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CARACTERIZAÇÃO E AVALIAÇÃO DAS PROPRIEDADES DOS ÓLEOS ESSENCIAIS DE *THYMUS CAESPITITIUS* BROT.; *THYMBRA CAPITATA* (L.) CAV.; *MYRTUS COMMUNIS* L. EM PREPARAÇÕES TÓPICAS

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Abstract

Today, consumers are looking for effective, safe and natural products that contribute to your health, wellness and beauty. As a result, the consumer created the need to develop new cosmetic products formulated with natural ingredients such as essential oils. These are complex mixtures of volatile, odoriferous and lipophilic secondary metabolites which are biosynthesized in specialized cells (secretory cells, epidermal cells, glandular trichomes), mainly present in aromatic plants. As natural ingredients, essential oils are a growing market trend for perfumery and cosmetic. In addition to the pleasant odor, they can also be important preservatives due to the strong antimicrobial activity. However, the essential oils are rather volatile and some of its constituents may cause skin sensitivity, for example, contact dermatitis and photoallergy. Thus, Annex III of Regulation No 1223/2009, provides a list of substances which cosmetic products must not contain out of the laid down restrictions.

Preservatives are added to cosmetic products so that they have an extended shelf-life, preventing the development of microorganism that cause diseases to consumers or harm the appearance of the product. However, legislation concerning chemical preservatives has undergone changes and updates as a result of the knowledge of sensitivity and toxicity problems inherent to these products.

This work aims at highlighting the potential of essential oils, with antimicrobial activity, isolated from native plants in Portugal: *Thymus caespititius* Brot., *Thymbra capitata* (L.) Cav., e *Myrtus communis* L., by including them in topical formulations, in order to assess their capacity as preservatives.

The three essential oils, obtained from *Th. caespititius*, *T. capitata* and *M. communis* were isolated from the aerial parts of different flowering plants by hydrodistillation, analyzed by gas chromatography for components quantification and by gas chromatography-mass spectrometry, for components identification.

All essential oils showed faint yellow colours. The isolated essential oils were complex mixtures, in which 64 constituents for *Th. caespititius*, 30 in *T. capitata* and 34 in *M. communis* were identified. Monoterpenes were the dominant class of compounds (76%, 97% and 91 %, respectively), whereas sesquiterpenes ranged between 2 and 16%, and phenylpropanoids from traces to 3%. α-Terpineol was the dominant compound *Th. caespititius* essential oil, while carvacrol dominated the *T. capitata*

essential oil. 1,8-Cineole was the major compound present in the essential oil of *M. communis*.

Based on chemical characterization of the three essential oils, including constituents considered as allergens (linalool, limonene, methyl eugenol, citronellol, geraniol and eugenol) present in Regulation 1223/2009, Annex III, two essential oils (*Thymus caespititius* and *Thymbra capitata*) out of three were chosen to be incorporated at 1% as bioactive ingredients in topical formulations and the three essential oils (*Thymus caespititius, Thymbra capitata* and *Myrtus communis*) were chosen to be incorporated at 0.5% in topical formulation, to be assessed for physical-chemical parameters: pH, viscosity, phase separation and particle size. As healthy skin pH is of approximately 5, and topical application to healthy skin products presenting pH of 4-6, the pH values for the emulsions of essential oil were within limits. Concerning viscosity, the addition of essential oils greatly increases the breakage resistance of the structure when compared to placebo. These results were in accordance with the analysis of particle size, since the incorporation of essential oils either 1 or 0.5% decreases the particle size. All emulsions were stable.

Antimicrobial activity was evaluated by the method of Kirby-Bauer and by the method of microdilution plate determining the minimum inhibitory concentration and minimum bactericidal concentrations for the three essential oils with or without dilution in DMSO and, for two controls: Dermosoft® OMP and benzyl alcohol. *Pseudomonas aeruginosa* ATCC 9027 was extremely susceptible to the essential oil of *T. capitata* without dilution in DMSO; *Staphylococcus aureus* ATCC 6538 showed greater susceptibility to essential oils *Th. caespititius* and *T. capitata* and, lower susceptibility to *M. communis* essential oil diluted in DMSO (1: 100). The essential oils of *Th. caespititius* and *T. capitata* were effective against fungi, showing better antifungal activity than Dermosoft® OMP for *Candida albicans* ATCC 10231. Three essential oils (*Th. caespititius*, *T. capitata* e *M. communis*) showed better activity against Gram-positive than Gram-negative bacteria, and these results are in accordance with previous studies. All essential oils showed better results than the two controls. In summary, *Th. caespititius*, *T. capitata* and *M. communis* essential oils were effective against fungi, Gram-positive and Gram-negative bacteria.

Regarding the challenge test, emulsions with 1% *T. caespititius* essential oil and *T. capitata* essential oil and emulsion with 0.5% *M. communis* essential oil are in accordance with the acceptance criteria A (2 Log_{10} reduction) for the fungi (*Candida albicans* ATCC 10231 and *Aspergillus brasiliensis*

ATCC 16404) as well as the bacteria (ATCCC 9027 *Pseudomonas aeruginosa* and *Staphylococcus aureus* ATCC 6538).

Th. caespititius and *T. capitata* essential oils showed potential as alternative preservatives for use in topical formulations. Although the essential oil emulsion with *M. communis* is in accordance with criteria acceptance of the challenge test (FP 9), the percentage of allergen: methyl eugenol exceeds in percentage used (0.5% essential oil) the maximum concentration permitted by law (Regulation nº 1223/2009, Annex III).

Keywords: essential oil, activity-antimicrobial, preservatives, emulsions.

Resumo

Hoje em dia, os consumidores procuram produtos eficazes, seguros e naturais que contribuam para a sua saúde, bem-estar e beleza. Como resultado, o consumidor criou a necessidade de desenvolver novos produtos cosméticos formulados com ingredientes naturais, tais como os óleos essenciais. Estes são misturas complexas de metabolitos secundários voláteis, lipófilos e odoríferos biosintetizados em estruturas especializadas (células secretoras, células epidérmicas, tricomas glandulares), presentes maioritariamente nas plantas aromáticas. Os óleos essenciais afirmam-se cada vez mais como uma tendência crescente no mercado da perfumaria e da cosmética. Para além de contribuírem com um odor agradável, podem também ser importantes agentes de conservação visto possuírem forte atividade antimicrobiana. Contudo, os óleos essenciais são bastante voláteis e de entre os vários constituintes de um óleo essencial, existem aqueles que produzem sensibilidade na pele provocando, por exemplo: dermatites de contacto e fotoalergia. Assim, o anexo III do Regulamento nº 1223/2009, apresenta uma lista de substâncias que os produtos cosméticos não podem conter fora das restrições previstas.

Os conservantes são adicionados aos produtos cosméticos para que estes tenham um prazo de conservação alargado, prevenindo o desenvolvimento de microrganismos que causem doenças ao consumidor ou, prejudiquem a aparência do produto. No entanto, a legislação referente aos conservantes químicos tem vindo a sofrer alterações e atualizações em consequência do conhecimento de problemas de sensibilidade e toxicidade inerentes a estes produtos.

Assim, este trabalho surge com o objetivo de valorizar os óleos essenciais com atividade antimicrobiana de plantas autóctones em Portugal: *Thymus caespititius* Brot., *Thymbra capitata* (L.) Cav., e *Myrtus communis* L., incluí-los em formulações tópicas, a fim de avaliar as suas capacidades como conservantes.

Os três óleos essenciais (*Th. caespititius*, *T. capitata*, *M. communis*) foram isolados das partes aéreas floridas das diferentes plantas por hidrodestilação, analisados por cromatografia gasosa para a quantificação dos seus componentes, e por cromatografia gasosa acoplada a espectrometria de massa para a identificação dos compostos. Todos os óleos essenciais apresentaram uma coloração

amarela, tendo sido identificados 64 constituintes em *Th. caespititius*, 30 em *T. capitata* e 34 em *M. communis*, sendo que, os monoterpenos foram a classe de compostos dominantes (76%, 97% e 91%, respetivamente), enquanto os sesquiterpenos variaram entre 2 e 16%, e os fenilpropanóides de vestigial a 3%. Para o óleo essencial de *Th. caespititius* o componente dominante foi α-terpineol, enquanto o óleo essencial de *T. capitata* apresentou como componente dominante o carvacrol. Já 1,8-cineole foi o composto maioritário do óleo essencial de *M. communis.*

Com base na caracterização química dos óleos essenciais em estudo, nomeadamente os constituintes referidos como alérgenos (linalool, limoneno, metil eugenol, citronelol, geraniol e eugenol) no Regulamento nº 1223/2009, anexo III, e com consequentes restrições na sua utilização, foram incorporados em emulsões óleo-em-água (O/A) na concentração de 1%, dois dos óleos essenciais: *Th. caespititius* e *T. capitata*, e na concentração de 0,5% os três óleos essenciais (*Th. caespititius*, *T. capitata* e *M. communis)*, realizando-se uma avaliação físico-química: pH, viscosidade, separação de fases e, tamanho da partícula.

