EO [5,6]. The difference between the subcategories of this group is based on the presence of an open chain of carbon atoms. Aliphatic hydrocarbons are linear chains that don't present an aromatic ring; Alkanes are linked together by a single bond between the two carbon atoms in their structures, while alkynes are linked by a triple carbon-carbon bond; Finally, the aromatics present a benzene ring (C_6H_6) [1].

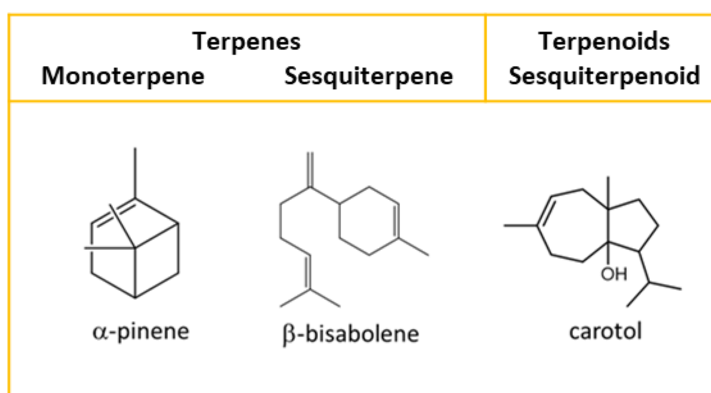

Figure 1- Examples of Terpene and Terpenoid structure

As to how the essential oils are obtained, they can be biosynthesized by most of plant organs as secondary metabolites, such as flowers, herbs, buds, leaves, seed, fruits, stems, twigs, bark, zest, wood, rhizome and roots [5,6].

In terms of extraction, the methods of plant extraction can be classified in two categories: the conventional and classic methods and the advanced and innovative ones, presented in Figure 2:

Plant Extraction Methods	
Conventional and Classical Methods	Advanced and Innovative Methods
Hydrodistillation Steam-distillation Hydrodiffusion Organic Solvent Extraction Cold Pressing Dry Distillation	Supercritical Fluid Extraction Subcritical Liquid Extraction Ultrasound Assisted Extraction Microwave Assisted Extraction Microwave Hydrodiffusion Microwave Steam Distillation Solvent Free Microwave Extraction

Figure 2- Plant extraction methods (Adapted from [13])

The traditional way of isolating the volatile compounds from a plant as essential oils is distillation, where during this process the plant is exposed to boiling water or steam, releasing the EOs through evaporation, then, by indirect cooling with water, the vapor mixture of water and oil condenses and proceeds the separation between the oil and water. Accordingly with the way that the water and the original matrix contact, there are three types of distillation, Hydrodistillation (HD), steam distillation and water/steam distillation [14,15].

Since the method used in this work is the hydrodistillation method, that is the one that we are going to develop about.

The hydrodistillation (Figure 3) is the most simple and old method used for the EO extraction. Is has become the standard method of extraction. The plant is immersed directly in the water, followed by boiling, presenting direct contact between the boiling water and the raw material[15]. The extraction device includes a source of heat topped by a vessel, where the water and plant material are collected, it also presents a condenser and a decanter to collect the condensate and separate de essential oil from the water [6,14].

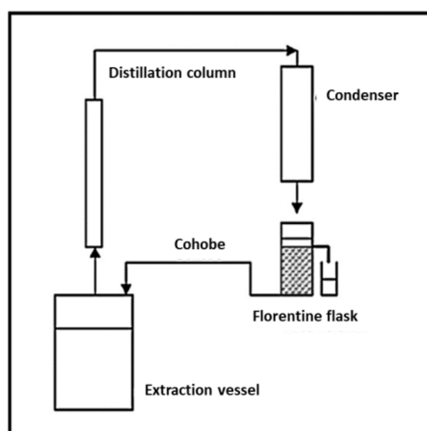

Figure 3- Hydrodistillation method [16]

The HD method presents the advantage that the water is immiscible with the majority of the terpene molecules of the oils, therefore after condensation, they can be easily separated by decantation. As disadvantages, it presents a long extraction time; can cause artifacts and chemical alterations of the terpenic molecules by continued contact with boiling water,

causing hydrolysis, cyclization among other; and overheating and loss of some polar molecules in the water extraction [6].

On a small scale, the Clevenger apparatus is recommended for the EOs yields by the third edition of the European Pharmacopoeia, as it allows the recycling of the condensates through a cohobate system. On an industrial scale, this method is still used, with an optimize variant, the turbodistillation, that allows the obtention of high yields by recycling the aromatic water, while reducing the distillation time due to the turbines. Because of its simplicity of installation since it does not require expensive equipment; being an easy method to implement and due to it selectivity, it is a good option for de industry [6].

1.1.2. **Antimicrobial activity**

On the modern days, many researchers have proven that species and herbs present antimicrobial activity, due to their essential oil's fractions, being able to inhibit the growth of wide range of pathogenic microorganisms [2,17].

In nature, the EOs are an important way to protect pants, they can act as antibacterial, antiviral, antifungals, insecticides and against herbivore, being lethal to many organisms or by inhibiting the production of methabolites[14]. The effectiveness of these properties variates along with the components of the oils as well as the bacteria [12].

The factors that affect the microbial survival and growth can be divided in three categories[18]:

Implicit and microbial factors, like the presence of the microorganism, its growth rate and lag phases, synergetic effects, and others.

Intrinsic factors, the physical and chemical factors, like nutrients, pH, water activity, presence of preservatives and other antimicrobial substances.

Extrinsic factors, such as the humidity, partial pressure of the oxygen, among others.

The antimicrobial activity of the essential oils and their components is well known, being either bacteriostatic, where the bacterial growth is inhibited but the microbial cells can recover their reproductive ability, or bactericidal, where the EO kills the bacterial cells[5]. Considering the large number of chemical groups of the essential oils, it is difficult to acknowledge an exact mechanism of action, for these reason it has been described that de EOs exhibit functional and structural damages of membrane of bacterial pathogens, by several targets in the cell [2,9]. For researchers, this activity depends on three factors: the characteristics of the essential oil (hydrophilic or hydrophobic), the chemical composition of the EO and the type of organism that it is going to attack [14]. Figure 4 presents the targets of EOs in bacterial cells.

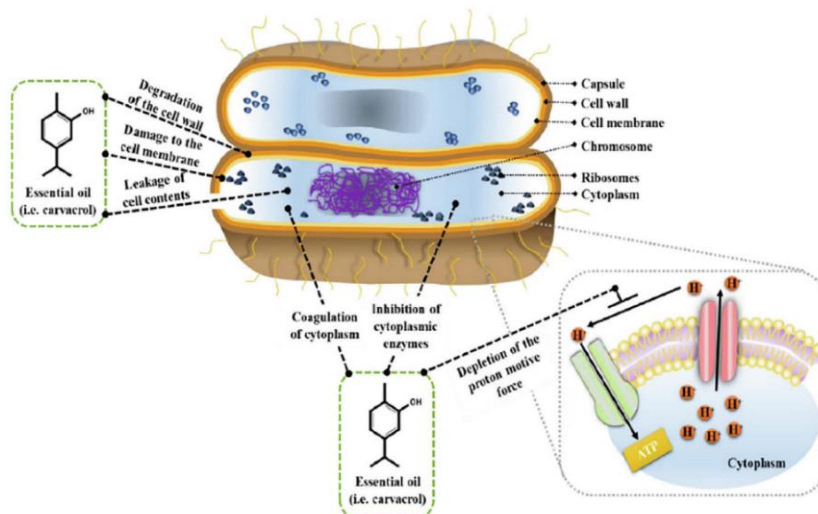

Figure 4 - Targets of Essential oils in bacterial cells [5]

Since the major composition of the essential oils is lipophilic, they act accordingly with their hydrophobicity and pass through the cell wall and cytoplasmatic membrane, by dividing into lipids in the cell membrane. These will case the disruption of the polysaccharides and phospholipid layer's structure, along with the fatty acids, disruption of the enzymatic systems and compromised genetic material of bacteria, causing the permeabilization of the membrane, which can lead to the leakage of ions and other molecules. Even though there is an amount of leakage that can be tolerated by the bacterial cells without the loss of viability, a larger loss of cell contents or critical output of molecules and ions can lead to the cell lysis [1,2,5,7,9,14]. The permeabilization of the cell membrane is also directly associated with the loss of ions and reduction of the membrane potential, which will cause the collapse of the proton pumps and depletion of the ATP pool, causing a cascade type of action [5,17]. The EOs' mechanisms on microorganisms can be seen in Figure 5.

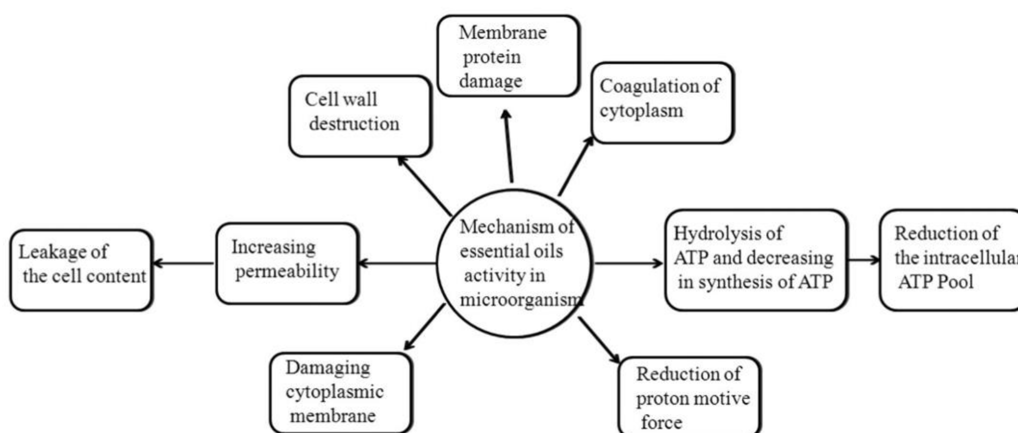

Figure 5 - Mechanisms of Essential Oils activity on microorganisms [1]

It is considered that the antimicrobial activity is mostly due to the presence of phenols, aldehydes, and alcohols. The phenolic compounds not only attack the cell walls and membranes, but also interfere with the membrane function, such as the electron transportation, nutrient uptake, protein, nucleic acid synthesis and enzymatic activity [2]. Compounds like cinnamaldehyde, citral, carvacrol, eugenol and thymol present significant

antimicrobial activity, followed by terpenes and ketones (e.g. β-myrcene, α-thujone or geranyl acetate) [1].

In terms of bacteria, we can divide them in Gram-positive and Gram-negative, and it is well known that the Gram-positive ones are more susceptible to the EOs action. In Gram-negative bacteria, the outer membrane is composed by a rigid double phospholipidic layer, that is linked with the inner membrane by lipopolysaccharides (LPS), which can limit the diffusion of hydrophobic compounds trough it [1,19]. On the other hand, Gram-positive bacteria do not present these layers, being instead surrounded by a thick peptidoglycan wall, that is not dense enough to resist to the small antimicrobial molecules, therefore, enabling the access to the cell membrane [19].

This type of activity can be determined *in vitro* by different methods, such as agar wells, disk diffusion, agar dilution and broth dilution [20].

As to the benefits of the essential oils antimicrobial activity implementation, there are a lot of areas where these properties can be administrated, being the basis of many applications in food preservation, aromatherapy, medicine, pharmaceutical, agronomic, sanitary, among others [17]. Here are some of the benefits:

Synergetic Reaction

Since a lot of bacteria have become drug-resistant, there is a need to find alternative methods to deal with these inconvenient, thus, since EOs are reported to present antimicrobial properties, their usage can be applied in various industrial fields [19]. Synergism is observed when the effect of the combination of substances is superior to the individual ones. There can be synergism between different essential oils or their components, such as the synergetic combination of oregano and basil EOs against *E.coli*, basil and bergamot EOs against *B. subtilis* and oregano and perilla EOs against *S. cerevisiae*, that significantly disrupted the integrity of cell membranes in comparison with the control groups [21]. It can also be a synergetic effect between EOs and antibiotics, like the combination of thymol and carvacrol with penicillin against *E.coli* and *S.typhimurium*, or the inhibition of protein synthesis and the damage of the bacterial cytoplasmatic membrane by combination of aminoglycosides and tea tree oil [19]. Other example of synergetic reactions is the incorporation of EOs based biocides (i.e Promax, based on *Thymus vulgaris*) in combination with traditional pesticides, which permits more flexible treatment periods, rising the shelf life of traditional fungicides, but also securing the safety of the farmers environment [1].

Mycotoxin Prevention

Food products are often contaminated by molds and their associated toxins, the mycotoxins. These are secondary metabolites, toxic to humans and animals, produced by specific filamentous fungi that contaminate agricultural products, reducing their quality, quantity in terms of nutrients composition. Food contaminated with mycotoxins, like aflatoxins, can be associated with risks of cancer, acute illness and sometimes fatality, due to the creation of reactive oxygen species. EOs from some aromatic plants can provide alternative options to the ones already used synthetic chemicals, presenting less harm to the human health and environment [22,23]. An example of these implementation is the suggestion of *Boswellia carterii* EO as a plant based preservative against storage fungi, aflatoxin production and in vivo efficacy in food systems [23].

Food preservation

Due to the EOs aroma, flavors, and natural antimicrobial content, they are very often used as food preservatives. Food industry has been using the extracts and EOs of aromatic plants due to their ability to control the growth of pathogenic microorganisms [1]. Some preservatives that present essential oils are already commercially available, like "DMC Base Natural", a food preservative produced in Spain, that comprises 50% essential oils from rosemary, sage and citrus and 50% glycerol [9]. There are also studies that demonstrate the efficacy of the use of the EOs, like Weissinger et. al (2001) reported, cinnamaldehyde and thymol oils are effective against six types of *Salmonella,* when administrated in hot air, at 50ºC, as fumigation [24].

Edible Biofilms/Coatings

Edible films are thin films used as barriers against extern elements, such as moisture, oils, gases, and vapors, in order to extend the shelf life of the product and also perform a support for antimicrobial, nutritional and antioxidant substances, such as EOs, which will not only prevent the growth of pathogenic and spoilage bacteria, by carrying the active ingredients and increasing their distribution in areas of microorganism proliferation, but also prevent the exchange of oxygen, water and carbon dioxide from the exterior [22,25,26]. The major difference between edible coatings and biofilms is that the biofilms are formed by a thin solid layer which is applied has a wrapping on the food product, however, the edible coatings are applied in a liquid form, normally by the submersion of the food product [25]. When the product is consumed, these edible films can be eaten together with the food or can be washed away. As an example of these application, there is Perdones et. al (2016) that demonstrated the combination of pure chitosan coatings with lemon EO in strawberry preservation, stimulate the formation of ester in a short period of time, and transfer terpene volatiles to the fruit, increasing the original fruit flavor. It also induces a better preservation

in terms of fungal decay, being a possible future alternative method to extend the life shelf of the strawberry [27].