O pH de pele saudável apresenta valores próximos de 5, e os produtos de aplicação tópica para pele saudável apresentarem um pH compreendido entre 4-6 assim, os valores de pH de todas as emulsões com óleo essencial encontram-se dentro dos limites referidos. Relativamente à viscosidade, a inclusão dos óleos essenciais aumenta significativamente a resistência da estrutura quando comparado com o placebo. O que se confirma com a análise do tamanho da partícula, visto que, de um modo geral, a inclusão dos óleos essenciais quer a 1%, quer a 0,5% diminui o tamanho das partículas. Todas as emulsões foram estáveis.

Avaliou-se a atividade antimicrobiana pelo método de Kirby-Bauer e pelo método da microdiluição em placa, determinando-se a concentração mínima inibitória e a concentração mínima bactericida para os três óleos essenciais com e sem diluição em DMSO e, para dois controlos: Dermosoft® OMP e do álcool benzílico. Verificando-se que: *Pseudomonas aeruginosa* ATCCC 9027 foi extremamente suscetível ao óleo essencial de *T. capitata* sem diluição em DMSO; *Staphylococcus aureus* ATCC 6538 apresentou maior suscetibilidade aos óleos essenciais de *Th. caespititius* e *T. capitata* e, menor suscetibilidade ao óleo essencial de *M. communis* diluído em DMSO (1:100). Os óleos essenciais de *Th. caespititius* e *T. capitata* foram eficazes contra os fungos, revelando melhor atividade antifúngica que Dermosoft® OMP para *Candida albicans* ATCC 10231. Os três óleos essências (*Th. caespititius,*

T. capitata e *M. communis)* apresentaram melhor atividade contra bactérias Gram-positivas do que bactérias Gram-negativas, estando estes resultados de acordo com estudos anteriormente realizados. Todos os óleos essenciais revelaram melhores resultados que os dois controlos. Sumariamente, os três óleos essenciais (*Th. caespititius*, *T. capitata* e *M. communis*) mostraram-se eficazes contra fungos, bactérias Gram-positivas e Gram-negativas.

Quanto ao teste de eficácia dos conservantes as emulsões com 1% de *Th. caespititius* e *T. capitata* e a emulsão com 0,5% de *M. communis* estão de acordo com o critério de aceitação A (redução de 2 Log_{10}) quer para os fungos (*Candida albicans* ATCC 10231 e *Aspergillus brasiliensis* ATCC 16404) quer para as bactérias (*Pseudomonas aeruginosa* ATCCC 9027 e *Staphylococcus aureus* ATCC 6538).

Os óleos essenciais de *Th. caespititius* e *T. capitata* revelaram-se potenciais conservantes alternativos para aplicação em formulações tópicas. Embora a emulsão com óleo essencial de *M. communis* cumpra com o critério de aceitação do teste de eficácia dos conservantes (FP 9), a percentagem do alérgeno metil eugenol, excede na percentagem utilizada (0,5% de óleo essencial) a máxima concentração permitida pela lei (Regulamento 1223/2009, anexo III).

Palavras-chave: óleo essencial, atividade-antimicrobiana, conservantes, emulsões.

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ABAP – 2,2'-azobis-(2-amidinopropane) dihydrochloride

BHT – Butyated hydroxytoluene

CFU – Colony forming units

CP – Cosmetic products

CSLI – Clinical and Laboratory Standards Institute

EC – European Commission

EMEA – European Medicines Agency

EO – Essential oil

EU – European Union

FDA – US Food and Drug Administration

GC – Gas chromatography

GC-MS – Gas chromatography coupled to mass spectrometry

HC – Health Canada

LC_{100} – Lethal concentration

MBC – Minimum bactericidal concentration

Mc – *Myrtus communis* L.

Mc EO –*Myrtus communis* L. essential oil

MEP – methyl-D-erythritol-4-phosphate

MIC – Minimum inhibitory concentration

MFC – Minimum fungicidal concentration

MH – Müeller-Hinton

O/W – Oil-in-water emulsion

PWD – Pine wilt disease

PWN – Pinewood nematode

SCCS – Scientific Committee on Consumer Safety

Thc – *Thymbra capitata* (L.) Cav.

Tc EO - *Thymbra capitata* (L.) Cav. Essential oil

Thc – *Thymus caespititius* Brot.

Thc EO - *Thymus caespititius* Brot. Essential oil

W/O – Water-in-oil emulsion

WHO – World Health Organization

Chapter 1. INTRODUCTION

The World Health Organization (WHO) has estimated that 56 million people died worldwide in 2012, and infectious diseases were responsible for one-third of all deaths. This situation is aggravated by the increasing number of disease causing microorganisms resistant to antibiotic therapy, which are able to recover and survive after antibiotic drug exposure through their ability to acquire and transmit resistance. Therefore, antibiotic resistance has become a public health problem of increasing magnitude, and the discovery and development of novel antimicrobial agents to address this problem is an important priority.

Plants are good options for obtaining a great variety of drugs; they are extensively used in folk medicine because they represent an inexpensive alternative, are easily accessible and can be applicable to various pathologies (Sardi *et al.* 2011).

Numerous scientific reports have shown that plants have a high potential to synthesize different antimicrobial substances which act as plant defence mechanisms and protect them against abiotic (UV radiation, drought, high or low temperatures, excessive soil salinity) and biotic (i.e. microorganisms, insects, and herbivores) stresses. Plant-derived antimicrobial agents can be classified into phenolics and polyphenolics, terpenoids, alkaloids, lectins, polypeptides, and polyacetylenes (Nabavi *et al.*, 2015). In fact, in traditional medicine, some plants and their essential oil (EO) were used to treat different conditions such as infectious diseases, inflammation, and used to improve the health and physical appearance of the human exterior, and protect a body part against damage from the environment since ancient times in human history (Carvalho *et al.*, 2015).

1.1 Essential oils

1.1.1 Traditional application of essential oils

Back to 1600 BC, in ancient Egypt, women used EO extracted from flower and pine trees with sweet and delicate odour to aesthetically improve their femininity (Oumeish, 2001). Cleoptera, Queen of Egypt (51 BC), was known to use all kinds of perfumes, especially in romantic occasions. Later, these Egyptian practices were inherited by the Greek and Roman women. In infused baths, they used the jasmine, lavender or ylang-ylang oils to relax physically and mentally (Carvalho *et al.*, 2015). The

Greek physician Hippocrates, known as the father of medicine, recommended often massage with essential oils and in his writings, refers to a vast number of medicinal plants (Cunha *et al.*, 2011).

The search of natural products for their healing potential is an idea coming from ancient times which has been taken up and developed in recent years. Emergence of drug resistant strains of pathogens, increase in the immunocompromised population and limitations of the available antibiotics/drugs have motivated people to use the complementary and alternative therapies, including the use of essential oils (Raut *et al.*, 2014).

1.1.2 Meaning of essential oils

EOs are complex liquid mixture of volatile, lipophilic and odoriferous compounds biosynthesized by living organisms, predominantly aromatic plants (Berger, 2007). They are a mixture of secondary metabolites produced in cytoplasm and plastids of plant cells and stored in secretory cells, cavities, canals, epidemic cells or glandular trichomes, present in different parts of the plants (Bakkali *et al.*, 2008). According to the European Pharmacopoeia, an EO is defined as "the product obtained by hydro-, steam- or dry-distillation or by expression (mechanical process without heating used for *Citrus* fruits) of a plant or of some parts". EOs are the complex mixture of several bioactive chemical components terpenes, terpenoids, phenylpropenes and phenolics showing lipophilic nature (Voon, *et al.*, 2012). They are synthesised in cytoplasm and plastids of plant cells via malonic acid, mevalonic acid and methyl-D-erythritol-4-phosphate (MEP) pathways.

Terpenes are hydrocarbon made up of several unit of isoprene (C_5H_8) while the terpenoids are biochemical modification of terpenes via enzymes that add oxygen molecules and move or remove methyl groups. In general terpenoids show higher antimicrobial activity than the terpenes (Burt, 2004). Phenylpropanoids are a diverse group of compounds derived from the carbon skeleton of phenylalanine that are involved in plant defense, structural support, and survival (Vogt, 2010), and they characterized by a C_6 (aromatic) - C_3 basic structure (Burlando *et al.*, 2010). The phenylpropanoid pathway serves as a rich source of metabolites in plants, being required for the biosynthesis of lignin, and serving as a starting point for the production of many other important compounds, such as the flavonoids, coumarins, and lignans (Fraser *et al.*, 2011).

The chemical variability of EOs, due to variable ecological and geographical conditions, age of the

plant, harvesting time and different methodology of their extraction (Bagamboula *et al.*, 2004) are the major issues for their application, for example: as natural preservatives. These apparent variations in the chemical profile of the EO may influence their potential biological activity (Burt, 2004). The possible synergisms between the different bioactive components of an EO are very difficult to identify because of their variable amount in intact EO. In general the major components of EO (>40-50% of total composition) cause antimicrobial activity but this hypothesis is not always true for all cases. Sometimes, the minor components of oil also play a significant role in enhancement of antimicrobial activity of intact EO (Prakash *et al.*, 2015).