Packaging Material

Active Packaging is a modern food packaging concept that joins the advances in food technology, safety, packaging, and material sciences in order to achieve the consumer expectations of fresher and safer products. It is defined as a system that in which the product, the package and the environment interact in a positive way. In this type of packaging, the antimicrobial agents can be incorporated into the packaging material, coated on the surface of packaging film, or added to the package in a sachet containing the antimicrobial compounds. The migration between the active compound can be reached by direct contact food/packaging or through gas phase diffusion from the inner packaging layer to the food surface [22,28].

Inside the active packaging, there are the bioplastics, that are considered as an environmentally friendly alternative to the non-biodegradable materials. For these plastics, are used biopolymers to substitute the synthetic polymers, like chitosan, alginates or proteins[11]. Chitosan, poly-β(1,5)-2-amino-2deoxy-D-glucose, is the second most abundant polysaccharide in nature, is a biodegradable cationic hydrocolloid with antimicrobial activity and film forming capacity obtained from the deacetylation of chitin from exoskeletons of crustaceans and insects, and in the cell wall of fungi and microorganisms [11,27]. The use of this biopolymer for the incorporation of the EOs, makes the overcome of the two greatest limitations of the direct incorporation of EOs intro the food matrices, being the cost and the intense aroma, that can have a negative impact in the sensory perception of applied food [29]. An example of this application is the use of chitosan films containing *Eucalyptus globulus* EOs for the packaging of sliced sausages, promoting a reduction of the microbial activity and control of the food-borne contamination in the food system [20].

1.1.3. **Antioxidant activity**

The search for natural antioxidants that are non-toxic also brought the researchers attention to the EOs. Since food deterioration is normally caused by oxidation processes, it is important to have a response against it, to postpone these irreversible changes on the food properties, lowering their shelf life and causing chronic diseases [20,23,30]. The oxidation is a multilateral reaction that can cause changes in terms of organoleptic and nutritional properties, quality, and the creation of potentially toxic molecules [5,30]. The main cause of the oxidation processes are the free radicals, or reactive oxygen species (ROS), chemical species that have on or more unpaired electrons, being highly unstable and causing damage to cellular macromolecules. Lipid oxidation is one of the factors that cause food's degradation, depending on if it presents high levels of fatty acids, which are more susceptible to lipid

oxidation. In visible terms, the lipidic oxidation process can be observed by color changes (discoloration), the appearance of odd flavors (rancid), but can also cause nutrient loss (vitamins, lipids and proteins) and production of toxic compounds [5,20,23].

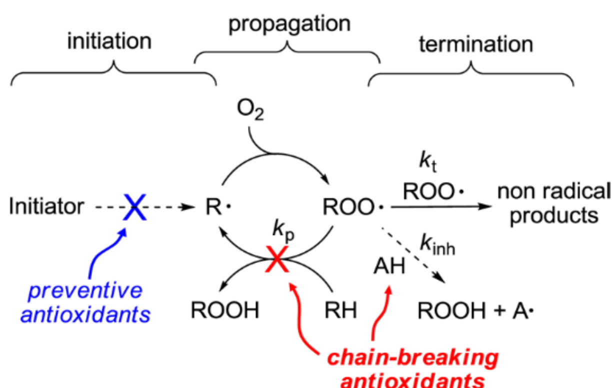

Figure 6 - Simplified mechanism of hydrocarbon autoxidation and antioxidant protection [30]

In food industry, antioxidants are defined as substances, that in small amounts are capable of preventing or delaying in significant ways the oxidation process [31]. By definition, antioxidants are compounds capable of slowing or retarding the oxidation process even in low quantities, commonly 1 to 1000mg/L. It is important to protect lipids, proteins, carbohydrates, and organic molecules that compose animal or vegetal tissue [30].

Oxidation occurs by a radical chain reaction mediated by peroxyl radicals (ROO·), parallel to the autoxidation of hydrocarbons. The process initiates with a ROS species, regardless origin, or structure (superoxide radicals, hydroxyl radicals, singlet oxygen and hydrogen peroxide), which will react with the lipidic substrate (RH), forming an alkyl radical R·, that will react at diffusion-controlled rate with oxygen in order to form peroxyl radicals. Cyclically, the peroxyl radicals will attack an additional molecule of substrate, forming it oxidize version (hydroperoxide ROOH) and another radical. This chain reaction will continue until two radicals quench each other, reaching the termination step. The number of cycles between initiation and termination is the “chain length” [30].

Compounds that are capable of causing damage into the chain reaction are called direct antioxidants and can be distributed in two categories, depending on their mechanism of action (Figure 6):

<u>Preventive antioxidants</u>, which interfere with the initiation process, by retarding the initial formation of radical species. These are able to donate a hydrogen atom rapidly to a lipid radical, forming a more stable radical. Some examples of these type of action are physical barriers preventing ROS generation or their access to important biological sites; chemical traps that absorb energy and electrons; quenching ROS; catalytic systems that neutralize or divert ROS, like antioxidant enzymatic superoxide dismutase, catalase, and

glutathione peroxidase; binding and inactivation of metal ions to prevent generation of ROS [30,32,33].

Chain-breaking antioxidants, which can slow or block the autoxidation by competing with the propagation reactions, namely, they react with peroxyl radicals faster than the oxidizable substrate in order to form species that do not propagate the oxidation chain. These react with the initiating radicals, inhibit the initiating enzymes, or reduce the oxygen level, without generating reactive radical species. Some examples are the deactivation of high energy species (singlet oxygen); absorbing UV light; scavenging and destruction of ROS, with ascorbic acid, tocopherols, uric acid, glutathione and flavonoids, with ; chelating metal that catalyzes free radical reaction or inhibiting enzymes, like peroxidase, NAPD oxidase, xanthine oxidase [30,32,33].

Antioxidant compounds can prevent, alter or even end the oxidative reactions at relatively low concentrations, therefore, EOs and their constituents can play an important role in this activity, since they can express antioxidant activity due to their ability to act as oxygen scavengers and allow the diffusion of active agents into coated food products [5,20]. The antioxidant potential of the EOs, in its majority, depends on their chemical composition, primarily of phenolic compounds and secondary metabolites with double bounds, due to their ability to donate hydrogen atoms to free radicals, turning them into more stable products [1,5].

In order to determine the antioxidant activity of the EOs, there are two common analytical methods: the FRAP (ferric-reducing antioxidant power) and DPPH (2,2-diphenyl-1-picrylhydrazyl) assay. FRAP assay is a method where antioxidants are used to reduce Fe^{3+} to Fe^{2+} in a low pH colorimetric reaction. The DPPH free radical method is used to determine the antioxidant properties of natural products, by showing their scavenging capacity [20]. It is also common the quantification of the total phenolic content of the EOs, normally using the Folin-Ciocalteu method. This method consist on the quantification of the phenolic groups and other potentially oxidizable groups present in the sample [31].

The antioxidant activities of the EOs can be applied just like the antimicrobial activity. One of the most abundant areas where this can be applied is in the food field, for extended shelf-life. In case of food packaging, the package can either release antioxidants into the food and package or absorb oxygen and other compounds from the food and surroundings. It also can be considered a more cost friendly and healthier alternative due being natural products [20]. An example of this property is the study of clove leaf essential oils, that provided higher antiradical activity against DPPH comparing with synthetic antioxidants (butylated hydroxytoluene – BHT and butylated hydroxyanisole – BHA), action as an iron chelator, and capacity to inhibit the generation of secondary products of lipid peroxidation [34].

1.1.4. **Other activities**

Besides the antimicrobial and antioxidant activities, there are others associated with essential oils, here are some examples:

Antiviral Activity [12]

Just as the antimicrobial activity, plants have been showing to possess antiviral properties as well. This capacity of inhibition of viral replication is considered to be due to the presence of monoterpenes, sesquiterpene and phenylpropanoid constituents of the EOs. This activity is believed to be restricted to the EOs ability to interfere with the viral envelope structures, preventing the absorption or entry of the virus into the host cells. An example of this activity is the dissolution of the HSV (herpes virus) envelope, by oregano essential oil, attenuating its infective ability.

Antidiabetic Activity [12]

Diabetic conditions include the inability of producing insulin or the incapacity to use it in order to regulate the normal levels of glucose in the blood, causing hyperglycemia or hypoglycemia. There is few research about the effect of EOs, comparing to other activities, being not well elucidated how this mechanism works. An example of the possible use of EOs as antidiabetic is the use of *Satureja khuzestanica* oil, in order to reduce the blood glycose levels in diabetic rats during the fasting period.

Anti-inflammatory Activity [7,12,32]

An inflammatory reaction is a protective response induced by the injured tissue or infection, in order to prevent invaders in the body, such as microorganisms or non-self cells, and to remove dead and/or damage host cells. The anti-inflammatory activity of EOs in its majority is attributed to their antioxidant activity, due to their ability to scavenge free radicals, since one of the inflammatory responses is the oxidative burst in various cells. Other possible attribution of these activity is the EOs interaction with signaling cascades that involve cytokines and regulatory transcription factor, and on the expression of pro-inflammatory genes. As an example of this activity is the EOs of *Aloe barbadensis*, *Illicium verum*, *Citrus aurantium*, *Cinnamomum zeylanicum*, *Juniperus communis*, *Lavandula officinalis*, *T. vulgaris* and *Cananga odorata*, that possess anti-inflammatory activity mediated trough mechanisms, such as the inhibition of COX-2 enzyme, pro-inflammatory cytokines, interleukin-1β and tumor necrosis factor-α, but also trough repressing pro-inflammatory genes.

Cancer Chemoprotective Activity [7,12,35]

The investigation of the potential activity against cancer of essential oils has drawn attention of the researchers, both in the EOs and their volatile constituents. Some foods are already known for presenting good sources of anticancer agents, such as garlic and turmeric. One of the major difficulties of chemotherapy is the treatment of malignant cell growth leading to cancer, however, some types of malignancies, such as glioma, colon cancer, gastric cancer, leukemia, among others, have been reported to be lowered after treatment with plant EOs. An example of this activity is geraniol from *Cymbopogon martini*, that has been reported to interfere with membrane functions, ion homeostasis and cell signaling events of cancer cell lines, inhibiting DNA synthesis, and reducing the size of colon tumors.

Allelopathic Activity [7,36]

Since 1996 that International Allelopathy Society as defined allelopathy as the science that studies the processes involving the secondary metabolites and how they influence the growth and development of agricultural and biological systems. The secondary metabolites involved in this are called allelochemicals. Allelochemicals allow the inhibition and/or stimulation of the germination and development of other plants, being an important mechanism between plants since it offers potential for selective biological selection. Volatile oils and their constituents have been studied for their use in weed and pest management. An example of this property is the study of *Hyssopus officinalis L., Lavandula angustifolia Miller, Majorana hortensis L.*, and others as possible herbicides, inhibiting the germination and radical growth of *Raphanus sativus L., Lactuca sativa L. and Lepidium sativum L.*

Insecticidal and Repellent Activity [12,37]

The insecticidal and repellent activity of the EOs have also been reported, since secondary metabolites naturally synthesized by plants are responsible for the response in case of insect pest. The difference between a repellent and an insecticide is that a repellent is a substance that acts locally, providing a vapor barrier for the insect to come in contact with the surface, while insecticides are able to kill the insect or larva. An example of this property is the usage of Gaultheria and Eucalyptus oils as insecticides against granary and flying insects.

1.2. *Daucus carota*

Botanical Aspects

The Apiaceae (Umbelliferae) family contains 466 genera and 3820 species, being one of the largest families of seed plants. The genus *Daucus* contains 25 species, being *Daucus carota* the most recognized. *Daucus carota*, Figure 7, is a biannual plant, with flowering season from June to August and that presents erect and branched stems, tough and furrowed, that

can achieve 1m high, with mostly white, small, and five-parted flowers arranged in umbrella-like inflorescence, the umbels [38–42].

Figure 7 - Daucus carota (https://jb.utad.pt/especie/Daucus_carota_subesp_carota, 25/05/2022, 10:55)

Daucus carota subsp. *sativus* is the only cultivated subspecies, the common carrot, and presents an important proportion worldwide. This domesticated form is assumed to be originated by selective breeding from an ancestral wild form of *Daucus carota* subsp. *carota*[43].

Mainly distributed in Europe, Asian and Africa, however, *Daucus carota*, also known as carrot, is commercially cultivated all over the word. Firstly, carrot was used for medical purposes, but then began to be used as food, due to its nutritive roots (vegetable) [40,44].

Traditional uses

Traditionally, *Daucus carota* was cultivated with the intention of being eaten raw or as a cooked vegetable, used in many dishes. Ancient Egyptians used this plant as a stimulant, carminative, diuretic, anthelmintic and for infantile diarrhea [42]. Fruits have been used as treatment of ancylostomiasis, dropsy, chronic kidney disease and bladder afflictions [45]. Other traditional application of this plant, is for hepatic and renal insufficiency, but also for skin disorders such as burns and furuncles [46].

Essential oil Composition

Essential oil composition of this species variates along the different subspecies and the location they are present in, depending on the subspecies, there are not a vast data of analysis. Examples of these varieties are *Daucus carota* subs. *maritimus*, in a Tunisian study, that presented as major components elemicin (31.4%), geranyl acetate (29.0%) and α-pinene (9.4%) [47], or *Daucus carota* subsp. *hispidus*, that also in a Tunisian study presented α-cadinol (13.6%), (E)-methyl-isoeugenol (11.4%), premnaspirodiene (11.4%) and carotol (9.8%) as major constituents [48]. *Daucus carota* subsp. *maximus*, accordingly to an Italian study presented as major compounds are carotol (44.7%) and β-bisabolene (12.7%), however, a Portuguese study reported α-pinene (10.0 – 25.9%), geranyl acetate (3.4 –

16.0%), cis-asarone (5.8 – 25.8%), (E)-methylisoeugenol (8.2 – 15.7%) and elemicin (4.9 – 13.6%) as major ones [43,49]. For *Daucus carota* subsp. *carota*, a German study compared Polish, French and Moroccan essential oils and reported as main component in all of them carotol, representing 19.6%, 25.9% and 33.4% respectively. The one collected in Poland had higher amounts of verbenol (14.9%), caryophyllene oxide (5.9%), α-pinene (4.6%) and trans-pinocarveol (4.6%). French oil presented α-pinene (9.5%), caryophyllene oxide (6.8%) and caryophyllene (5.5%). Finally, Moroccan oil presented caryophyllene oxide (8.7%) and β-bisabolene (7.7%) as major components. The previously referred Portuguese study also reported this subspecies, being α-pinene (13.0 – 27.1%) and geranyl acetate (28.7 – 65.0%) as higher percentage components [43,50]. Lastly, an Indian study referred as major components of *Daucus carota* subsp. *sativus* carotol (10.2 – 58.5%), α-pinene (21.2 – 41.2%), myrcene (6.4 – 14.1%) and limonene (4.4 – 12.7%) [44].

Properties

In terms of properties, carrots produce a variety of phytochemicals, like α-tocopherol, carotenoids, phenolics, and polyacetylenes, that provide essentially antioxidant effects. They are also very nutritive, providing provitamin A, vitamin C, D, E, K, B1, B6, and biotin and minerals like magnesium, potassium, calcium, phosphorus, sodium, and others [41]. Table 1 presents a summary of the biological activities of this species.

Table 1 - Summary of Biological Activities of Daucus carota [39,44]

Biological Activities of *Daucus carota*
Antibacterial; stomach/gastroprotective effects; anti-inflammatory; antifungal; anthelmintic; hepatoprotective; cytotoxic; antioxidant; cardiovascular; antidiabetic; wound healing; anti-atherogenic

Applications

Daucus carota essential oils are a very valuable product and can be used as flavoring agents, as fragrance in perfumes, cosmetics and soaps, but also in the pharmaceutical industry due to properties such as antidiuretic, antimicrobial and anti-inflammatory [39,51].

1.3. ***Aloysia citrodora*** **(Lúcia-Lima)**

Botanical Aspects

Aloysia citrodora Palau, Figure 8, also known as, also known by *Aloysia triphylla (L'Her.)* Britton and *Lippia citriodora* (Lam.) and commonly called Lemon Verbena, Lúcia Lima in

Portuguese, is a member of the Verbenaceae family and *Aloysia* genus, that consists in 200 species of herbs, shrubs, and small trees. It is a perennial herb, native to Chile and was presented to Europe around 1784. It is widely grown in Chile, Argentina, South African, Mediterranean, and European countries. It is a bush with white flowers and fruits, with an intense scent sweet, lemon-like, and lightly floral. It is also well known for its characteristic aromatic odor and taste [52–56].

Figure 8 - Aloysia citrodora (https://jb.utad.pt/especie/Aloysia_triphylla, 08/03/2022, 18:30)

This plant is a perennial shrub with up to 3m height, striate and scabrous branches, and lanceolate 7–10 cm margined leaves with short petioles. The tiny flowers have white or light blue color which appear on a hairy calyx with four tips in the panicle-like spikes. The petals form a 4–5mm funnel at the base which covers 2 long and 2 short stamens [57].

Traditional uses

Reports about its usage date back to the 17th century, and its leaves were used in order to make infusions for refreshing drinks, liquor flavoring and soft drinks [57]. It is also traditional in Mexico to traditional medicine for stomach disorders, depression and anxiety [58]. Brazilian local populations used *A. citrodora* as a natural medicine to treat tropical diseases [59]. Tincture and essential oil usage as bactericide purposes are also common in this plant, but the majority of its traditional uses if around the teas and infusions, due to A. citrodora properties referred later on. Focusing on Portugal uses, its leaves are used as an infusion for diarrhea, insomnia and rheumatism [57,60,61].

Essential oil Composition

The following table (Table 2) presents the composition of *Aloysia citrodora* essential oils, from different parts of the plant, as well as different locations, with the components that present percentages higher than 5%:

Table 2 - Summary of the mains compounds from Aloysia citrodora, according to the bibliography

Plant material	Location	Composition	References
Leaves	Iran	Geranial (23.24%) and neral (16.55%), γ-terpinene, Caryophyllene oxide (5.51%), α-curcumene (5.21%), Spathuneol (5.18%)	52
Unspecified	Italy	Limonene (36.7%), Sabinene (24%), citronellal (12%)	62
Aerial	Kairouan, Tunisia	Geranial (25.15%), Neral (17.22%), Limonene (7.27%), Geraniol (5.57%), ar-curcumene (5.24%)	54
Aerial	Korba, Tunisia	Geranial (26.85%), Neral (14.81%), Limonene (6.34%), Geraniol (6.02%)	
Aerial	Siliana, Tunisia	Geranial (27.41%), Neral (15.60%), Limonene (7.52%), Geraniol (5.87%), ar-curcumene (5.22%)	
Aerial	Gabes, Tunisia	Geranial (25.15%), Neral (17.22%), Limonene (7.27%), Geraniol (5.57%), ar-curcumene (5.24%)	
Unspecified	France	Geranial (18.46%), Limonene (17.34%), Neral (12.04%), β-caryophyllene (6.31%), Farnesene (5.66%)	63
Aerial	Morocco	Limonene (23.40%), Geranial (10.42%), Neral (7.30%), Cineole (7.17%), β-caryophyllene (5.19%)	53
Unspecified	Argentina	Neral (13 – 31.5%), Geranial (3.3 – 29.2%), Limonene (2.9 – 21.3%), Caryophyllene oxide (1 – 10.5%), Spathulenol (0.9 – 11.1%)	55
Aerial	Morocco	Citral (14.21%), β-caryophyllene (10.71%), 1,8-cineole (9.1%), Citronellol (8.87%), iso-menthone (6.43%), α-bergamotene (5.33%), menthonol (5.10%)	64

In terms of oil composition, the major component is citral, which corresponds to a mixture of two isomeric acyclic monoterpene aldehydes: geranial (citral A/α, *trans*-citral) and neral (citral B/β, *cis*-citral) (Figure 9) that also provides a typical flavor to the plants [65,66].

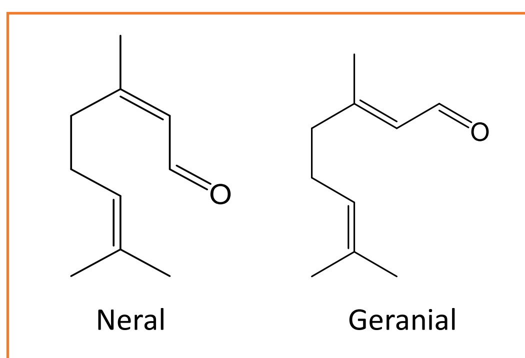

Figure 9- Citral components: Neral and Geranial

Properties

As to the properties of *A. citrodora* EOs, it is known that presents properties like aromatic, antispasmodic, stomachal and digestive, sedative, carminative, anti-inflamatory, analgesic, hipoglycemic, anti-migrane, tonicardic and antipyretic, for this reason, it is common to use *A. citrodora* for treatments of diseases related to microbial infection, anti-inflamation and

anti-oxidative stress. Its leaves, due to essential oils, also present carminative properties, effects against fever, painkiller action and against anemia, as well as gastrointestinal disorders, it and also antioxidant, antimicrobial, antifungal properties [52–56,64]. Table 3 presents a summary of the biological activities of this specie.

Table 3 - Summary of Biological Activities of Aloysia citrodora [57]

Biological Activities of *Aloysia citrodora*
Antioxidant; antinociceptive; anti hyperpropulsice; homeostatic stability effects; growth improvement; improvement blood parameters; sedative; anticonvulsant; antihyperalgesic; anxiolytic; anticolitis; anti-inflammatory; spasmolytic effect; muscle relaxant; antigenotoxic; larvicidal; anti-obesity related metabolic disturbances; ocular-protective; insecticidal; cytoprotective; anti-cancer; cardiovascular effect;

Applications

As to application, we can divide into four categories: food, pharmaceutical, fragrance, and domestic use. As to the food industry, it is used for non-alcoholic drinks, eupeptic tea, and flavoring agent. For the pharmaceutical industry, can be used as an additive in dietary supplements, and applicated as a carminative, antispasmodic and sedative, due to its properties. As to the fragrance industry, it is also used as an aromatic ingredient, such as flavoring curries or wines and for food seasoning and herbal remedy in domestical uses [52,54].

1.4. *Cymbopogon citratus* (Erva Principe)

Botanical Aspects

Cymbopogon citratus (DC.) Stapf (Figure 10) commonly known as lemongrass or Erva Principe in Portugal is an aromatic member of the Poaceae family, originated from West India. It is a perennial herb cultivated at large scale in the tropics and sub-tropics that presents a tall can that can achieve 2.5 meters heigh, with green and linear leaf lamina and produces flowers when achieved the mature stage of growth [66–69].
The resources possible from this plant are fiber, leaves and essential oil. It is very rich source of lignocellulose, due to the high percentages of cellulose, hemicellulose and lignin in it[69,70].

Figure 10 - Cymbopogon citratus (https://jb.utad.pt/especie/Cymbopogon_citratus#imagem-43828, 11/05/2022, 14:00)

Traditional uses

The use of infusions of this plant has been reported for many years, due to it medicinal value. It is normally consumed as tea, and in cultures like the Northern Philippines and Nigeria, they believe that this tea can help with indigestion, stress, colds and cure many diseases [71]. They also can be used as a component of deodorant products like perfumes, soaps, candles or even insect, snake and reptile repellents [72]. In Ayurvedic medicine, this plant can be found as an anti-obesity and antihypertensive method, since it is used to regulate the levels of lipids, glucose in the blood [72].

Essential oil Composition

Similarly, to *Aloysia citrodora* essential oil, *C. citratus* also presents as major constituents Geranial and neral.

In Table 4 are present the composition of *Cymbopogon citratus* essential oils, from different parts of the plant, as well as different locations, with the components that present percentages higher than 5%:

Table 4 - Summary of the mains compounds from Cymbopogon citratus, according to the bibliography

Plant material	Location	Composition	References
stalk	Malaysia	Geranial (32.10%) Neral (22.36%), limonene (5.71%) and geraniol (5.40%)	70
All plant	Selangor	Geranial (45.95%), Neral (31.13%), β-myrcene (7.68%)	69
Leaves	Faso	Geranial (48.10%), Neral (34.60%), β-myrcene (11.00%)	73

Leaves	Brazil	Geranial (39.70%), Neral (29.60%), β-myrcene (14.10%), Geraniol (5.70%)	66
All plant	Vietnam	Geranial (33.85%), Neral (29.4%), β-myrcene (12.15%)	74
All plant	Kenya	Geranial (39.53%), Neral (33.51%), β-myrcene (11.41%)	75
Leaves	Algeria	Geranial (42.16%), Neral (31.52%), β-myrcene (7.45%)	76
Leaves	Brazil	Geranial (31.89%), β-myrcene (25.37%), Neral (24.62%), 4.5-epoxy-carene (6.00%)	77
All plant	Iran	Geranial (39.16%), Neral (30.95%), Limonene (5.83%)	78
Leaves	Benin	Geranial (39.50%), Neral (35.50%), β-pinene (10.10%)	79

Properties

Recent studies indicate the possibility of C. citratus EO present effectiveness as an inhibitor of biodegrading oxidation and storage contaminating microbes [80]. Previous studies have reported the benefit from applicating this plant with medical purposes, due to properties like antibacterial, antifungal, antiprotozoal, anti-carcinogenic, anti-inflammatory, antioxidant, cardioprotective, antitussive, antiseptic among others [71].

C. citratus also provides mineral constituents like sodium, potassium, magnesium, iron, zinc and phosphorus [72].

Table 5 - Summary of Biological activities of Cymbopogon citratus [71,81,82]

Biological Activities of *Cymbopogon citratus*
Aromatherapy; food preservative; hypocholesterolemic; antidiabetic; antimicrobial; diuretic; hypoglycemic; hypotensive; anti-inflammatory; antioxidant; anticarcinogenic; hematological; neuropharmacological; analgesic; antinociceptive; Insecticide repelling; astringent; antipyretic; cardioprotective; cell signalling and transport; hemodynamic;

Applications

The leaves can be used as flavoring both for food and beverages, and also as a condiment. Due to its rose aroma developed by the citral components, is well known in the perfume, cosmetic and food and flavoring industries [83]. It can be used un aromatherapy and cosmetology, but also for making non- alcoholic beverages and baked foods as flavoring and preservative agent [71]. Due to its antimicrobial and anti-insecticidal activities, it can be applicated in order to control pathogens and insect plagues [72]. Another application of this oil is for the isolation of the major components, the citral, mixture of geranial and neral [84].

1.5. Aim

The aim of this study is to characterize different essential oils and to evaluate their application into chitosan based edible films for application in food industry. The following species were used:

- *Daucus carota* (subspecies *maritimus, hispidus, maximus, carota* and *sativus)*
- *Aloysia citrodora*
- *Cymbopogon citratus*

The study was divided into two parts:

- For the *Daucus carota* subspecies were extracted the oils, by hydrodistillation method, followed by their characterization by Gas-Chromatography coupled with Mass Spectrometry (GC-MS), followed by the study of their respective phenolic and flavonoid content, as well as antioxidant activity (Figure 11).

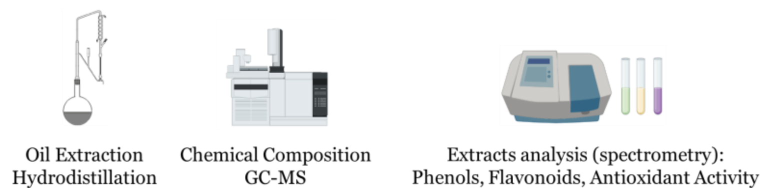

Figure 11 - Daucus carota aim

- For the *Aloysia citrodora* and *Cymbopogon citratus*, the essential oil was characterized by GC-MS, followed by phenolic content and antioxidant activity study. After characterization, they were prepared chitosan based edible films with the respective oils, for further migration and contact assay (Figure 12).