1.1.3 Essential oils application of cosmetics

Now, the EOs are gaining remarkable interest for their potential multipurpose use as antioxidant, antibacterial and antiseptic agents (Cannas *et al.*, 2013) and promising and interesting for cosmetic products: for example, EO with anti-bacterial or anti-fungal activities allow reducing the use of preservatives components in a product (Kohiyama *et al.*, 2015). These biological activities of EO attract the attention of scientific community towards the development of eco-friendly botanical antimicrobials. Being natural in origin, EO and their components are considered environment favourable, and user friendly (Prakash *et al.*, 2015).

Essential oils are part of the composition of cosmetic products, mainly to create a pleasing odor, however, can combine their odoriferous property with the fact that 1) are biodegradable natural products, 2) have low toxicity to mammals, and 3) can perform simultaneously the functions of more than one of its synthetic equivalents (Figueiredo *et al.*, 2007).

Currently, the use of EO is as noticeable as pervasive and these substances have been generally recognized as safe and are widely accepted by consumers (Costa *et al.*, 2013). European Medicines Agency (EMEA), US Food and Drug Administration (FDA) and Health Canada (HC) published guidelines about safety assessment of plants products, including the description the herbal substance, herbal preparation or combinations thereof and their clinical or non-clinical data. In summary, these guidelines ensure greater safety to the final consumer.

The present work has, among others, the aim of the valorization of essential oils obtained from Portuguese plants: *Thymus caespititius* Brot.; *Thymbra capitata* (L.) Cav.; *Myrtus communis* L. as

preservatives in cosmetic formulations.

1.2 *Thymus caespititius* Brot.

The *Thymus* genus is one of the most taxonomically complex in the Lamiaceae family and includes 250–350 taxa (species and varieties) of wild growing evergreen species of herbaceous perennials, aromatic herbs, and subshrubs, native to Southern Europe, North Africa, Asia, and Australia (in Clasiglia *et al.*, 2015; Nabavi *et al.*, 2015; Costa *et al.*, 2013). Eleven *Thymus* species have been recorded for Portugal included in five sections of this genus (Figueiredo *et al.*, 2001), among which *Thymus caespititus* Brot.

In the genus *Thymus* the volatile oils and the polyphenols are mainly responsible for the pharmacological properties of the plants. All *Thymus* species produce essential oils, and several representatives are important herbs and spices used in all parts of the world (Pinto *et al.*, 2014).

1.2.1. Geographic distribution

The western Mediterranean region is considered to be the centre of origin of the genus *Thymus* and usually a region with a Mediterranean climate and flora. Macaronesia consists of five archipelagos belonging to three countries, Cape Verde (Cape Verde Islands), Portugal (Azores, Madeira and Savage Islands) and Spain (Canary Islands) (Figueiredo *et al.*, 2010). *Thymus caespititius* is a Portuguese aromatic species endemic of the north-western Iberian Peninsula, including the Azores and Madeira archipelagos (Trindade *et al.*, 2009; Figueiredo *et al.*, 2008; Miguel *et al.*, 2004).

1.2.2. Botanical description

Th. caespititius is a low creeping shrub, with woody, radiant branches. Its branches thickly covered with linear to spatulate leaves (5-10 x 0.7-1.2 mm) and the bracts are equal to the leaves. The vegetative branches are pilous. The corolla (< 5mm) is bilabiate and has purple intense color or whitish inflorescences (Castroviejo, 1986) (Fig. 1.1).

(a)  (b) 

Fig. 1.1 *Thymus caespititius* Brot. plant (a) and its aerial parts (b) (courtesy of Ana Cristina Figueiredo of Lisbon University).

1.2.3. Phytochemistry

The main chemical classes of the compounds occurring in the EO, obtained from the plants belonging to the genus *Thymus*, are terpenes, terpene alcohols, phenolic derivatives, aldehydes, ketones, ethers, and esters. The EO chemical composition varies depending on the species and chemotype considered. As regards the genus *Thymus* plants, there are different chemotypes, depending on the dominant component of the EO (Nabavi *et al.*, 2015).

The Ibero-Macaronesian *Th. caespititius* is characterized by a high chemical polymorphism mainly among the EO isolated from individuals collected in mainland Portugal, in the Azores and Madeira archipelago (Figueiredo *et al.*, 2010). EO of plants vegetate in mainland Portugal are characterized by their high chemical homogeneity. The main feature of these EO is their great wealth in α- terpineol (>30%) and the presence of large number of sesquiterpenes, particularly oxygenated. On the other hand, in the Azores archipelago vegetating plants have enhanced chemical polymorphism. Whereas in the mainland the EO were of the α-terpineol chemotype, in the Azores archipelago, carvacrol, thymol, α-terpineol, sabinene, carvacrol/α-terpineol, α-terpineol/T-cadinol and carvacrol/thymol chemotypes were recorded (Figueiredo *et al*., 2008; Trindade *et al.*, 2008; Trindade *et al.*, 2009; Lima *et al.*, 2010). For plants that vegetate in Madeira archipelago there was, as in the Mainland, chemical homogeneity, with a predominance of α-terpineol in the EO (in Figueiredo *et al.*, 2007).

1.2.4. Ethnobotany

Thyme is a largely used medicinal plant. In ancient times it was used by the Egyptians as unguents for embalming and then by the Greeks and Romans for its therapeutic purposes (Barros *et al.*, 2010). Thyme is used for its expectorant, spasmolytic and antiseptic properties and infusions are used for treating ulcers, dermatitis and rheumatic pains. Commonly used as a condiment for typical Mediterranean cuisine in Portugal, Spain, Italy, Cyprus, and Greece (in Casiglia *et al.*, 2015) and thyme species are also used for flavoring purposes, namely of cheeses, stews, soups, meats, fishes, dressings, honey and even chocolates (Figueiredo *et al.*, 2010). *Thymus caespititius* commonly designated in Portugal by "Alecrim da Serra" (Rivera *et al*., 1995), despite its frequent use as a spice, infusions of the flowers or from the dry plant are used internally as tonic, stomachic, and spasmolytic, or externally as antiseptic, rubefacient, and parasiticide (Table 1.1), (Figueiredo *et al.*, 2008).

The demand for EO from *Thymus* species is increasing for perfumery, cosmetic and medicinal uses (Costa *et al.*, 2013).

Table 1.1 - Application of different *Th. caespititius* parts and essential oil.

***Th. caespititius* part**	**Tradicional application**	**References**
Leaves	**Food industries**: Flavoring to cooked foods and are also used to prepare an aromatic infusion.	Casiglia *et al.*, 2015
Flowers	Infusions	Pinto *et al.*, 2014
Essential oil	**Food industries**: flavor, food preservative and food industries; **Cosmetic**: To flavor toothpastes, mouthwashes, and cough medicines; An ingredient of perfumes and cosmetic.	Casiglia *et al.*, 2015; Figueiredo *et al.*, 2010; Mohammed *et al.*, 2010

1.2.5. Pharmacological activity of Portuguese *Thymus caespititius*

In recent years, the pharmacological activity of Portuguese *Th. caespititius* has been studied for many purposes; among which: antioxidant, antifungal and nematicidal activities.

Dandlen *et al.*, 2010 tested the antioxidant activity of 6 Portuguese *Thymus* spp. essential oils and its chemotypes. The authors concluded that the EO from *Th. caespititius* was significantly more effective to scavenger hydroxyl radicals when compared with others *Thymus* spp., and the EO of a single species can show different antioxidant abilities, depending on its chemotype. Whereas Miguel *et al.*, 2004, tested the antioxidant capacity the essential oil from *Th. caespititius*, in the

absence/presence of radical inducer 2,2'-azobis-(2-amidinopropane) dihydrochloride (ABAP), α-tocopherol and butylated hydroxytoluene (BHT). *Th. caespititius* EO showed, at 250 and 500 mgL $^{-1}$, the highest antioxidant index, comparable to or higher than that of α-tocopherol and BHA, respectively, but still lower than that of BHT, at the same concentrations. The authors still demonstrated that *Th. caespititus* EO, which had no phenolic compounds, showed the highest antioxidant activity demonstrating that the presence of this type of compound is not obligatory for the antioxidant activity.

In Pinto *et al.*, 2014, the antifungal activity of the EO from *Th. caespititius* was evaluated against *Candida* spp., *C. neoformans*, *Aspergillus* spp., and dermatophytes. The *Th. caespititius* were collected in the flowering stage in north Portugal and, as expected, the EO was predominantly composed oxygen-containing monoterpenes (41%) being α-terpineol (36%) the major compound. Thus, the antifungal activity concerning filamentous fungi, dermatophytes showed higher susceptibility (minimum inhibitory concentration - MIC 0.16–0.32 μL/mL) and *Aspergillus* species were the most resistant (MIC 1.25-5 μL/mL, *A. flavus* being the less susceptible strain). Regarding yeast cells, MIC values ranged from 0.64 to 2.5 μL/mL for *Candida* spp. and *C. neoformans* showed the lowest MIC value for the EO of 0.16 μL/mL. Moreover, the EO revealed fungicidal activity against *Candida* spp., *C. neoformans*, and dermatophytes, with minimum fungicidal concentration (MFC) values equal to, or just one Log_2 dilution above, the MIC. The exceptions are *Aspergillus* strains, showing highest values of MFC, two or more times above the MIC. Besides the fungistatic or fungicidal activity, the EO displayed an important inhibitory effect on germ tube formation in tested strains of *C. albicans* at sub-inhibitory concentrations. In this study, the fungicidal activity displayed by *Th. caespititius* EO confirms its potential as an antifungal agent against a wide spectrum of fungal species implicated in mycoses, particularly dermatophytosis.