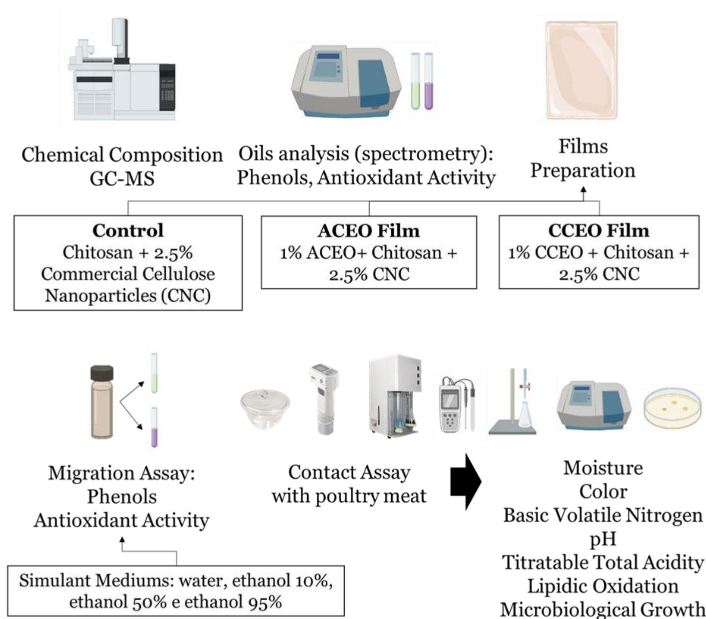

Figure 12 - Cymbopogon citratus and Aloysia citrodora aim

Chapter 2
Methodology

Chapter 2 – Methodology

2. Methodology

2.1. Material

2.1.1. Plant material

Plant material was collected from various parts of Portugal, provided by Banco Português de Germoplasma Vegetal (BPGV). Flowering and mature umbels were preserved at dry place and room temperature. Five subspecies of *Daucus carota* were collected: *D. carota* subsp. *maritimus*; *D. carota* subsp. *hispidus*; *D. carota* subsp. *maximus*; *D. carota* subsp. *sativus* and *D. carota* subsp. *carota*. Roots of *D. carota* subsp. *sativus* and leaves of *D. carota* subsp. *maritimus*; *D. carota* subsp. *hispidus*; *D. carota* subsp. *maximus* also provided. All samples were collected from more than ten individuals.

2.1.2. Essential oils

Essential oils from *Cymbopogon citratus* and *Aloysia citrodora* used in this work were commercial oils provided by Biomater – Soulful Farming. These oils are from a Portuguese company, extracted also in Portugal.

2.2. Methods

2.2.1. Essential oil isolation/extraction

For the essential oil isolation of the umbels of *Daucus carota* subspecies was used the hydrodistillation method. A 10g of plant per 200mL of water were collected in a distillation flask and was used a Clevenger apparatus for 2 hours.

2.2.2. Extracts obtention

To obtain the extracts, two methods were performed: The hydrodistillation method for the umbels and extraction by Soxhlet for the roots and leaves, using two possible.

2.2.3. Essential oil analysis and chemical composition

Essential oil analysis and chemical composition proceeded by GC-MS, using an Agilent 7890A GC coupled with an Agilent 5975C inert XL mass selective detector. For the separation of the volatile compounds was used an DB-5 J&W GC capillary column (5%

phenylmethylpolysiloxane, 30m length, 0,25mm diameter and 0,25µm film), being helium the mobile phase, at 1mL/min. The components identification was obtained by comparison of the retention times and mass spectra, which were also compared with the ones at the data system library NIST and Wiley.

2.2.4. Total phenols

2.2.4.1. Extracts

The total phenolic content was examine using a colorimetric assay with Folin-Ciocalteu's reagent, following the method described by Sain and Hillis, 1959, using a UV-Vis Spectrophotometer Thermo Scientific Evolution 160, at a 765nm wavelenght[85].

For the calibration curve with gallic acid, was used a base solution of gallic acid 1mg/mL at a 25mL volumetric flask. Then proceeded to dilutions using methanol, with the following concentrations: 0, 100, 200, 300, 400, 500, 800, 1000 mg/L.

Using a test tube, add 50µL of the diluted gallic acid solutions, 450µL of distilled water and 2.5mL of Folin-Ciocalteu's reagent, and let it react for 5 minutes. Then add 2mL of a sodium carbonate aqueous solution (75mg/L) and wait 90 minutes.

Total phenols were expressed in mg of gallic acid (GA) per gram of dry biomass (mg GA eq. /g dry biomass) [85].

2.2.4.2. Essential oils

The total phenolic content was analyzed in four simulator mediums: water, ethanol 10%, ethanol 50%, ethanol 95%. For the calibration curves was used a mother solution of gallic acid at 1g/L, followed by dilutions of 200, 100, 50, 25, 10, 5, 2.5, 1.25 mg/L, for each medium. Using a teste tube, add 3mL of H_2O Mili Q, 1mL of sample and 0.25mL of Folin-Ciocalteu's reagent, then were shaken in the vortex and added 0.75mL of sodium carbonate 5% and incubated for an hour at the dark, room temperature.

The phenol concentration in each medium was determined by the Folin-Ciocalteu's method, at a 760nm wavelength [86].

2.2.5. Total Flavonoids

The total flavonoids content of the extracts was examine using the colorimetric method described by Latoui (2012), using a UV-Vis Spectrophotometer Thermo Scientific Evolution 160, at a 415nm wavelenght[85].

For the calibration curve, were used different concentrations of quercetin, diluted using methanol, at the following concentrations: 0, 10, 50, 100, 150, 200, 300, 350, 400 mg/L.

On a test tube add 50µL of the sample, 200µL of methanol and 1.25mL of distilled water, then add 0.075mL of 5% sodium nitrate solution and wait 5 minutes to react. Add 0.15mL of 10% aluminum chloride and wait 6 minutes. Finally, add 0.5mL of 1.0M sodium hydroxide and distilled water into a final volume of 3mL.

Total flavonoids were expressed in mg of quercetin (Q) per gram of dry mass (mg Q eq. /g dry biomass).

2.2.6. Antioxidant activity

The DPPH· radical presents a purple color when at its higher absorbance, at 517nm. By accepting an H from an antioxidant, or being reduced, the radicals start to give place to the correspondent hydrazine, passing its color from purple to yellow. This way, the antioxidant activity can be measured by the evaluation of the diminution of DPPH· absorption at 517nm.

2.2.6.1. Extracts

For the calibration curve, were used different concentrations of DPPH, diluted a mother solution of DPPH 0.2mM, using methanol, to the following concentrations: 0.02, 0.04, 0.06, 0.08, 0.10mM[85].

For the extract, prepare a 25mL sample of 2mg/mL in methanol a effectuate the following dilutions at 5mL volume: 50, 100, 200, 250, 500, 1000, 1500 µg/mL.

The procedure consists of adding 0,1mL of extract and 3,9mL of DPPH. To the blank sample we use 0,1mL methanol diluted in 3,9mL DPPH. To correct the colored samples, prepare a solution with 0,1mL extract and 3,9mL methanol. Incubate at ambience temperature for 90 minutes before reading de absorbance, at a 517nm wavelength.

Obtaining the calibration curve of each sample dilution, proceed to calculate the Antioxidant activity (AAI), where the IC50 corresponds to the concentration that can inhibit 50%, and can be obtained by the sample dilutions curve:

$$AAI = \frac{Final\ DPPH\ concentration\ (\mu g.mL^{-1})}{IC50\ (\mu g.mL^{-1})}$$

Equation 1 - Antioxidant Activity

2.2.6.2. Essential oils

For the antioxidant activity analysis, it was prepared a DPPH· solution (24mg/L ethanol). For the calibration curves, were prepared mother solutions of 1.4mg/mL of ascorbic acid, followed by 0.14, 0.035, 0.0252, 0.0175, 0.00875, 0.004375 mg/mL dilutions in the four mediums (water, ethanol 10%, ethanol 50%, ethanol 95%).

To analyze the antioxidant activity, add 1mL of sample in 3mL of de DPPH· solution and incubate for 20 minutes at room temperature, in the dark. Read the absorbance at 517nm wavelength. Obtaining the calibration curve, it is possible to then calculate the inhibition percentage (I%), that corresponds to the difference between absorbances with and without sample:

$$I\% = \frac{(Abs0 - Abs1)}{Abs0} x\,100$$

Equation 2 - Inhibition percentage (I%)

2.2.7. Chitosan Films Preparation

For the biofilm's preparation, the process was based by the Siripatrawan and Harte (2010) and Dias *et al.* (2014) methodology, with some adjustments [87,88]. First was prepared the filmogenic solution (FS), by dissolving 1.5g of high molecular wight chitosan, from Sigma Aldrich (Germany) in a 1% (v/v) solution of glacial acetic acid, with agitation for 24h, at room temperature. After full dissolution of the chitosan, where prepared three different films, a control one and two with EOs, being *Aloysia citrodora* (ACEO) and *Cymbopogon citratus* (CCEO).

The control film was prepared with 200mL of FS, 10 drops of glycerol, for plasticization functions, and 0.0525g (2.5%) of commercial cellulose nanoparticles (CNC) as a reinforcement, with 2 cycles of ultraturrex agitation for 5 minutes and 15 minutes of ultrasonic bath. The EOs films were prepared the same way, with the exception that there were also added 6 drops of tween, as emulsion agent, and 2mL (1% v/v) of the respective EO [89]. Between the additions, there were made 3 cycles of ultraturrex agitation for 5 minutes and 15 minutes of ultrasonic bath.

2.2.8. Migration assays

The *in vitro* quantification of the diffusion of the antioxidant compounds from bionanocomposites trough time can be analyzed by diffusion tests [90,91].

For the migration assay, it was studied how the active compounds behave in the different simulant mediums (water, ethanol 10%, ethanol 50% and ethanol 95%), and their released to the simulant medium.

Small 1cm^2 squares of biofilms were placed inside amber vials with 4mL of the simulant mediums and incubated at 40ºC, for the following hours: 2, 4, 8, 18, 24, 48, 96, 168 and 240.

In each time the biofilms were removed from the vials and the antioxidant activity and total phenols determination was measured in the simulants medium [90].

2.2.9. Contact Assay

For the contact assay analysis, the biofilms (Chitosan + CNC; Chitosan+CNC+ACEO and Chitosan+CNC+CCEO), in duplicate, were used to wrap 30g of poultry meat previously grinded. The wrapped meat with the biofilms was contained, as well as a control without film, at a 4ºC temperature for 0, 3, 6, 9 and 13 days. In each day the following analysis were made:

2.2.9.1. Moisture content

For moisture determination, based on the AOAC (2016) [92] method, 1g of poultry meat was weighted in a previously weighted crucibles. Samples were placed in an oven at 103 ± 2 ºC for about 12hours. After that time, the crucibles were placed in a desiccant until achieving room temperature, for new weighting.

For moisture content calculation, the following equation is used, where P1 represents the weight of the crucible + meat before oven, P2 the weight of crucible + meat after oven and PF the weight of the crucible alone.

$$\% Humidity = \frac{P1 - P2}{P1 - PF}$$

Equation 3 - Moisture content (%)

2.2.9.2. Color

For color determination of the poultry meat sample, was determined with CIE-L*a*b* coordinates, using a colorimeter CR410 (Minolta Co., Tokyo, Japan), with a D65 light source, and a visual angle of 10º [93]. The coordinate L * corresponds to the luminosity, going from 0 (black) to 100 (white); a* corresponds to the chromaticity of green, going from green to red and b* the chromaticity of blue, going from blue to yellow. Sample is placed in a petri

plaque and measured three times, all in different positions, in order to evaluate the Hue angle and color variation [94]:

$$\Delta E = \sqrt{(L0 - L)^2 + (a0 - a)^2 + (b0 - b)^2}$$

Equation 5 - Color variation

$$Hue\ angle = tan(\frac{b*}{a*})^{-1}$$

Equation 4 - Hue Angle

2.2.9.3. Basic Volatile Nitrogen

For basic volatile nitrogen determination, based on the AOAC (2016)[92] method, 5g of poultry meat were weighted and added to 100mL of deionized water at 50ºC for 15 minutes with agitation. Further filtration is required using qualitative filter paper. After the filtration process, 25mL of filtrated are added to a distillation tube with 75ml of distilled water with 3 drops of phenolphthalein 0.1% and some drops of NaOH 6N, to neutralize de medium. On an 250mL Erlenmeyer were placed 50mL of boric acid 2% and 0.5mL of boric acid indication solution. Both flasks are placed in a distiller (distillation tube on the left and Erlenmeyer on the right

This process occurs by the solutions vapor drift, being the distilled collected in the boric acid solution, when the color turns from purple to green. Further, proceeds with a titration with HCl 0.02N until color turns into purple again.

For this calculation, the following equation is used, expressed in grams of nitrogen per 100g of meat:

$$Basic\ Volatile\ Nitrogen\ (\frac{gN}{100g\ meat}) = \frac{V_T\ x\ 0{,}02\ x\ 1{,}401}{m}\ x\ \frac{100}{V_E}$$

Equation 6 - Basic Volatile Nitrogen

Where, V_T corresponds to the HCL volume (mL) used in the titration, m to the meat weight and V_E to the volume of extract used.

2.2.9.4. pH

The pH analysis is based on the determination of hydrogen ions activity. The pH value can present acidic values (below 7) or basic values (above 7) [95].

For the pH analysis, the methodology used was accordingly with AOAC (2016) [92], where 5g of poultry meat are weighted and added to 50mL of deionized water at 40ºC for 15 minutes with agitation. Further filtration is required using qualitative filter paper, before

determination with a pH digital meter, previously calibrated with a pH 4 and a pH 7 solutions.

2.2.9.5. Titratable total acidity

For acidity analysis we based on the AOAC (2016) [92] method, were we use the previously filtrated extract from the pH, and add 3 drops of phenolphthalein 0.1%, as indicator. The titratable total acidity is determined by a titration using NaOH 0.1N. The results are expressed in g of oleic acid per 100g of meat.

For this calculation, the following equation is used, where V corresponds to the NaOH volume and M to the meat mass (g):

$$Titrable\ total\ acidity = \frac{V\ x\ 0{,}1\ x\ 28{,}2}{M}$$

Equation 7 - Titrable Total Acidity

2.2.9.6. Lipidic Oxidation

For the determination of the oxidative state of the samples was used the thiobarbituric acid reactive substances (TBARS) method, according to Rosmini *et al.* (1996) [96]. For the calibration curve, in order to quantify the malonaldehyde (MDA), there were prepared solutions of 1,1,3,3-tetraethoxypropane (TEP), in eight tubes, from 0 to 50µmol prefacing with TCA until 5mL and added 5mL of 2-thiobarbituric acid (TBA) 0.02M and let for 30min in a water bath at 90°C.