For the nematicidal activity, Barbosa *et al.*, 2010 tested initially the toxic effect of 27 EO was evaluated at 2mg/mL and after 24h, mortality of nematodes was significantly higher than 90% for the EO obtained, among others, from *Th. caespititius*. According to their lethal concentration (LC_{100}) values, the EO of *Th. caespititius* were clearly those to select for fractionation and bioassay guided search of highly active compounds able to effectively control pinewood nematode (PWN).

In Faria *et al.*, 2013, the nematicidal activity were separately assessed for the chemotypes from Portuguese *Th. caespititius*. The chemotypes rich in carvacrol and/or thymol showed high nematotoxic

activities while α-terpineol-rich chemotypes showed corrected mortalities <60%. The occurrence of chemotypes must be taken into account when choosing a nematotoxic EO bearing-species, since EO particular chemotype proved to be determinant in nematotoxic activity. For example, *Th. caespititius* rich in *p*-cymene showed lower LC_{100} values comparatively to the related EO.

1.3 *Thymbra capitata* (L.) Cav.

The common name thyme includes species of the genus *Thymus* L. and *Thymbra capitata* (L.) Cav. Given their morphological resemblance, some authors have included *T. capitata* in the genus *Thymus* as *Th. capitatus* (L.) Hoffmanns. et Link. However, the latter has been re-classified as *Thymbra capitata* (L.) Cav. (Figueiredo et al, 2010).

Thymbra capitata (L.) Cav. [=*Thymus capitatus* (L.) Hoffm. et Link. = *Coridothymus capitatus* Rch. f.] (Figueiredo *et al.*, 2008) is known as Spanish oregano or conehead thyme (Figueiredo et al, 2010).

1.3.1. Geographic distribution

Thymbra capitata (L.) Cav. is a circum-Mediterranean plant, it grows widespread, for example: in southern Portugal (Algarve) (Oliveira *et al.*, 2012; in Figueiredo *et al.*, 2007), in the South of Italy (between 0 and 600 m above sea level) (Miceli *et al.*, 2006), in Tunisia (it grows associated with *Teucrium polium* L., *Olea europaea* L., *Quercus coccifera* L., *Rosmarinus officinalis* L., *Ceratonia siliqua* L., *Dactylis glomerata* L., *Fumana thymifolia* L. and *Lygeum spartum* L.) (Ali *et al.*, 2013). This species is typical of *garrigues*, dry slopes and Mediterranean pine forests and it is considered a good indicator of the dry Mediterranean area (in Miceli *et al.*, 2006; Tommasi *et al.*, 2007, Ali *et al.*, 2013), therefore it is present in different bioclimatic zones extending from the subhumid to the upper arid, on sandy and often on rocky soils, under a rainfall varying from 300 to 1000 mm/year, but needs full sun to develop to their full potential (Ali *et al.*, 2013; Nabavi *et al.*, 2015).

1.3.2. Botanical description

T. capitata is a Mediterranean endemic, 10-40 cm height, aromatic perennial plant. It is characterized by its branches thickly branched with linear-lanceolate and glabrous leaves (1-10 cm).

Flowers with conical corolla, with by on the outside, and of purple or white colors (Fig. 1.2). It is a hermaphrodite and possesses a capacity for asexual reproduction by vegetative propagation. It is characterized by being a very aromatic plant. (Casiglia *et al.*, 2015; Nabavi *et al.*, 2015; Ali *et al.*, 2013; Miguel *et al.*, 2010, Castroviejo, 1986).

(a)  (b) 

Fig. 1.2 *Thymbra capitata* (L.) Cav. plant (a) and its aerial parts (b) (courtesy of Graça Miguel of Algarve University).

1.3.3. Phytochemistry

The main chemical classes of the compounds occurring in the EO, obtained from the *T. capitata* are terpenes, terpene alcohols, phenolic derivatives, aldehydes, ketones, ethers, and esters. Carvacrol (phenolic monoterpene), thymol (phenolic monterpene derivative of *p*-cymene), *p*-cymene (biological precursor of carvacrol), γ-terpinene (isomeric hydrocarbons) are the major compounds of *T. capitata* EO (Fig. 1.3) (Nabavi *et al.*, 2015) and are responsible for its properties. There is some evidence that minor components have a critical part to play in biological activities, possibly by producing a synergistic effect between other components. Several studies have focused on the antimicrobial activity of the EO of *T. capitata* in order to identify the responsible compounds as described in Pharmacological Activity (1.3.5). Phenolic terpenes seem to play an outstanding role. These phenolic terpenes join to the amine and hydroxylamine groups of the proteins of the bacterial membrane altering their permeability and resulting in the death of the bacteria (Bounatirou *et al.*, 2007).

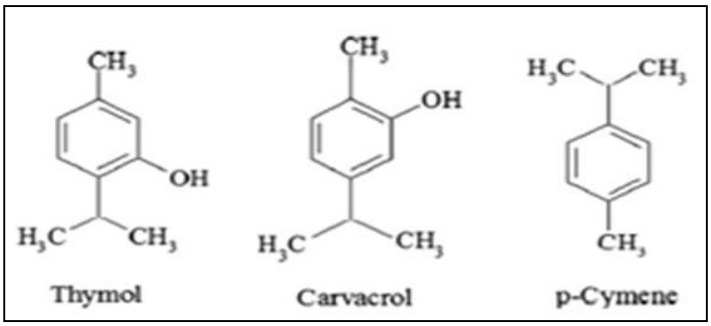

Fig. 1.3 – Chemical structure of the some substances occurring in *Thymbra capitata*: thymol, carvacrol and *p*-cymene (Adapted from: Nabavi *et al.,* 2015).

The EO chemical composition varies depending on the species and chemotype considered (Nabavi *et al.*, 2015). *T. capitata* EO usually shows a great chemical homogeneity characterized by high carvacrol amounts (≥50%) and lower amounts of their biogenetic precursors (γ-terpinene and *p*-cymene). Nevertheless, thymol rich EOs also occurred, although they have only been described for plants collected in mainland Italy (in Figueiredo *et al.*, 2010). Despite the higher number of samples studied in Portugal the thymol type has not been identified (Bounatirou *et al.*, 2007; in Figueiredo *et al.*, 2007, Miceli *et al.*, 2006).

1.3.4. Ethnobotany

Thymbra capitata EO is among the world's top 10 EO also used as a preservative for food purposes (Ehivet *et al.*, 2011). *T. capitata* is commonly used as a culinary spice (Bounatirou *et al.*, 2007), as a condiment to season and to improve the organoleptic of food, and as a source of antioxidant compounds for food conservation (Hosni *et al.*, 2013) and this species is also used for flavoring purposes, namely of cheeses, stews, soups, meats, fishes, dressings, honey and even chocolates (Figueiredo *et al.*, 2010). In Portugal, *T. capitata* is used in some Portuguese traditional meat dishes, particularly in rabbit meat (Miguel *et al.*, 2010; Faleiro *et al.*, 2005).

In traditional medicine (Table 1.2), it is used as antiseptic being used for the treatment of cutaneous infections, such as acne, and in mouthwashes against gum infections (Oliveira *et al.*, 2012; Figueiredo *et al.*, 2008) and it also used as astringent, spasmolytic, expectorant and antitussive (De Lisi *et al.*, 2011; Hosni *et al.*, 2013).

The EO from the plant, called “Spanish oregano oil”, is used in perfumery and cosmetic, but should not be used in aromatherapy because it is highly irritant to the mucous membranes (Casiglia *et al.*, 2015).

The demand of terpenes and phenols from *T. capitata* is increasing for cosmetic, pharmaceutical and food industries. However the use of material often comes from natural populations, which are suffering from increasing anthropic pressures (Ali *et al.*, 2013).

Table 1.2 - Application of different *T. capitata* parts and essential oil.

***T. capitata* part**	**Traditional application**	**References**
Leaves	**Food Preparation –** raw in salads or added as a flavouring to cooked foods and also used to prepare an aromatic tea for its antioxidant and spasmolytic activities. **Medicine -** infusion and decoction are used against diarrhea, digestive and respiratory system disorders.	Casiglia *et al.*, 2015; Ali *et al.*, 2013; Bounatirou *et al.*, 2007
Flowers	**Medicine –** infusion and decoction are used against diarrhea, digestive and respiratory system disorders.	Ali *et al.*, 2013
Essential oil	**Food Preparation –** flavouring baked goods; condiments; beverages and ice-creams. **Cosmetic –** to flavor toothpastes, mouthwashes, and cough medicines, as well as being an ingredient of perfumes and cosmetics.	Casiglia *et al.*, 2015; Figueiredo *et al.*, 2010

1.3.5. Pharmacological activity of Portuguese *Thymbra capitata*

Some studies published in the last years involve *Thymbra capitata* essential oil tested for nematicide (Faria *et al.*, 2013; Barbosa *et al.*, 2010), antifungal (Oliveira *et al.*, 2012; Salgueiro *et al.*, 2004), and antioxidant properties (Miguel *et al.*, 2003; in Figueiredo *et al.*, 2008; Faleiro *et al.*, 2005).

Faria *et al.* (2013) studied the use of EOs and decoction waters, isolated from 84 plant samples, were tested against *Bursaphelenchus xylophilus*, in direct contact assays. The main components of *T. capitata* EO (>10%) were carvacrol (68%) and γ – terpinene (11%). The results obtained suggest that the EO fractions with oxygen- containing molecules, and some components, namely: carvacrol and γ – terpinene may be responsible for EO nematotoxic activity. Nevertheless, despite the overall low activity of the essential oils hydrocarbon fraction, this type of components also seems to contribute, in several cases, to the EO pinewood nematode total nematotoxic activity, probably by additive and/or

synergic interactions between EO fractions or compounds. These results are in accordance with results from Barbosa *et al.* (2010).

Salgueiro *et al.* (2004), tested the antifungal activity of the EO of *T. capitata.* The study of its chemical composition revealed a high content in carvacrol and previous *in vitro* studies showed that sub-inhibitory concentrations of *T. capitata* EO were able to inhibit yeast germ-tube formation; both the EO and its major compound exhibited fungicidal effect against *Candida* spp., resulting from a direct damaging of the cytoplasmic membrane. Oliveira *et al.*, (2012) demonstrated *T. capitata* EO, rich in carvacrol (75%), showed a potent anti-*Candida* effect (MIC=0.32μL/mL). Oliveira *et al.*, (2012) studied the anti-*Candida* activity of Portuguese *T. capitata* EO and demonstrated potent activity upon pre-formed *Candida* biofilms (MIC=0.32 μL/mL). This EO was able to disrupt the biomass and inhibit the metabolic activity of distinct *Candida* spp. pre-formed biofilms. Concentrations similar to MIC were able to disrupt the biofilm matrix and inhibit the metabolism up to 50%.

Previous works on Portuguese *T. capitata* EOs showed antioxidant activity. Faleiro *et al.*, (2005) demonstrated the *T. capitata* EO mainly constituted by carvacrol, with an antioxidant index of 96%. The presence of available hydrogen atoms from phenol seems to be responsible for the good barrier against the primary oxidative process. Also, Miguel *et al.*, (2003) studied the antioxaidant activity of aerial parts of *T. capitata* EO and the results indicated that similar antioxidant activities were found for carvacrol or for the essential oils used, indicating an absence of synergistic or antagonistic effect promoted by the remaining components present in the EO isolated from *T. capitata*.

1.4 *Myrtus communis* L.

Myrtus communis L. (Myrtaceae), is an evergreen shrub that grows mainly in Mediterranean climates (Gardeli *et al.*, 2008; Ghasemi Pirbalouti *et al.*, 2010). Commonly known as myrtle, it belongs to the Myrtaceae family, which comprises approximately 145 genera and over 5500 species (Aleksic *et al.*, 2014) and includes economically important trees or shrubs, in wood production (*Eucalyptus* spp.), as ornamental (*Callisnetum* spp., *Eucalyptus* spp., *Leptospermum* spp., *Melaleuca* spp., *Myrtus* spp. and *Rhodomyrtus* spp.), as a source of spices (*Syzygium aromaticum*, *Pimenta dioica*), in the production of edible fruits (*Feijoa sellowiana*, *Myrciaria cauliflora*, *Psidium guajava*, *Syzygium jambos*,

S. malaccense), production of EO, used as flavouring, antiseptic and aromatherapy (*Eucalyptus* spp. and *Melaleuca* spp.) (Figueiredo, 2013).

1.4.1. Geographic distribution

M. communis is a common part of the typical Mediterranean flora. It is native to southern Europe, North Africa and west Asia. It is also distributed in Southern America, northwestern Himalaya and Australia (Aleksic *et al.*, 2014). Myrtle grows spontaneously Iran, Spain, France, Greece, Turkey, Tunisia, Algeria, Morocco, Croatia and Montenegro, among others countries with Mediterranean climate (Berka-Zougali *et al.* 2012; Jerkovic *et al.* 2002; Mahmoud *et al.* 2010; Mimica-Dukić *et al.* 2010, Senatore *et al.* 2006). In Italy, it grows along the coasts and on the internal hills and it is abundant especially on the islands, where it represents one of the most characteristic species (Cannas *et al.* 2013). In Portugal, myrtle grows wild in mainly in the central and southern parts of the country. Less frequent in the north, it is also present on the Madeira island and on 5 of the Azores islands (Faial, Pico, S. Jorge, Stª Maria and S. Miguel) (Figueiredo, 2013).

1.4.2. Botanical description and taxonomy

Myrtle is an evergreen sclerophyll shrub or small tree, 1.8–2.4 m height, with small foliage and deep fissured bark (Mendes *et al.* 2001). It is characterized by its branches thickly covered with lanceolate and glossy leaves, forming almost impenetrable thickets. Their leaves have different size (3–5 cm) (Figueiredo, 2013; Aleksic *et al.*, 2014). The white flowers are delicate and fragrant, displaying beautiful yellow stamens. The fruit is a berry blue-black color when ripe (Senatore *et al.*, 2006; Mahmoud *et al.* 2010; Figueiredo, 2013) (Fig. 1.4).

Fig. 1.4 *Myrtus communis* L. aerial parts (courtesy of Ana Cristina Figueiredo of Lisbon University).

1.4.3. Phytochemistry

In general, the leaves of *Myrtus communis* contain tannins, flavonoids such as quercetin, catechin and myricetin derivatives and volatile oils (Ahmet Cakir, 2004; Baytop, 1999). The EO of *M. communis* is extracted from the leaves, branches, fruits and flowers (Sumbul *et al.,* 2011), and the main compounds of the EO are: 1, 8-cineole, α-pinene, methyl eugenol, terpineol, *trans*-carveol, *cis*-carveol, geraniol, methyl geranate, α-terpinyl acetate, neryl acetate, β-caryophyllene, myrcene, sabinene, *p*-cymene, *cis*-terpinene, linalyl acetate, car-3-ene, phellandrene, methyl eugenol, methyl butyrate, methyl benzoate, benzyl alcohol, isobutyl butyrate, myrtenyl acetate, limonene, α-terpineol, linalool, eucalyptol, *p*-cymol, β-pinene, camphene, butyl butyrate and myrtenol (in Sumbul *et al.*, 2011). Some terpenoid compounds such was myrtenyl acetate, 1,8-cineole, limonene and linalool, are present in leaf (0.19-0.37%), fruits (0.03-0.13%) and flowers essential oils (0.21-0.26%) but in different proportions (Sumbul *et al*., 2011).

The fruits of this plant are mostly composed of volatile oils, tannins, sugars, flavonoids and organic acids such as citric and malic acids (Ahmet Cakir, 2004; Baytop, 1999).

Portuguese myrtle EO are characterized by high content of limonene+1,8-cineole (25.9 (berries)–39.5 (leaves)%), and myrtenyl acetate (6.6% (berries)–24.8% (leaves)) as the major components. α-pinene (9.7% (berries)–21.5% (leaves), and linalool (6.2% (leaves)–36.5% (berries) are also present at high content. All three parts of the plant show the same components, varying in proportions. Thus, Portuguese myrtle EO belongs to the group of myrtles EO which are characterized by the presence of

myrtenyl acetate as one of the major components (Pereira *et al.*, 2009).

1.4.4. Ethnobotany

Myrtles find wide use in cooking and cosmetics in many Mediterranean countries, revealing their aromatic characteristics. In cooking, fresh flowers are used in salads or garnishes, while the leaves and berries are used in meat dishes. In some countries, the leaves are used to wrap the chesses during the curing process. In some countries (Italy, Portugal, for example) berries and leaves from myrtle are used to produce well-known liquors (in Aleksic *et al.*, 2014; Figueiredo, 2013), it used for, among other purposes, to alleviate the symptoms of colds (Figueiredo, 2013). The flowers buds, flowers and fruits, properly dried, can be preserved in vinegar for culinary use, or in suitable oil (sweet almond oil, for example) for cosmetic purposes. In cosmetics and perfumery, myrtle is used for the essence or the EO. The fragrant water obtained from flowers, known as Angel water or Myrtle water was particularly famous in the seventeenth century. In 2007, an internationally recognized cosmetics brand launched as part of a tribute to the Mediterranean flora, four fragrances, among which stands out the myrtle (L'Occitane by Myrtle). The EO, also, are used as food preservative.

Not only but also, myrtle is used in folk medicine. Leaves and fruits myrtle are frequently consumed as an infusion and decoction for the treatment of many types of infectious diseases: urinary, respiratory, gastrointestinal, skin, and others.