For the sample determination, were measured 5g of poultry meat to an Erlenmeyer and added 10mL of TCA 7.5% (w/v) and shake for 45min. The supernatants were filtered using qualitative filter paper. The volume of 5mL of the filtrate were collected into a tube flask and added 5mL of TBA and let for 30min in a water bath at 90°C. Sample absorbances were measured at 530nm in a UV/VIS spectrophotometer. Results are expresses in mg of malonaldehyde per gram of sample.[93]

To calculate the TBARS Index, the equation bellow is used, where C corresponds to the absorbance divided by the slope of the calibration curve, M the meat mass in g and H, the moisture content (%):

$$TBARS\ Index(\frac{mg\ MDA}{Kg\ sample}) = 72{,}06\ x\ C\ x\ (\frac{10 + (M\ x\ H)}{Mx\ 5})$$

Equation 8 - TBARS Indez

2.2.10. Microbiological Growth

For the evaluation of the microbiological growth in the poultry meat, three types of parameters were selected: total viable microorganisms, mesophilic (ISO 4833-1:2013) and psychotrophic (ISO 17410:2001) and *Enterobacteriaceae* (ISO 21528-2:2017). During this analysis all the procedures was carried in a laminar flow chamber Steril-Helios, Italy, in order to prevent possible contaminations.

In a sterile flask, place 1 g of poultry meat and 9mL of saline solution (0.1% m/v tryptone and 0.85% m/v sodium chloride), proceed to homogenize and carry through successive decimal dilutions with vortex agitation in between. When achieved the pretended dilutions, proceed to inoculate in a petri dish 1mL of sample with further addition of growth medium (Plate Count Agar – PCA for the total viable microorganisms and Violet Red Bile Glucose – VRGB for *Enterobacteriaceae)*. Upon the medium dries, place the petri dishes incubating, being mesophlic microorganisms in a oven at 30ºC for 72 hours and *Enterobacteriaceae* for 24h. For psychotrophic microorganisms place in the freezer at 7ºC for 168 hours.

Chapter 3 Results
and Discussion

Chapter 3 – Results and Discussion

3. Results

3.1. Daucus carota

3.1.1. Essential oil isolation/extraction

After the essential oil extraction, obtained by the hydrodistillation method, were obtained the yields percentage (v/w), present at Table 6. The highest yield was for *Daucus carota* subsp. *maximus*, with 0.45%, followed by *Daucus carota* subsp. *maritimus* (0.39%), *Daucus carota* subsp. *hispidus* (0.33%), *Daucus carota* subsp. *carota* (0.23%), and finally, with the lowest yield, but also, the lowest mass, *Daucus carota* subsp. *sativus* (0.06%).

The obtained yields where extremely low, which made it impossible for further antimicrobial and antioxidant activity tests. For that reason, for *Daucus carota* subspecies trials, were used the extracts provided by the hydrodistillation method.

Table 6- Yields obtained by the extraction of the Daucus carota subspecies oils

Subspecies	Plant mass (g)	Oil Volume (mL)	Yield (%)
D. carota subsp. *maritimus*	282.69	1.10	0.39
D. carota subsp. *hispidus*	54.37	0.18	0.33
D. carota subsp. *maximus*	85.14	0.38	0.45
D. carota subsp. *carota*	140.57	0.32	0.23
D. carota subsp. *sativus*	31.06	0.02	0.06

3.1.2. Essential Oils Chemical composition

For the essential oil composition, where found that *Daucus carota* subspecies present the percentages showed (Table 7). The main compound, with percentage above 5%, found in *Daucus carota* subsp. *maritimus* were geranyl acetate (33.9%), *trans*-methyl-isoeugenol (9.3%), α-pinene (7.2%), cis-asarone (6.9%), elemicin (6.0%) and β-pinene (5.5%). For *Daucus carota* subsp. *hispidus* the main compounds were geranyl acetate (41.1%), caryophyllene oxide (9.1%) and *trans*-methyl-isoeugenol (7.0%). For *Daucus carota* subsp. *maximus*, the major compounds were *cis*-asarone (47.1%), geranyl acetate (14.8%), elemicin (13.1%) and *trans*-methyl-isoeugenol (6.8%). For *Daucus carota* subsp. *carota* the major compounds were geranyl acetate (32.2%), *cis*-asarone (29.6%), *trans*-methyl-isoeugenol (9.8%) and α-pinene (6.5%). Finally, *Daucus carota* subsp. *sativus* presented as major components carotol (53.8%) and daucol (26.7%).

Table 7- Chemical composition of the essential oils from Daucus carota subspecies

		% *Daucus carota* subspecies				
Compounds	RI*	*maritimus*	*hispidus*	*maximus*	*carota*	*sativus*
α - pinene	936	7.2	2.0	1.0	6.5	-
Camphene	950	0.5	-	-	-	-
Sabinene	973	1.5	4.3	2.3	1.2	-
β - pinene	978	5.5	1.7	1.9	4.2	-
β - myrcene	989	1.1	0.5	-	0.7	-
α -terpinene	1017	-	-	-	-	-
p - cymene	1024	0.3	0.4	-	-	-
Limonene	1029	2.0	1.3	0.9	1.1	-
γ-terpinene	1059	-	0.5	-	-	-
cis - Linalool Oxide (furanoid)	1075	0.5	-	-	0.7	-
trans - Linalool Oxide (furanoid)	1083	0.3	-	-	-	-
α - terpinolene	1086	-	-	-	-	-
Linalol	1099	2.2	1.7	-	1.2	-
α - campholenol	1124	0.3	0.7	-	-	-
trans - Pinocarveol	1140	1.2	3.3	0.8	0.9	-
trans - Verbenol	1144	0.3	3.3	-	-	-
cis - Verbenol	1145	1.7	2.3	-	1.3	-
Sabina ketone	1157	-	0.7	-	-	-
Pinocarvone	1160	0.6	1.0	-	-	-
p-mentha-1,5-dien-8-ol	1166	0.5	0.5	-	-	-
4-Terpineol	1177	0.4	2.4	1.5	-	1.3
α - terpineol	1189	0.5	-	-	-	-
Myrtenal	1192	1.3	3.2	-	-	-
Verbenone	1206	0.6	-	-	-	-
Carvone	1242	-	0.4	-	-	-
Linalyl acetate	1255	1.9	-	-	-	-
Bornyl acetate	1283	-	0.9	-	-	-
α - terpinenyl acetate	1347	0.4	-	-	-	-
Geranyl acetate	1379	33.9	41.1	14.8	32.2	2.5
β - selinene	1486	0.3	-	-	0.8	-
trans - methyl - isoeugenol	1495	9.3	7.0	6.8	9.8	0.7
β - bisabolene	1508	3.4	4.0	3.9	2.2	0.5
Elemicin	1553	6.0	-	13.1	4.1	-
Spathulenol	1576	-	0.4	-	-	0.3
Caryophyllene oxide	1581	1.9	9.1	4.5	2.5	1.2
Carotol	1595	2.2	-	-	0.7	53.8
Daucol	1639	0.6	-	-	-	26.7
cis - asarone	1679	6.9	3.3	47.1	29.6	3.3

*RI – Retention Index on DB-5-MS

3.1.3. Total phenols (TPC)

Phenolic compounds are the most common secondary metabolites in plants. They present a common structure with an aromatic ring with one or more hydroxyl substituents [97].

From the calibration curve, with an absorbance measured at 765nm, we obtain the following line:

$$Abs = 0.0004x + 0.0062 \ (R^2 = 0.9982) \quad \text{Equation 9}$$

Important to refer, that the majority of the studies around the *Daucus carota* subspecies focus on the roots of the plant, becoming a difficulty to compare some results with the literature.

With the previous line, were calculated the total phenols in mg of gallic acid per g of dry biomass, obtaining the following results:

3.1.3.1. Umbels (extract)

Table 8 presents the total phenols obtained by the umbels extracts of *Daucus carota* subspecies. The subspecies with the highest phenol content was *Daucus carota* subsp. *maritimus* (50.17 mg/g), followed by *Daucus carota* subsp. *sativus* (32.81mg/g), *Daucus carota* subsp. *hispidus* (31.92 mg/g), *Daucus carota* subsp. *maximus* (25.33 mg/g) and, with the lowest *Daucus carota* subsp. *carota* (20.36 mg/g).

Comparing the obtained results to previous studies we can see that our sample presented much lower values among the TPC of the *Daucus carota* subsp. *carota,* to the ones presented by Jorge Silva, 2020, that presented 118.1 mg/g DW of total phenols, however, the sample used in the described study was a phenolic-enriched extract [98]. Seemingly, no previous data was found for umbels extracts from the other subspecies.

Table 8-Total phenols from Daucus carota umbels obtained by aqueous extracts

Subspecies	Phenols (mg/L)	Dry mass (g/L)	Total phenols (mg/g)
maritimus	592.0	11.80	50.17
hispidus	199.5	6.25	31.92
maximus	249.5	9.85	25.33
carota	309.5	15.20	20.36
sativus	239.5	7.30	32.81

3.1.3.2. Roots (extract)

For the root extracts were used a Soxhlet method, where only the *Daucus carota* subsp. *sativus* was analyzed. In order to understand in each medium, the phenol content was better, both aqueous medium and methanol were used. Results shown (Table 9) that for phenolic content, the aqueous medium was better, showing almost the double of total phenols. According to the literature, studies with this subspecies' extracts have shown, lower phenolic content, however, the root part used in other studies was the consumed part, the carrot, and in this extraction were used the non-consumed part. Studies presented total phenols 35.97 mg/100g FW for carrots in aqueous medium [99] or between 81.25 mg/Kg and 113.69 mg/Kg in 80% ethanol [40]. Due to the difference between the expression of the values in FW (Formula Weight) and DW (Dry Weight), it is not possible to compare the results.

Table 9- Total phenols from Daucus carota subsp. sativus roots, obtained by aqueous and methanolic extracts

Subspecies	Phenols (mg/L)	Dry mass (g/L)	Total phenols (mg/g)
Sativus H_2O	57	9.2	6.20
Sativus MeOH	87	25.2	3.45

H2O – aqueous extract; MeOH – methanolic extract

3.1.3.3. Leaves (extract)

For the leave extract TPC (Table 10), the extraction was realized by the Soxhlet method, using methanol as medium, for the three subspecies of *Daucus carota*. The samples presented values between 15 and 21 mg/g of total phenols, being *Daucus carota* susp. *maritimus* the one with the highest phenol content (20.63 mg/g). Similarly, with the umbels analysis, there were no previous studies reporting the TPC of this subspecies, however, Ksouri et at. 2014 reported a TPC for methanolic leaves extract from *Daucus carota* subsp. *carota* of 13.83 mg/g, being a value around the ones presented by our subspecies [100].

Table 10- Total phenols from Daucus carota leaves obtained by methanolic extracts

Subspecies	Phenols (mg/L)	Dry mass (g/L)	Total phenols (mg/g)
maritimus MeOH	49.5	2.4	20.63
hispidus MeOH	212.0	11.6	18.28
maximus MeOH	44.5	2.8	15.89

MeOH – methanolic extract

3.1.4. Total Flavonoids (TFC)

Flavonoids group is the largest group of phenolic compounds present in plants. This consist in 15 carbon atoms arranged in two aromatic rings and one three carbon bridge [97].

From the calibration curve, with the absorbance measured at wavelength of 415nm, we obtain the following line:

$$Abs = 0.0014x + 0.0461 \; (R2 = 0.9811) \; \text{Equation 10}$$

With the previous line, we calculated the total flavonoids in mg of quercetin by g of dry biomass, obtaining the following results:

3.1.4.1. Umbels (extract)

For the Daucus carota subspecies TFC (Table 11), *Daucus carota* subsp. *maritimus* presented the highest level of flavonoids, with 29.05 mg/g, followed by *Daucus carota* subsp. *hispidus* (12.56 mg/g), *Daucus carota* subsp. *sativus* (12.22 mg/g), *Daucus carota* subsp. *maximus* (12.03 mg/g) and, with the lowest, *Daucus carota* subsp. *carota*, with 8.92 mg/g. This results among with the ones from the TPC seem accurate, since all samples presented lower values of flavonoids, being this a subcategory of the phenols. The obtained values mean that 57.90% of the phenols of *Daucus carota* subsp. *maritimus* are flavonoids, as well as 39.34%, 47.49%, 43.81% and 37.24% respectively for the remaining subspecies.

Table 11 - Total flavonoids from Daucus carota umbels obtained by aqueous extracts

Subspecies	Flavonoids (mg/L)	Dry mass (g/L)	Total Flavonoids (mg/g)
maritimus	342.79	11.80	29.05
hispidus	78.50	6.25	12.56
maximus	118.50	9.85	12.03
carota	135.64	15.2	8.92
sativus	89.21	7.30	12.22

3.1.4.2. Roots (extract)

As to the TFC content of the *Daucus carota* subsp. *sativus* roots (Table 12), it presented more similar values between mediums, being the aqueous one still higher than the methanol. In this trial, *Daucus carota* subsp. *sativus* in aqueous medium presented 1.55 mg/g of TFC, which corresponds to 25% of the TPC, compared to the methanolic medium, that corresponds to 31% of the TPC, that shows a higher level of flavonoids present when the medium is methanol. According to the literature, studies with this subspecies' extracts using the consumed part of the root presented percentages of around 15% of total flavonoids in proportion with the phenol content [99].

Table 12 - Total flavonoids from Daucus carota subsp. sativus roots, obtained by aqueous and methanolic extracts

Subspecies	Flavonoids (mg/L)	Dry mass (g/L)	Total Flavonoids (mg/g)
Sativus H_2O	14.21	9.2	1.55
sativus MeOH	27.07	25.2	1.07

H_2O – aqueous extract; MeOH – methanolic extract

3.1.4.3. Leaves (extract)

The leaves TFC (Table 13) was analyzed in three subspecies, *maritimus, hispidus and maximus*, all in methanolic medium. The results shown that *Daucus carota* subsp. *maritimus* presented the highest levels of flavonoids (13.07 mg/g), corresponding to 63.35% of the TPC, followed by *Daucus carota* subsp. *hispidus*, with 5.78 mg/g (31.62% TPC) and *Daucus carota* subsp. *maximus,* with 4.31 mg/g and 27.12% TPC. Similarly, to the TPC, there were no studies found using these subspecies leaves, Ksouri et at. 2014 reported a TFC for methanolic leaves extract from *Daucus carota* subsp. *carota* of 1.98 mg/g, a value below the ones reported by our subspecies, which might indicate that the subspecies *maritimus, hispidus* and *maximus* present higher levels of flavonoids in comparison with *Daucus carota* subsp. *carota* [100].

Table 13 - Total flavonoids from Daucus carota leaves obtained by methanolic extracts

Subspecies	Flavonoids (mg/L)	Dry mass (g/L)	Total Flavonoids (mg/g)
Maritimus MeOH	31.36	2.4	13.07
Hispidus MeOH	67.07	11.6	5.78
Maximus MeOH	12.07	2.8	4.31

MeOH – methanolic extract

3.1.5. Antioxidant Activity

The definition of an antioxidant is a molecule that is capable of slowing or preventing the oxidation of other molecules, thus, it is important to investigate the antioxidant activity of the EOs in order to understand if they present a good activity in preventing oxidation caused damages [101].