Since ancient times, different parts (leaves, fruits, flowers and branches) and EO of the myrtle plant traditionally have as sorted specific applications (Table 1.3).

Table 1.3 - Application of different *M. communis* L. parts and essential oil.

***M. communis* part**	**Traditional application**	**References**
Leaves	**Food preparation** – liquors, flavoring meat and sauces; infusion and decoction used for relieve symptoms of colds **Perfume and cosmetic preparation** – hair tonic and stimulant; **Medicine** – infusion and decoction used as antiseptic, anti-inflammatory agent, laxative, analgesic, haemostatic agent and externally for wound healing, antiseptic and anti-inflammatory agent, and as a mouthwash, for the treatment of candidiasis antiseptic, disinfectant, hypoglycemic agents, therapy of urinary diseases.	Ahmet Cakir *et al.*, 2004; Aleksic *et al.*, 2014; Baytop, 1999; Bouzabata *et al.*, 2015; Dukié *et al.*, 2010; Gortzi *et al.*, 2008; Mansouri *et al.*, 2001; Mulas *et al.*, 2000; Serce *et al.*, 2010.

M. communis part	Traditional application	References
Fruits	**Food preparation** – liquors used for relieve symptoms of colds and as disinfectant and antiseptic; flavoring meat and sauces; infusion used as a diuretic; **Medicine** – used orally for infectious disease such as diarrhea and dysentery and externally for skin diseases and wound healing infectious disease including diarrhea and bloody diarrhea; hypoglycemic agents.	Ahmet Cakir *et al.*, 2004; in Aleksic *et al.*, 2014; Baytop, 1999; Dukié *et al.*, 2010; Figueiredo , 2013; Mansouri *et al.*, 2001.
Branches	The burned branches used as disinfectants and flavourings environment; **Medicine**: remedy for asthma, eczema, psoriasis, diarrhea, gastrointestinal disorders and urinary infections, when administrated orally; applied by inhalation and externally. Young branches are approved to be stimulant, antiseptic, astringent and hypoglycemic.	In Aleksic *et al.*, 2014; Figueiredo, 2013.
Flowers	**Food preparation –** fresh flowers used in salads and garnish; **Cosmetic –** fragrant water **Medicine**: against varicose veins and for preparing capillary lotions for external use;	in Aleksic *et al.*, 2014; Figueiredo, 2013.
Essential oil	Perfumary; properties balsamic, antiseptic and sedative; food preservative; used in the treatment of lung disorders.	Ahmet Cakir, 2004; in Aleksic *et al.*, 2014; Bouzabata *et al.*, 2015; Figueiredo, 2013.

1.4.5. Pharmacological activity of Portuguese *Myrtus communis*

The pharmacological activity of Portuguese *M.communis* EO was only described for the nematicide potential in Faria *et al.* (2013).

The Portuguese pine forest has become dangerously threatened by pine wilt disease (PWD), caused by the pinewood nematode (PWN), *Bursaphelenchus xylophilus*. Synthetic chemicals are the most common pesticides used against phytoparasitic nematodes but its use has negative ecological impacts. Phytochemicals may prove to be environmentally friendly alternatives. Thus, Faria *et al.* (2013), used the EO and decoction waters, isolated from 84 plant samples, were tested against *Bursaphelenchus xylophilus*, in direct contact assays. Of the 84 plant samples, one include the Portuguese *M. communis.* The fresh, flowering phase aerial of *M. communis* colleted in Algarve, Portugal was distilled and analyzed. The main components (>10%) were 1,8-cineole (37%), α-pinene

(24%), limonene (13%). The results obtained for the myrtle EO do not showed a nematotoxic activity effective.

1.5 Cosmetics

Cosmetics are a mixture of many materials and an understanding of physical chemistry is essential in designing, manufacturing and ensuring the stability of cosmetics (Mitsui, 1998). Thus, all cosmetic formulation is basically composed of excipients or vehicles of variable nature, in which specific elements are introduced (sometimes said to be “active”), preservatives, dyes (if necessary), and perfumes. According to the product and its intended use, the vehicle may be in the forms: liquid, solid or semisolid (Barata, 2002).

1.5.1 Purpose of cosmetics

Cosmetics are becoming of more importance in daily life; they are used regularly by increasing numbers of people and very large quantities are consumed each year.

The most obvious purpose of cosmetics is protection of the body from the elements of nature, such as heat and sunlight. Early people painted themselves with oils or mixtures of oils, clays and plant materials to protect themselves against dryness from cold, burns from strong sunlight, and irritation from insect bites. Additionally, cosmetics were used for religious purposes. Fragrant woods for example were burnt to produce smoke and incense that would ward off evil spirits. Further protection was afforded to an individual by painting the body to guard against evil (Mitsui, 1998).

It is understood by product cosmetic “any substance or mixture intended to be placed in contact with the *external* parts of the human body (epidermis, hair and capillary system, nails, lips and external genital organs) or with the teeth and the oral mucosa, in the aim exclusively or mainly to cleaning them, perfuming them, changing their appearance, protecting them, keeping them in good condition or correcting body odors” (SCCS, 2012). Thus, all cosmetic products must be safe and can not change or damage the physiological balance of the skin.

In Portugal, cosmetic products are legislated according with Regulation (EC) Nº 1223/2009, Deliberation n.º 15/CD/2013, Decreto Lei n.º 189/2008, September 24th, as it stands,

namely articles 10, 20, 22, 23, 24, 25, 29 as well as penalties applicable (Infarmed, 2016).

1.5.2 Cosmetics and Skin

1.5.2.1 Structure and functions of skin

Measuring a surface area of approximately 1.5–2.0 m^2, skin can be considered the largest organ of the body. The anatomy of the skin consists of three primary layers: the epidermis, the dermis, and the hypodermis- a subcutaneous adipose tissue (Burlando *et al.*, 2010). Various appendages, such as hair, nails, and glands (sweat and sebaceous), are also found in the skin (Mitsui, 1998).

The epidermis is composed of several cell layers about 0.1-0.3 mm thick. From the external surface inwards, these layers are called the horny layer (*stratum corneum*), granular layer, spinous layer, and basal layer (Mitsui, 1998). The *stratum corneum* functions as a protective physical and chemical barrier and is only slightly permeable to water. This tissue comprises 10-15 layers of flattened, keratinized cells which, together with the intercellular lipid matrices (mainly ceramides, cholesterol, and fatty acids), form the principle barrier to the ingress of exogenous substances (Marti-Mestres, 2000). Thus, the epidermis, provides waterproofing and serves as a barrier to infections (Burlando *et al.*, 2010).

The dermis is composed of connective tissue below the epidermis and is characterized by an extensive extracellular matrix containing high levels of collagen and elastin, matrix proteins conferring strength, firmness, and elasticity to the tissue. The dermis is a connective tissue hosting hair bulbs and skin glands (Burlando *et al.*, 2010), and it is highly perfused by blood and lymph vessel networks that provide an efficient means of drug removal into the systemic pool Marti-Mestres, 2000).

The hypodermis is a deep cutaneous layer characterized by fat or adipose tissue. This tissue extends deeply to contact the connective sheaths of muscles and skeletal system, and acts as a thermal insulator, a reserve of fuel for the body's metabolism, and a cushion that smoothes the potentially damaging effects of bumps and other traumatic events (Burlando *et al.*, 2010).

The skin represents the contact between inner and outer environment and carries out many functions: it catches information from exterior, regulates homeostasis (temperature, blood dynamic), absorbs vitamins and hormones and, most of all, it is a fundamental protective barrier of internal organs against prejudicial effects of environmental and xenobiotic agents. Selective permeability,

capacity of self-reparation and renovation, flexibility and resistance are all characteristic that make skin a multifunctional barrier (Mitsui, 1998).

1.5.3 Use of Emulsion Formulations in Dermatology

Cosmetic emulsions such as lotions and creams are rarely simple two-phase oil and water systems, and their study and development is one of the most difficult and complex subjects. Such preparations often contain several interacting excipients and may be composed of additional phases to oil-and-water (Marti-Mestres, 2000; Ribeiro *et al.,* 2004).

Common oily phase ingredients used in emulsions are: polar oils; waxes; emulsifying and co-emulsifying agents, among others. On the other hand, humectants, hydrophilic thickeners are used in the water phase. Preservatives, antioxidants, fragrances, colourants, UV filters are also used as ingredients in cosmetic emulsions.

In the interface, surfactants are used to emulsify. They are very important for the formulation since they are responsible for the production homogeneous and stable oil-in-water (O/W) or water-in-oil (W/O) emulsions (Barata, 2002). In aqueous systems containing surfactant/fatty alcohol combinations, the additional phases generally form when the emulsifier, in excess of that required to form a monomolecular film at the oil droplet interface, interacts with continuous phase water to form a gel network of vastly swollen bilayer structures. This swollen network controls the rheological properties of the emulsions (Ribeiro *et al.,* 2004).

Both oil-in-water and water-in-oil emulsions are extensively used for their therapeutic properties and/or as vehicles to deliver drugs and cosmetic agents to the skin. The emulsion facilitates drug permeation into and through the skin by its occlusive effects and/or by the incorporation of penetration-enhancing components (Raposo *et al.,* 2014). As well as, facility control of their properties (Xu *et al.,* 2006).