From the calibration curve, with an absorbance measured at a 517nm wavelength we obtained the following lines:

$$Abs = 0.0246x - 0.0052 \ (R^2 = 0.9996) \quad \text{Equation 11}$$

The table below (Table 14) presents the possible antioxidant effects possible, accordingly with the AAI, calculated by Eq. 11:

Table 14 - Antioxidant effects and respective values [102]

AAI	Antioxidant Effect
< 0,5	Weak
0,5 a 1	Moderated
1 a 2	Strong
> 2	Very Strong

For the umbels Antioxidant Effect in each subspecies were prepared samples with different concentrations of DPPH and sample in it. The concentration with the highest percentage inhibition (%I) provide us the curve that give us the IC50 value in order to proceed to calculus of the AAI [85].

The following results were obtained:

3.1.5.1. Umbels (extract)

For the umbels Antioxidant Effect (Table 15), the best DDPH concentration varies, being 0.08mM for *Daucus carota* subsp. *maritimus*, 0.04mM for *Daucus carota* subsp. *hispidus* and *Daucus carota* subsp. *sativus*, and 0.02 mM for *Daucus carota* subsp. *maximus* and *Daucus carota* subsp. *carota*. As to antioxidant effect of *Daucus carota* subsp. *maritimus*, it presented an AAI of 0.917, with an IC50 of 34.56 µg/mL, being the only umbel sample with moderate antioxidant effect. The other samples of *Daucus carota* subspecies presented weak antioxidant effect, with *Daucus carota* subsp. *hispidus* with the highest value, almost at moderate. Since no umbel extracts studies of AAI were found in literature, we can compare the IC50 of Daucus carota L. essential oil for aerial parts, by Mohammedi *et al.* 2015, being between 96.4 and 202.0 µg/mL [103]. These higher values of IC50 might indicate that the majority of the compounds that provide the antioxidant activity stay in the essential oil, being the extract that remains lower in terms of antioxidants.

Table 15 - Antioxidant activity from Daucus carota umbels obtained by aqueous extracts

Subspecies	[DPPH] mM	Concentration (µg/mL)	IC50 (µg/mL)	AAI
maritimus	0.08	31.68	34.56	0.917
hispidus	0.04	14.64	34.82	0.421
maximus	0.02	7.24	24.54	0.295
carota	0.02	7.20	25.79	0.279
sativus	0.04	13.95	36.47	0.383

3.1.5.2. Roots (extract)

For roots' extract antioxidant activity (Table 16), the concentration of DPPH in both aqueous and methanolic sample were 0.02 mM, though the antioxidant effect in both samples presented very different values, showing in this case that the methanolic extract presents higher antioxidant capacity. According to the literature, studies with this subspecies' extracts using the consumed part of the root presented percentages of DPPH Inhibition of 13%, unfortunately, the calculation method is not the same to compare the results [99].

Table 16 - Antioxidant activity from Daucus carota subsp. sativus roots, obtained by aqueous and methanolic extracts

Subspecies	[DPPH] mM	Concentration (µg/mL)	IC50 (µg/mL)	AAI
Sativus H_2O	0.02	5.293	76.67	0.069
Sativus MeOH	0.02	0.129	11.48	0.475

H_2O – aqueous extract; MeOH – methanolic extract

3.1.5.3. Leaves (extract)

For the leaf's extracts, as to the roots (Table 17), all DPPH concentration to the samples was 0.02 mM, and shown in *Daucus carota* subsp. *maritimus* and *Daucus carota* subsp. *hispidus* weak antioxidant effect, 0.463 and 0.375, respectively, and moderated effect for *Daucus carota* subsp. *maximus*. Previous studies reported IC50 for *Daucus carota* subsp. *carota* as 124.1 µg/mL and 83 µg/mL for leaf extract of *Daucus carota L.*, showing that our samples presented lower inhibition concentrations of 50% compared to the studies, and for thar reason, better activity [98,103].

Table 17 - Antioxidant activity from Daucus carota leaves obtained by methanolic extracts

Subspecies	[DPPH] mM	Concentration (µg/mL)	IC50 (µg/mL)	AAI
maritimus MeOH	0.02	4.642	10.02	0.463
Hispidus MeOH	0.02	6.675	17.81	0.375
Maximus MeOH	0.02	5.374	7.21	0.746

MeOH – methanolic extract

3.2. *Aloysia citrodora* and *Cymbopogon citratus*

3.2.1. Essential Oils Chemical composition

For the essential oil composition, where found that *Aloysia citrodora* and *Cymbopogon citratus* present the percentages showed on the table 18. The main compound, with percentage above 5%, found in *Aloysia citrodora* were geranial (21.2%), followed by neral (16.5%), limonene (9.9%) and ar-curcumene (6.0%). For *Cymbopogon citratus*, the main

compounds were geranial (36.3%), neral (26.1%) and β-myrcene (17.1%). The main constituents present to be the same as the ones reported from previous studies, as presented in tables 2 and 4 of the introduction.

Table 18- Chemical composition of the essential oils from Aloysia citrodora and Cymbopogon citratus

Compounds	RI*	*% Aloysia citrodora*	*% Cymbopogon citratus*
a-thujene	929	0.1	-
α-pinene	936	0.6	-
Camphene	950	tr	-
Sabinene	973	1.5	-
β-pinene	978	0.1	-
Oct-1-en-3-ol	980	tr	-
6-methylhept-5-en-2-one	986	0.6	0.6
β-myrcene	989	0.2	17.1
2,3-dehydro-1,8-cineole	990	-	tr
3-hexen-1-ol, acetate, (Z)	1004	tr	-
p-cymene	1024	0.1	-
Limonene	1030	9.9	0.3
1,8-Cineole	1032	4.0	0.1
β-cis-Ocimene	1038	0.2	0.4
β-trans-Ocimene	1048	1.3	0.3
γ-Terpinene	1060	tr	-
cis-sabinene hydrate	1067	0.3	-
α-terpinolene	1087	tr	-
Linalool	1099	1.4	1.0
trans-p-mentha-2,8-dienol	1128	0.1	-
cis-limonene oxide	1134	0.1	-
Camphor	1143	0.7	-
Citronellal	1154	0.2	0.3
Borneol	1166	0.4	-
Terpinen-4-ol	1177	0.4	tr
p-cymen-8-ol	1184	1.4	-
α-terpineol	1190	1.0	-
trans-carveol	1217	0.1	-
Citronellol	1228	-	0.4
Nerol	1229	1.5	-
Neral	1242	16.5	26.1
Geraniol	1255	1.4	2.3
Linalool acetate	1255	-	2.3
Geranial	1270	21.2	36.3
Lavandulyl acetate	1289	0.1	-
2-undecanone	1293	0.1	0.2
Myrtenyl acetate	1329	0.3	0.1
α-copaene	1376	0.6	0.1
Geranyl acetate	1380	1.6	0.9
β-bourbonene	1384	0.5	0.1

Methyl eugenol	1402	0.1	-
α-cedrene	1412	0.5	0.1
trans-caryophylene	1420	4.4	1.1
Trans-α-bergamotene	1435	-	0.1
α-Humulene	1453	0.6	0.1
Allo-aromadendrene	1460	0.6	0.1
Geranyl propanoate	1477	0.3	tr
ar-curcumene	1482	6.0	1.2
Bicyclogermacrene	1494	0.4	0.3
β-bisabolene	1508	0.2	tr
γ-cadinene	1513	0.6	-
δ-cadinene	1523	0.2	-
(E)-nerodiol	1561	0.7	0.1
Spathunelol	1576	2.0	0.2
Caryophylene oxide	1581	3.0	0.1

*RI – Retention Index on DB-5-MS; tr – traces of less than 0.05%

3.2.2. Total phenols

From the calibration curve, with an absorbance measured at 760nm, we obtain the following lines and respective concentration values for the simulant mediums, water, ethanol 10%, ethanol 50% and ethanol 95%, respectively. in mgEGA/L:

$$Abs = 0.0132x - 0.0029 \ (R^2 = 0.9997) \ \text{Equation 12}$$
$$Abs = 0.0215x - 0.0319 \ (R^2 = 0.996) \ \text{Equation 13}$$
$$Abs = 0.02x - 0.0198 \ (R^2 = 0.9981) \ \text{Equation 14}$$
$$Abs = 0.0151x - 0.0028 \ (R^2 = 0.9991) \ \text{Equation 15}$$

Table 19 - Total Phenols from the essential oil (mg EGA/L)

Sample	Concentration + SD (mg/L)			
	Water	**Ethanol 10%**	**Ethanol 50%**	**Ethanol 95%**
ACEO	216 ± 5	255 ± 34	288 ± 59	-
CCEO	109 ± 18	110 ± 6	133 ± 5	268 ± 11

As it can be observed in table 19, ACEO presented increased concentrations of gallic acid equivalents from the most polar solution (H2O) to the one with lower polarity (ethanol 95%). The ethanol 95% medium in the ACEO presented high levels of turbidity, making the results obtained not viable for the calculation of equivalents. As to the CCEO results, the medium with higher levels of EGA was ethanol 95%, with 268 mgEGA/L, for more than 50% in comparison with the ethanol 50%, the lower was water with 109 mgEGA/L . The analyses of these results might indicate that in more alcoholic extracts with less polarity, there is a higher concentration of phenols, as in concordance with the literature, since increasing the water content in a solvent cause the decrease of the total phenolic content[104]. According to

the literature, have been reported phenolic content of AC of 342.9 mgEGA/g extract [105], but seems to be no register of the essential oils analysis in the simulating mediums, like presented in this study. Referring to CCEO, according to the literature there have been studies reporting phenolic content of 149.2 mgEGA/100mL EO and 1846.92 mgEGA/L EO leaves [106,107]. The results presented in this study were lower compared to the ones presented by the literature, which might be due to the solvent used for the extractions.

3.2.3. Antioxidant Activity

From the calibration curve, with an absorbance measured at 517nm, we obtain the following lines and respective percentage inhibition values for the simulant mediums, water, ethanol 10%, ethanol 50% and ethanol 95%, respectively:

$$Abs = 2740.3x - 1.021 \ (R^2 = 0.9996) \quad \text{Equation 16}$$

$$Abs = 2589.3x - 5.5049 \ (R^2 = 0.9855) \quad \text{Equation 17}$$

$$Abs = 2871.9x - 1.5353 \ (R^2 = 0.9641) \quad \text{Equation 18}$$

$$Abs = 5056.9x - 2.7936 \ (R^2 = 0.9893) \quad \text{Equation 19}$$

As observed at the table 20, ACEO presented high percentages of inhibition for all the simulant mediums, being at 1:20 dilution 77% for water, 78% for ethanol 10% and, 78%, ethanol 50%. CCEO presented higher values compared to the previous oil. The percentage of inhibition for the 1:20 dilution, presented values from 83 to 89% among the simulant mediums, being ethanol 95% the highest and water the lowest.

Table 20 - Antioxidant Activity (%Inhibition) from the essential oils in oils extracted by different solutions in a proportion 1:20. Results presented are relative to original EO

Sample	Inhibition Percentage			
	Water	Ethanol 10%	Ethanol 50%	Ethanol 95%
ACEO	77 ± 12	78 ± 1	77 ± 0.4	60 ± 3
CCEO	83 ± 2	85 ± 15	87 ± 7	89 ± 5

According to the literature, Ali et. al 2011 reported a % of inhibition of ACEO of 22.1% and 34.2% for 1:200 and 1:100 dilutions, respectively [64]. Hashemi et. al 2018 reported AC scavenging activity up to 80%, depending on the concentration and extraction method used [52]. As to antioxidant activity of CCEO, Vázquez-Briones et. al 2015 reported percentages of inhibition of 55.57% and Mirghani et. al 2012, values up to 89% inhibition at ratio 1:2 [106,107].

3.2.4. Migration Assay

Migration assays should follow legal proceedings that are stablished by the country where they are performed, in order to protect the consumer safety. Accordingly with this this work proceeded in 4 mediums, each of them representing a food simulant, water for aqueous foods, 10% ethanol for alcoholic beverages and foods with hydrophilic properties, 50% ethanol for foods with alcoholic content above 20% and that present lipophilic properties, and finally, 95% ethanol for fatty foods [91,108].

For the migration assay, were analyzed the TPC and Antioxidant Activity, with the following results:

3.2.4.1. Total phenols

With the TPC of the migration assay, we were able to identify the quantity of phenolic content that was capable to migrate from the film to the medium. The results presented some irregularities, due to the chitosan low water resistance and hydrophilic nature [93].

Results in Figure 13 shown that ACEO films presented more difficulty in the aqueous and ethanol 10% mediums to migrate without the film being dissolved in the medium, as it can be observed by the lack of data in the figure, nonetheless, presented very significant levels of phenolic migration for ethanol 50% (around 4 to 9 mgEGA/L), in a slow grade of migration, but even higher for ethanol 95% (from 8 to 23 mgEGA/L), with high phenolic liberation in the first 8 hours, presenting degradation afterwards, as observed by the decreased levels of the phenols. It can be observed a higher affinity to the mediums with higher levels of ethanol.

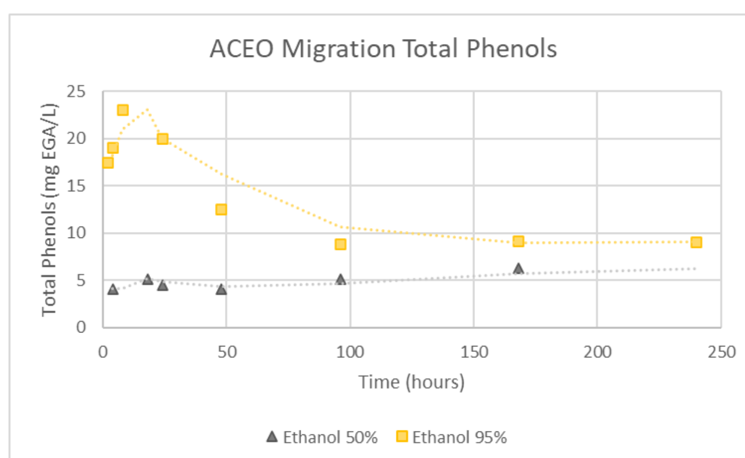

Figure 13 - ACEO Total Phenolic content migration

CCEO, Figure 14, presented a more consistent migration throughout the mediums, even at water and ethanol 10% with low degradation of the film. This oil shown a slow and consistent migration throughout the trial. In this case, there is no significant degradation of the phenols, being consistent the levels of phenolic content over time.

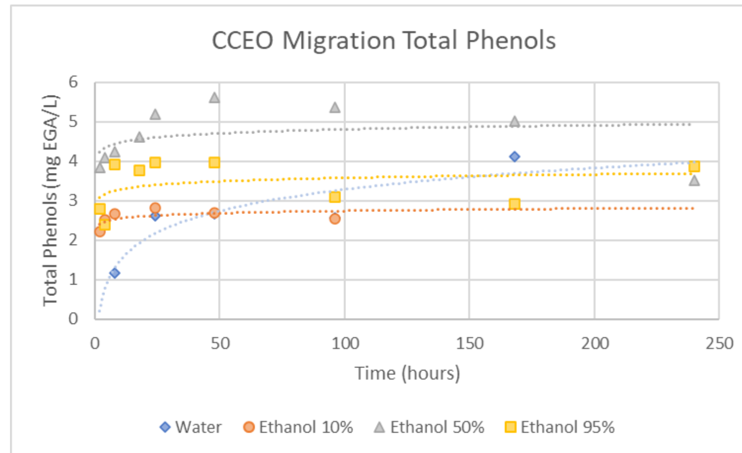

Figure 14 - CCEO Total Phenolic content migration

These results indicate a possibility of addition of the ACEO for more alcoholic and fatty foods, namely, more hydrophilic foods. For the CCEO, it might be a useful antioxidant to use in more hydrophobic foods, since it presented consistent results of migration throughout the trial, with little dissolution of the film. The interaction between the EOs and the chitosan polymer is important in order for the phenols present in the film can also protect it against oxidation, but also improve the barrier properties of the film, retarding the contact of the food in the package with water, oxygen and light, as a shield to protect the product [90].

3.2.4.2. Antioxidant Activity

The antioxidant activity study of the migration assay allows to identify the capacity of the migrated compounds to the medium to inhibit the oxidation procedure. Results presented values below 20% inhibition in all the sample trials.

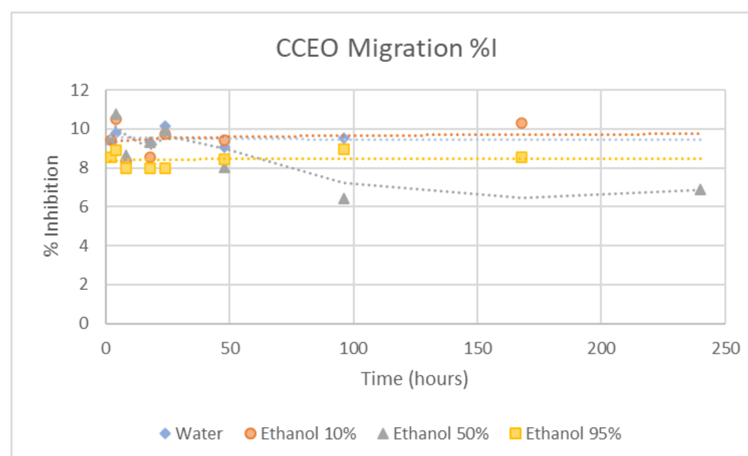

Figure 15- CCEO %Inhibition migration

Starting with the CCEO film, Figure 15, it presented around 8 to 10% percentage of inhibition over the hours, in all the simulant mediums, with the exception of the ethanol 50%, due to the degradation of the phenolic content, causing a decrease also in the inhibition percentage. The higher inhibition percentage was around the 8 hours for all the simulant mediums, being the ethanol 50% the one with highest maximum pick, at 8h, followed by ethanol 10%.

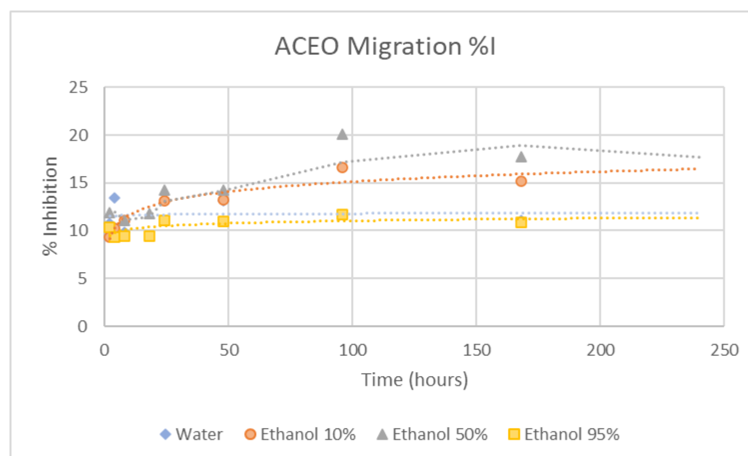

Figure 16 - ACEO %Inhibition migration

As to the ACEO, Figure 16, the results presented some more percentage of inhibition, going from 9 to values up to 20%. In this oil, the highest percentage of inhibition occurred later for the ethanol 10% and 50%, being around 96 hours the maximum, showing a slower liberation compared to the CCEO.

These results indicate that CCEO and ACEO present an inhibition percentage that can be helpful to the package material since it provided consistent inhibition percentages to the films.

The antioxidant activity present in the EO films may be attributed to the presence of the phenolic compounds and terpenoids, that demonstrate their redox properties by different mechanisms, such as hydrogen donation, free radical scavenging activity, among others [109].

3.2.5. Contact Assay

3.2.5.1. Moisture content

The moisture present in the food can influence both the sensorial quality and the stability of the product, since the deterioration process is related to the content of water present [93]. The results (Table 21) show that the unwrapped meat (without film) presented an increasing level of moisture along the days, increasing by around 3% in the 13 storage days, however, all the meat wrapped in films presented a decrease in the moisture. Since chitosan presents hydrophilic properties, it is possible that the film absorbed some of the meat water, explaining the decrease [110]. Comparing the Ch film with the EOs ones, it is observed that ACEO film present a similar decrease in terms of moisture, in contrast to CCEO film, that presented a lower degrease in terms of moisture, being in between the moisture achieved at the 13th by the unwrapped sample and the samples wrapped in chitosan and chitosan + ACEO. The behavior of CCEO can be explained by a higher hydrophobicity character given to the chitosan film, that reduced its water absorption, but that should be confirmed with physical tests on the films, such as swelling and water permeability [29].

Previous studies reported that the film water absorption can increase the thickness of the meat, which can alter the experience of the consumer in terms of taste and texture, however, the reduction of the water content is helpful to delay microbial growth [111].

Table 21 - Contact Assay: Moisture content of meat (%)

Time (days)	Unwrapped	Ch	CCEO	ACEO
0	73.9 ± 0.1	73.9 ± 0.1	73.9 ± 0.1	73.9 ± 0.1
3	75.3 ± 0.8	71.0 ± 0.2	72.6 ± 0.6	70.0 ± 3.6
6	75.2 ± 0.7	71.3 ± 0.6	71.0 ± 0.3	69.7 ± 0.1
9	75.9 ± 0.9	68.9 ± 0.7	69.4 ± 0.9	67.1 ± 3.8
13	76.6 ± 0.9	67.7 ± 0.7	70.4 ± 0.7	67.1 ± 0.7

3.2.5.2. Color

Since one of the first things the consumer notices when searching for food is their appearance, it is important to find a package that maintain the common color of the product. In this case, the “reddish”/pink and bright color of the meat is and indicator that it is fresh and one of the factors that can cause the consumer to exclude other pieces if not resemblant to this [112]. The color of the poultry meat depends on numerous factors, for instance, age, gender, genetics, diet, intramuscular fat, moisture, among other [113].

The initial Hue angle from the meat without film was 40 and increased to 48 by the end of the 13 days (Table 22). The wrapped meat with EOs films were the ones that presented a higher Hue angle change a long time, to a more yellowish tone, especially ACEO. Meat with EOs also presented a higher Hue angle than chitosan film, that maintained a similar variation with the unwrapped meat. Contrarily to what has been shown by other essential oils studies, for example, ginger essential oil, by Souza et. al 2018, the Hue angle of the unwrapped meat (70º) was 15º higher than the wrapped meat with EO [29], or by Pires et. al 2018, that rosemary EO also presented 15º lower values for the wrapped meat (69º), as well as 10º lower for ginger EO [93]. This results indicate that the Hue angle of the unwrapped meat was lower in our study than the ones reported by the literature, but also that the EO provided an increase of the Hue values, nonetheless, this color variation might be due to the natural color of the essential oil that migrated to the meat [113]. In terms of the variation in color (table 23), to the unwrapped meat at day 0, there is an increase with time, more pronounced with chitosan and CCEO. In this case, the changes in color of the meat, due to ACEO, and compared with unwrapped meat at day 0 were less pronounced.

Table 22 - Contact Assay: Hue Angle of the meat

Time (days)	Unwrapped	Ch	CCEO	ACEO
0	40 ± 5	40 ± 5	40 ± 5	40 ± 5
3	46 ± 3	46 ± 1	46 ± 4	48 ± 1
6	41 ± 6	46 ± 1	49 ± 2	52 ± 0
9	47 ± 3	49 ± 2	52 ± 1	61 ± 1
13	48 ± 2	50 ± 1	53 ± 6	61 ± 0

Table 23 - Contact Assay: Color variation (ΔE) of the meat

Time (days)	Unwrapped	Ch	CCEO	ACEO
3	2.10 ± 1.49	7.54 ± 1.68	8.55 ± 2.25	5.58 ± 0.15
6	5.42 ± 2.27	7.74 ± 3.04	9.19 ± 1.86	6.47 ± 0.13
9	4.52 ± 0.54	7.92 ± 0.77	9.53 ± 1.19	5.84 ± 0.63
13	4.77 ± 0.83	7.87 ± 2.02	8.92 ± 1.05	6.18 ± 1.23

3.2.5.3. Basic Volatile Nitrogen (BVN)

One of the ways to identify the stage of degradation of a product is by their spoilage mechanisms, one of them being the degradation of proteins and other compounds that present nitrogen (N) in it. This mechanism causes the accumulation of organic amines that can be identified by the total volatile basic nitrogen. Basic volatile nitrogen will cause not only changes in color and flavor, but can also be toxic in elevated quantities, affecting the safety of the product [114].

The unwrapped meat presented the highest increase of BVN that went from 2.1 to 14.7 g of N per 100g of meat (Figure 17). Contrarily to this, all the wrapped meat presented similar nitrogen values to the initial ones, probably significant in terms of slower times of the degradation of the proteins and amines, increasing the shelf life. In a way similar to what occurred with our samples, Rezaeifar et. al 2020, presented results with a significant increase in the control with no film, being the wrapped in chitosan and with ACEO levels of BVN lower by around 80% in these samples, reporting a possibility of the phenolic compounds decreasing the growth of microorganisms and consequently preventing the spoilage and breaking down of the proteins [115]. For CCEO, the evolution of BNV presented by Hosseinzadeh et. al 2020 were similar to ours, for 0.5% and 1.5% CCEO films, but BNV data were smaller than the ones from this study because it was a study with minced meat [116].

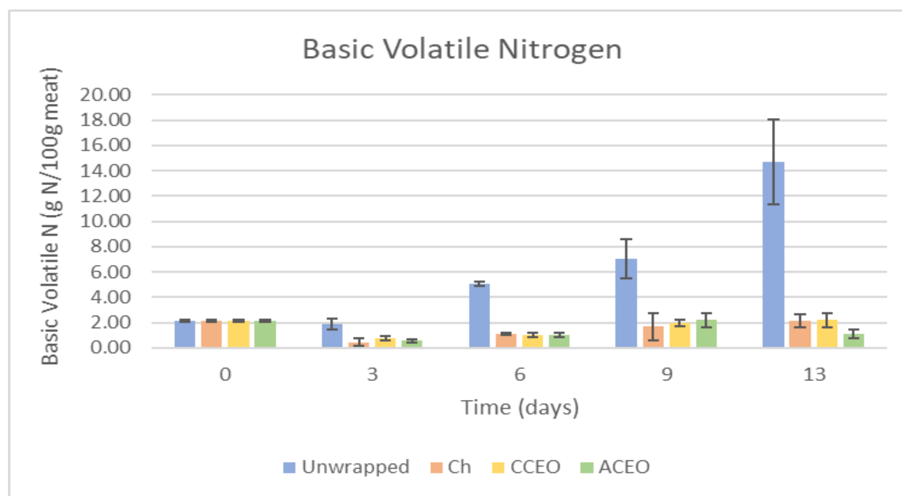

Figure 17- Contact Assay: Basic Volatile Nitrogen values (g N/100g meat)

3.2.5.4. pH

The pH value regulates the reactions that follow the food production and deterioration process, this way, it is relevant to keep track of this value in order to maintain a safe and well-preserved product. The muscle pH after the animal is slaughtered, and converted into meat, can drop from around 7.2 to values around 5, since the glycogen is converted to lactic acid. pH values normally range from 5 to 7, being in between 5.7 to 6 for higher quality products [93,117].

The initial pH value for the sample meat was 5.95, achieving a 7.51 pH at the 13th day wrapped (Table 24). The deterioration of the meat increased the pH, as expected. The increase in pH values may be attributed to the growth of microorganisms that produce volatile basic components [29]. Different results were presented by the wrapped meat, that maintained similar values to the initial day, being the CCEO wrapped the one with more stable results.

Previous study by Rezaeifar et. al 2020, reported using 1% Aloysia citrodora EO (Lúcia Lima) in edible coatings for trout presented pH values similar to the control chitosan, although slightly more acidic, just like our results shown [115]. But in this study, the application of ACEO also reduced a bit the pH of the meat, which was attributed to the migration of the oil to the meat. As to coatings with sodium alginate and 0.2% and 0.5% ACEO in chicken breast, presented by Hosseini et. al 2021, reported no significant difference in comparison with the chitosan control [118]. As to Cymbopogon citratus (Erva Principe) EO, Hosseinzadeh et. al 2020, reported that minced meat wrapped in films with 0.5% and 1.5% presented a pH reduction of 0.17 and 0.22 respectively, compared to the chitosan control [116]. A similar reduction in pH was also observed in this study. Which was also attributed to the migration of the EO to the meat. Wrapping the meat in pristine chitosan also maintained the pH, and there was a small reduction in pH along the time, which can be attributed to the chelation of some free fatty acids released from the meat to the chitosan (to the amine groups). Therefore, the maintenance of the pH indicates the efficiency of the films in the extension of the shelf life of the products.