In the formulation of an emulsion for topical drug delivery, many factors must be considered. Drug stability, specific product use, site of application, and product type must be combined in a multiphase dosage form that will readily allow drug release when the formulation is applied to the skin. The potential of the vehicle to release the drug is dependent on the physicochemical properties of the specific diffusant and the matrix of chemicals that have been combined together to form the emulsion.

Safety, stability, and effective preservation must be combined with optimum drug delivery in the total formulation, which must retain aesthetic acceptability to the patient for compliance considerations (Martin-Mestres, 2000; Raposo *et al.*, 2014).

1.5.4 Essential Oils in Skin Care Preparations

In this era, consumers are looking for effective, safe and natural products that contribute for their health, wellness and beauty. As a result, consumer creates the need for development of new cosmetic products formulated with natural and nutraceutical ingredients. Nowadays, a significant number of cosmetic products that combine natural ingredients and innovative delivery systems have been developed. As natural ingredient, plant essential oils for perfumery and cosmetics are a growing market trend, being used in skin creams, balms, shampoos, soaps and perfumes (Carvalho *et al.*, 2015). Data from European Union (EU) indicate that for the period 2002-2009, EO market grew at 3% per year. However, with the expansion of the European market for natural ingredients, Europe is the largest consumer and producer of cosmetic products. Thus, between 2010 and 2014, European imports of EO increased by an annual 5% in terms of value, as cosmetic manufacturers are increasingly interested in new EO whereas conventional cosmetics are starting to contain more natural products (CBI, 2016).

Several authors propose the use of essential oils as natural conservation agents for cosmetic preparations, alone or in combination with other preservatives. They have suggested that the use of these essential oils into formulations, besides offering a pleasant aroma, could assure a protection against bacteria and fungi by impregnating the cavities in the interior of the product with antimicrobial substances. Consequently, the added essential oil can enhance the dermato-cosmetic properties of the final product, not only by protecting the consumer against bacterial or fungal infections, but also by contributing to the preservation of the formulation (Manou *et al.*, 1998).

However, EO have a short life, as they are volatile and reactive in the presence of light, heat, moisture and oxygen. Several researchers have investigated alternatives to overcome these challenges, and microencapsulation has been considerate as one of the most effective techniques (Carvalho *et al.,* 2015). Also, a number of EO have a potential to produce skin sensitization such as dermatitis allergic or irritant contact and phototoxicity/photoallergy (Antignac *et al.,* 2011). Thus, in

Regulation EC Cosmetics Regulation, annex III is present the list of sensitising substances with restriction use in cosmetics. Human skin exposure to known or suspected photo-phytodermatitis-causing substances should be kept at minimum and particular care should be applied in the safety assessment of plants or plant products from species (Antignac *et al.,* 2011).

1.5.5 Cosmetic application of preservatives

During the life cycle of a cosmetic product (CP) microbiological contaminants may appear during production and/or filing and/or even during use by the consumer. From the very moment that the product is opened until its last use by the consumer there is a permanent microbial contamination, variable and growing. It is then necessary that the product is properly preserved to ensure consumer safety, maintain quality and product specifications so as to ensure handling of the same with the quality and expected hygiene. It is essential a routine microbiological analysis of each batch of the finished product that will enter the market. The parameters analyzed, the methods used and the results obtained in each batch must be specified in reports to be included in the product information file (PIF) later.

There are several factors that can influence the microbial stability of a product. Among these factors are the concentration of alcohol, glycerine, water activity (a_w) and pH (Berthele *et al*., 2014; Ghalleb *et al*., 2015). However, it is possible to ensure the microbiological stability of a product with a specific combination of selected parameters (Ghalleb *et al.,* 2015). The efficacy of preservation of a CP development should be verified experimentally so as to ensure its microbiological stability and its correct preservation during storage and respective use (Siegert, 2012). Thus, the risk of microbiological any CP depends on its composition. If the preservative system used is not effective, there may be growth of bacteria and fungi, and or cause problems in the health of consumers, changes in pH, color and odor of the product. There is extreme importance to perform the preservative efficacy test used and also toxicological tests in order to ensure the safety of the CP (Ghalleb *et al*., 2015). For this reason, the Scientific Committee on Consumer Safety (SCCS) issues opinions about some substances that may raise some questions in terms of consumer safety. This is the case of parabens and triclosan, both used as preservatives in CP and can lead to problems the endocrine level if their use in such products does not take into account certain aspects.

The evaluation of the antimicrobial protection of CP starts by the artificial contamination of the finished product, followed by the assessment of the contamination reduction to levels below the limits. Despite being mentioned the obligation to perform this test in order to prove the microbiological stability of the product, the Regulation does not specify the procedure for the test. The SCCS recommendation: *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Candida albicans* (of an official reference stock in the EU) and specific germs which are known to lead to spoilage of CP. There are several methods for evaluating the efficacy of preservative used. Among them are: European and US Pharmacopoeias method, schülke KoKo test and the method of ISO 11930: 2012.

The evaluation of the antimicrobial protection of CP according to European and US Pharmacopoeias are different in microorganism test and acceptance criteria for topical preparations. The European pharmacopoeia uses the following test microorganisms: *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Candida albicans* and *Aspergillus brasiliensis*, and the acceptance criteria requires that test is performed at 2, 7, 14 and 28 days with Log reduction or no increase. US Pharmacopoeia uses the following test microorganisms: *Pseudomonas aeruginosa, Echerichia coli*, *Staphylococcus aureus*, *Candida albicans* and *Aspergillus brasiliensis*, and the acceptance criteria requires that test is performed only after 14 days (Fungi no increase and bacteria have a reduction of 2 Log reduction) and after 28 days (no increase for fungi or bacteria). But, the additional use of spoiling microorganisms required by the SSCP is not covered by these tests (Siegert, 2012).

The schülke KoKo test is specifically verified for the assessment of CP. The germ spectrum includes typical product spoiling microorganisms (*Enterobacter gergoviae*, *Echerichia coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Pseudomonas putida*, *Kocuria rhizophila*, *Staphylococcus aureus*, *Candida albicans*, *Asperillus brasiliensis*, *Penicillium funiculosum*) besides the pathogen germs as required by the SCCP. By inoculation of a mixed germ spectrum, a symbiotic growth according to the natural conditions is possible. To future equivalent detection of bacteria as fungi, the tests are carried out on tryptone-soy-agar as well as on sabouraud-dextrose-agar. A wrong assessment of the efficacy of the preservative by inhibition of the growth of one microorganism by the growth of other germs is impossible as growth of microorganism means that the sample is not sufficiently preserved (Siegert, 2012).

ISO 11930: 2012, first published in April 2012, is a standard that comprises a preservation efficacy

test method and a procedure for evaluating the overall antimicrobial protection of a cosmetic product which is not considered low risk, based on a risk assessment described in ISO 29621. According to this latter ISO, if the CP has low microbiological risk, such as products with an above alcohol content of 20% and organic solvents and singles use products or do not come in contact with air (Ghalleb *et al*., 2015), the evaluation of the antimicrobial protection of a CP is not required. It is noted the difference in respect of CP designed to be used in the area adjacent to the eye, and these must be tested with a large number of species. It is also noted that the preservative stability not be taken into account for this ISO. But, this includes a diagram to help decide the best way to evaluate the microbiological risk.

In addition to the use of standard ISO 11930: 2012 on the efficacy of the preservatives are also used the standards ISO 16212: 2008 and ISO 21149: 2006 for the microbiological control of CP (Siegert, 2012). Both standards were created in the context of associated with cosmetic microbiology. ISO 16212:2008 appears with the purpose of providing guidelines for enumeration of yeasts present in cosmetics when determining the number of colonies on selective agar medium after aerobic incubation. While, ISO 21149: 2006 provides guidelines for counting and detection of mesophilic aerobic bacteria present in CP. This can be affected by colony counting on agar medium after aerobic incubation, or to verify the absence of bacterial growth after the medium enrichment. This method may be the most appropriate for some types of CP such as the products immiscible in water.

The test efficacy antimicrobial preservation ensures safe production and storage but this is not enough for the period after opening. It is necessary to additional information such as the type of packaging, results obtained with similar products and habits and practices of consumers.

In cosmetics, the quality control must ensure that the microbial load in the product does not compromise the quality of the final product neither the consumer safety. There are several key factors to the achievement of adequate levels of microbial quality of the final product, employment of an ideal preservative is essential and requires careful consideration as to the type of product, its use, formulation characteristics and microbial challenge. The final selection of the preservative must be a compromise between the antimicrobial efficacy and compatibility with the product, and this selection quite a difficult work for the pharmaceutical and cosmetics industry.

Preservatives are substances added to CP to increase its useful life, preventing the development of

microorganisms that can cause disease or harm the appearance of the final product (Barata, 2002).

Cosmetic products fall under the general requirements of the European Commission (EC) Cosmetics Regulation 1223/2009, whereby the toxicological profile of all used ingredients and detailed knowledge of the product-specific exposure are required as fundamental for the safety assessment. As imposed by the European legislation, cosmetics must be safe for the consumer.