Table 24 - Contact Assay: pH values of meat

Time (days)	Unwrapped	Ch	CCEO	ACEO
0	5.95 ± 0.04	5.95 ± 0.04	5.95 ± 0.04	5.95 ± 0.04
3	6.30 ± 0.06	6.26 ± 0.01	6.40 ± 0.06	6.33 ± 0.02
6	6.78 ± 0.19	5.63 ± 0.01	5.78 ± 0.01	5.51 ± 0.07
9	6.73 ± 0.35	5.49 ± 0.04	5.77 ± 0.01	5.44 ± 0.03
13	7.51 ± 0.16	5.58 ± 0.04	5.72 ± 0.01	5.39 ± 0.01

3.2.5.5. Titratable acidity

Titratable acidity measures the total acid concentration present in food, and is determined by titration of intrinsic acids with a standard base and can be used as a complement to the pH analysis since it is a better indicator of the microbiological stability of certain foods [93,119].

The unwrapped meat presented the highest decrease of oleic acid equivalents, which is in accordance with the pH values observed, since they became less acidic (Table 25). The wrapped meat presented a smaller increase of value, in accordance with the pH values also.

Table 25 - Contact Assay: Total Titratable Acidity (g oleic acid/100g meat)

Time (days)	Unwrapped	Ch	CCEO	ACEO
0	1.06 ± 0.07	1.06 ± 0.07	1.06 ± 0.07	1.06 ± 0.07
3	1.58 ± 0.16	0.98 ± 0.10	0.84 ± 0.17	0.89 ± 0.23
6	0.62 ± 0.01	0.83 ± 0.15	0.91 ± 0.16	0.87 ± 0.06
9	0.82 ± 0.59	0.90 ± 0.16	1.05 ± 0.21	1.11 ± 0.12
13	0.38 ± 0.07	1.40 ± 0.07	1.13 ± 0.25	1.39 ± 0.08

3.2.5.6. Lipidic Oxidation (TBARS Index)

The TBARS assay is used to quantify the malonaldehyde (MDA) present in the sample and indicates the lipid rancidity in meat products [93].

In terms of results, in all the samples an increase of the malonaldehyde concentration was observed, being the highest at the unwrapped meat, but also, very similar to the meat wrapped with pristine chitosan to day 9 (Figure 18). Until day 9, the application of EOs to the film prevented the lipidic oxidation of the meat, once it can be seen the lower values, either in CCEO and ACEO. CCEO wrapped presented a slightly lower MDA concentration, being the lowest the sample wrapped with film without EOs, at day 13. Rezaeifar et. al 2020, with the edible coating of trout and its chitosan control, shown that ACEO presented a good effect on the antioxidant activity of the coating, reducing the TBARS value from 2.78 to 1.67 mg MDA/Kg meat [115]. Hosseini et. al 2021, also shown effective results from the application of ACEO to coating, due to the presence of geranial, neral and limonene as antioxidants [118]. Olorunsanya et. al 2010 reported that, in various concentrations of CCEO (0.5, 1 and 1.5% in films), it always shown an effective improvement of the lipidic stability during storage [120]. In this study, both essential oils were effective until day 9, but at day 13, the difference to chitosan was neglectable, probably due to the oxidation of the EO's their selves.

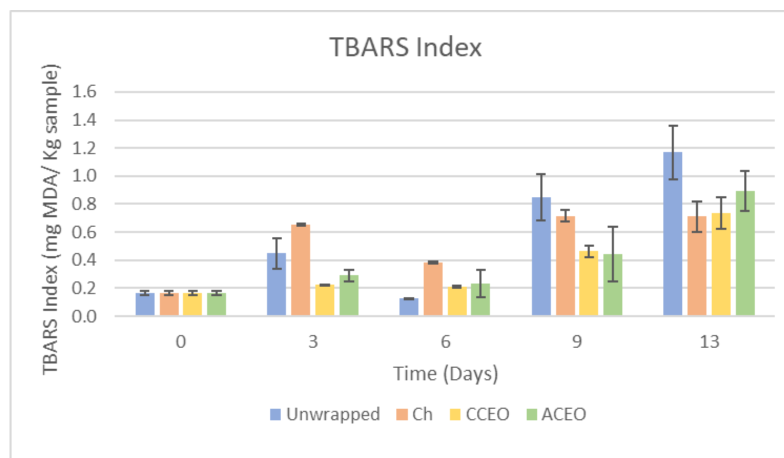

Figure 18 - Contact Assay: TBARS Index values (mg MDA/Kg meat)

3.2.5.7. Microbial Growth

The use of refrigeration been long known to present unfavorable effect to bacteria. This common way of storage of food is at low temperatures, due to the delay of the of alterations in terms of development and spoilage of bacteria. Since meat is considered one of the most perishable foods, refrigeration is used for storage after slaughter, distribution and then at retail [121,122]. Spoilage of meat is associated with microbial growth and as well as biochemical and enzymatic deterioration [93]. In this assay were analyzed psychotrophic microorganisms, that present growth as temperatures around 7ºC or less and present both gram-positive

(lactic acid bacteria) and gram-negative bacteria (*Pseudomonas spp.*, *Enterobacteriaceae*) [93,122], but also mesophilic microorganisms, with optimum growth between 30-39ºC, which are the group of some pathogens like *Streptococcus aureus*, *Salmonella spp.* and *Escherichia coli* [123].

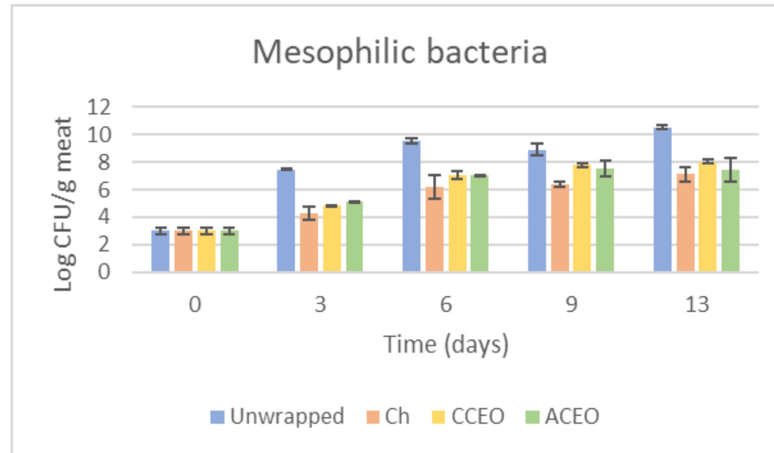

Figure 20 - Contact Assay: Mesophilic bacteria

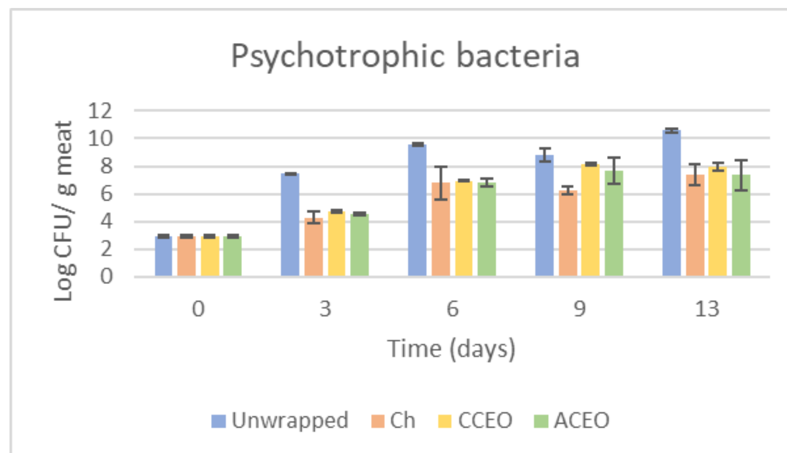

Figure 19 - Contact Assay: Psychotrophic bacteria

In terms of the mesophilic microorganisms (Figure 19) and and psychotrophic microrganisms (Figure 20), results show that both mesophilic and psychotrophic microorganisms presented a growth along the 13 days, and that in the wrapped meat the growth was slower compared to the unwrapped meat, as expected. All the wrapped samples presented throughout the 13 days similar behavior, with no differences among films with and without EOs. This reduced growth of microorganisms wrapped in chitosan is attributed to the intrinsic antimicrobial activity of the chitosan [29].

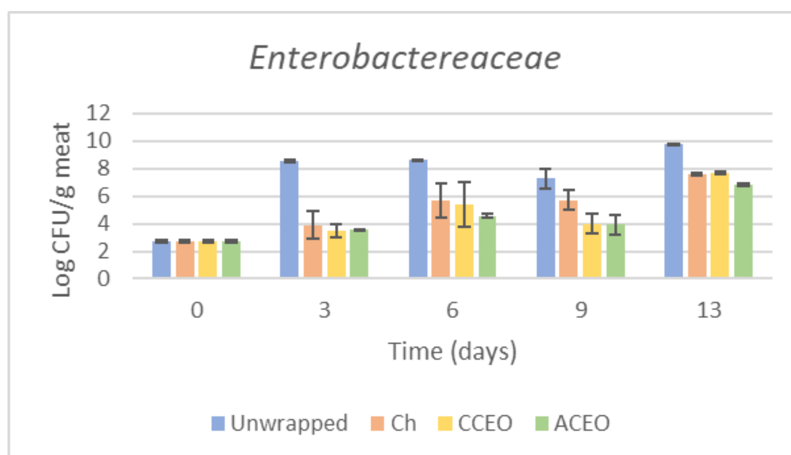

Figure 21 - Contact Assay: Enterobactereaceae

In terms of *Enterobacteriaceae*, the behavior presented a small variation, showing at the 13th day that ACEO presented lower growth values (6.8 log (CFU/g)) compared to the chitosan film and the CCEO film, that presented 7.6 and 7.7 log (CFU/g), respectively (Figure 21). Indeed, there was a trend that ACEO presented lower microbial growth then CCEO and pristine chitosan although not statistically significant.

In general, all the wrappers were effective in controlling the microbial growth of the analyzed microorganisms, showing capacity to increase the shelf life. Previous investigations also reported similar behavior to this essential oils as seen in this study, reporting around 6 log(CFU/g) for mesophilic bacteria in poultry wrapped with ACEO, near 7 log(CFU/g) for psychotrophic and around 4 log(CFU/g) for *Enterobacteriaceae*, although not being possible to compare to a chitosan film without oil [118]. Trout samples wrapped with edible ACEO presented levels of 4.56 log (CFU/g) compared to a control chitosan (6.19 log (CFU/g)) at 12 days of storage for the total volatile countable microorganisms, and 4.89 log(CFU/g) for *Enterobacteriaceae* with ACEO, being this 1.3 lower than the chitosan control [115]. CCEO in minced meat presented values for total bacteria a little below 8 log(CFU/g) for 0.5 and 1.5% EO, compared to chitosan and unwrapped meat, that almost reached 9 log(CFU/g), psychotrophic presented similar values for the EO, but with chitosan and unwrapped around 8.5 log(CFU/g), followed by *Enterobacteriaceae* with 8.5 to 9.5 log(CFU/g) difference between them [116].

Chapter 4
Conclusions

Chapter 4 – Conclusions

4. Conclusions

4.1. *Daucus carota*

From this study it was able to understand that *Daucus carota* from Portugal present a variety in terms of essential oils chemical composition among the subspecies, being the most prominent compound Geranyl acetate in three of the subspecies, *maritimus, hispidus* and *carota*, but also *cis*-asarone for *Daucus carota* subsp. *maximus* and carotol and daucol for *Daucus carota* subsp. *sativus*. For future research, in order to enrich the study of these oils it is necessary to use higher amounts of plant mass, in order to obtained higher yields for possible evaluation of antioxidant properties and phenol content.

As to the *Daucus carota* extracts, it was possible to understand that the umbels were the part of the plant that presented the highest phenol content, as well as flavonoid content, although with weak to moderate antioxidant effect, most likely due to the method used, hydrodistillation, capture the majority of the volatile compounds into the essential oils. Comparing the roots from *Daucus carota* subsp. *sativus* in two different mediums, aqueous and methanolic, was possible to identify a higher content of phenols in the aqueous sample, however, the methanolic one presenting higher levels of flavonoids as well as better antioxidant activity, which might indicate a correlation between the flavonoid content and the antioxidant effect. Finally, in *Daucus carota* leaves, the phenol content between the subspecies *maritimus, hispidus* and *maximus* were relatively similar, despite the variation in terms of TFC, since subspecies *maritimus* presented more than half of the phenolic content in flavonoids. Curiously, the subspecies *maximus*, that presented lower phenol content, was the one with the highest antioxidant activity.

For future perspectives with this species, it would be very interesting to understand better the content variation between these plants oils and how they could be applied in order to embrace their properties.

4.2. *Aloysia citrodora* and *Cymbopogon citratus*

From this study it was possible to identify that the compounds from the essential oils of *Aloysia citrodora* and *Cymbopogon citratus* from Portugal are very similar to the ones previously reported from other sources, being for both geranial and neral the main compounds, followed by limonene for *Aloysia citrodora* and β-myrcene for *Cymbopogon citratus*. In terms of phenolic content and antioxidant activity, both oils shown some activity, being the ethanolic mediums the ones with more emphasis. As to the contact assay of the films with the essential oils, this have shown that they can be helpful in terms of

slowing down the deterioration speed of the poultry meat, since presenting decreased levels of the pH values and reduction of the deterioration and microbial growth process. In the first days of the storage, until 9th day, the EOs helped to preserve the lipidic oxidation. In terms of the microbial growth, although differences were not significant, ACEO helped to reduce the *Enterobactereacea* growth in the poultry meat, better than CCEO. The wrappers, in comparison with the unwrapped meat, provided a decrease in the moisture content, turning the meat a little more dry and making it less conditional for bacterial growth. The films with EOs provided a more yellowish color, probably due to the components present in the oils. Wrapping the poultry meat with the films also helped to maintain the pH levels and it was observed a decrease in the nitrogen levels by 62% compared to the unwrapped meat. The wrapping with chitosan also helped to lower lipidic oxidation and the microbial growth. The study provided the information that fresh poultry meat protected by bioplastics can improve the shelf-life time, and the incorporation of the essential oils facilitated the retard of the oxidation process, compared with chitosan + CNC control.

For future research it would be interesting to repeat the previous tests at some other essential oils concentrations, to see if there is any variation of results, but also to test the antimicrobial activity of these oils against specific pathogens and make more trials in order to adapt their use to be applied in the industry. Also, testing the EOs in composites but as edible packaging to other food matrices would be also interesting to study.

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