List of preservatives allowed in CP is presented Annex V of the regulation. If the manufacturer wants to use a preservative that is not covered by this annex, an authorization must be obtained. If the Commission considers that the use of preservatives present in Annex V may be a risk to consumer safety, can modify annex properly, after consulting the SCCS. The Commission may amend this annex and add preservatives or modify the information contained in it, for the benefit of technical and scientific progress.

The safety of a CP is determined based on the safety assessment of its ingredients, which is performed using literature data, *in vitro* tests and human tests since, in EU, finished CP are no longer tested in animals.

Successive amendments and therefore, updates the legislation of chemical preservatives relate to the existence of problems of sensitivity and toxicity inherent in these products, so there is need to investigate plant-derived molecules more innocuous and equally effective. And yet, the limited number of antifungal drugs, these drugs and resistance to the high cost of some of these drugs demonstrate the need for the discovery of new antifungal constituents (Cannas *et al.*, 2013).

Ingredients for the preservation of CP require high standards of safety and compatibility. Just a few of the numerous well-known and permitted preservatives are used in the majority of products.

Since single molecules (whether synthetic or natural products) typically have a unique mechanism of action, which facilitates the occurrence of resistance and that herbal preparations such as an EO based on interactions between their different constituents its use may have an advantage as regards the appearance of a smaller number of resistors and a greater spectrum of antimicrobial activity (Oliveira *et al.*, 2012).

Preservatives, in addition to having effective antimicrobial activity should be selectively toxic and effective against bacteria (Gram-positive and Gram-negative) and fungi. However, due to inherent characteristics, the vast majority of synthetic origin preservatives may give feedback to the user, such

as local irritation, contact dermatitis, hypersensitivity and others (Lee *et al.*, 2007). Moreover, also the fact that many of the substances of synthetic origin, used in cosmetics are provided with a degree of toxicity, and thus can be replaced with advantage by herbal products. Plant medicinal or in addition to the constituents of greater activity has other compounds which can contribute to influence its action on skin tissue increasing for example or by preventing their absorption changes (Cunha *et al.*, 2011).

The combination of several preservatives brings many benefits compared with the use of a single preservative. By using more than one preservative, the amount of each can be lowered, thus decreasing the possibility of developing contact dermatitis. Moreover, the use of more than one preservative may increase the spectrum of antimicrobial activity. Future solution possibly is the use of a set of preservatives, in preference to just one (Lundov *et al*., 2011).

Among methods of preservation, botanical materials and natural molecules offer different possibilities of use. Even though these materials may entail higher costs, they are frequently preferred with the aim of enhancing the dermocosmetic properties of products (Burlando *et al.*, 2010). Thus, the market for cosmetics and pharmaceutical products with components of plant or mineral origin has really grown in recent years.

Therefore, the increasing need for alternatives to current preservatives, as well as the need created by the consumer of cosmetic products with natural ingredients leads to look for alternatives that guarantee also a long shelf life to the finished cosmetic product. The present study meets this reality choosing three Portuguese plants [*Thymus caespititius* (Thc), *Thymbra capitata* (Tc) and *Myrtus communis* (Mc)] to obtain from them essential oils to be used as an alternative to current preservatives. The essential oils obtained were characterized and their antimicrobial activity assessed. Then, new emulsions containing 1% and 0.5% (w/w) of EO were produced, characterized and preservative efficacy was assessed.

Chapter 2. MATERIAL AND METHODS

2.1. Plant material

Collective and/or individual samples, from cultivated and wild-growing plants, were collected from mainland Portugal and at the Azores archipelago (Portugal) (Table 2.1). A voucher specimen of each plant species was deposited in the Herbarium of the Botanical Garden of Lisbon University, Lisbon, Portugal.

Table 2.1. Plant species scientific names, arranged in alphabetic order of the corresponding plant family, plant part used for hydrodistillation and collection place.

Family / species	Code	Plant part	Collection place
Lamiaceae / Labiatae			
Thymbra capitata (L.) Cav.	Tc	fresh, flowering phase aerial parts	Algarve
Thymus caespititius Brot.	Thc	fresh, flowering phase aerial parts	Azores, Minho
Myrtaceae			
Myrtus communis L.	Mc	fresh, flowering phase aerial parts	Algarve, Lisboa

2.2. Essential oil isolation

The essential oils (EO) were isolated by hydrodistillation for 3 h, using a Clevenger-type apparatus, Figure 2.1, according to the European Pharmacopoeia (Council of Europe, 2008). Hydrodistillation was run at a distillation rate of 3 mL/min. Before chemical analysis and biological activity assays, the essential oils isolated from each species collective and/or individual samples, obtained at different locations, Table 2.1, were pooled taking into account that they had the same chemotype. The EO were stored at -20ºC in the dark until analysis.

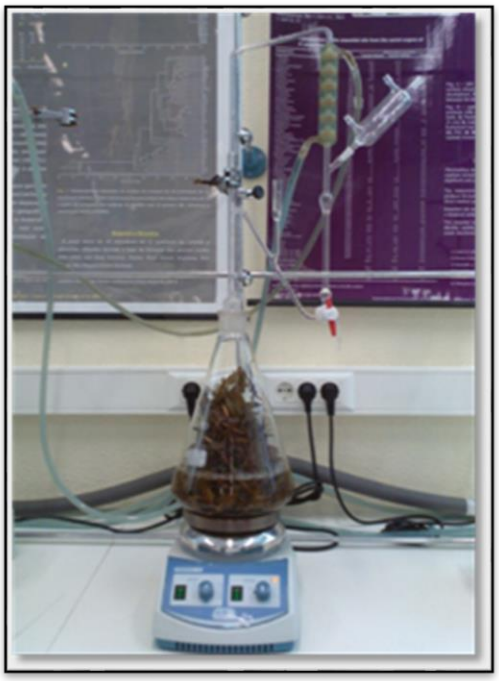

Fig. 2.1. Aspect of the hydrodistillation Clevenger-type apparatus used for isolation of *Thymbra capitata*, *Thymus caespititius and Myrtus communis* essential oils.

2.3. Essential oil analysis

Essential oils were analyzed by gas chromatography (GC), for component quantification, and gas chromatography coupled to mass spectrometry (GC-MS) for component identification. Component quantification was performed by normalization and internal standard methods in view of their uses during the experimental assays.

2.3.1. Gas chromatography (GC)

Gas chromatographic analyses were performed using a Perkin Elmer Clarus 400 gas chromatograph (Perkin Elmer, Shelton, CT, USA) equipped with two flame ionization detectors (FIDs), a data handling system and a vaporizing injector port into which two columns of different polarities were installed: a DB-1 fused-silica column (30 m x 0.25 mm i.d., film thickness 0.25 μm; J & W Scientific Inc., Rancho Cordova, CA, USA) and a DB-17HT fused-silica column (30 m x 0.25 mm i.d., film thickness 0.15 μm; J & W Scientific Inc.). Oven temperature was programmed, 45-175°C, at 3°C/min, subsequently at 15°C/min up to 300°C, and then held isothermal for 10 min; injector and detector

temperatures, 280°C and 300°C, respectively; carrier gas, hydrogen, adjusted to a linear velocity of 30 cm/s. The samples were injected using split sampling technique, ratio 1:50. The volume of injection was 0.1 μL of a pentane-essential oil (1:1) solution.

For routine quantification of EO components, the percentage composition was computed by the normalization method from the GC percentage peak areas, calculated as mean values of two injections from each EO, without using correction factors.

Besides percentage peak areas, real concentration of each EO constituents was determined, based on literature response factors (Costa *et al.* 2007). From each of the EO a 100μL batch was sampled which was spiked with 10μL of the pure internal standard, *n*-tridecane for Thc and Tc EO and *n*-dodecane for Mc EO, prepared at a concentration of 1mg/μL of distilled *n*-pentane. Response factors were used for the absolute quantification of EO constituents, based on the following formula:

$$Amount\ of\ component\ i = \frac{RFi \times AREAi \times STD\ AMOUNT}{AREAs \times SMP\ AMOUNT}$$

Where RF_i is the literature relative response factor of component *i* (Table 2.2), $AREA_i$ is the peak area of component *i*, $AREA_s$ is the peak area of the standard component, STD AMOUNT is the amount of the standard in the sample, SMP AMOUNT is the amount of sample. The amount of component *i* is a concentration expressed in grams of compound *per* 100 grams of essential oil.

Table 2.2. Literature response factors (RF) (Costa *et al*. 2007) used in the absolute calculation of each essential oil constituent.

Compounds	**RF**
Monoterpene hydrocarbons	1.03
Oxygen-containing monoterpenes	1.30
Sesquiterpene hydrocarbons	0.98
Oxygen-containing sesquiterpenes	1.30
Phenylpropanoids	1.30

2.3.2. Gas chromatography-Mass Spectrometry (GC-MS)

The GC-MS analyses were performed using a Perkin Elmer Clarus 600 gas chromatograph, equipped with a DB-1 fused-silica column (30 m x 0.25 mm i.d., film thickness 0.25 μm; J & W Scientific,

Inc.), and interfaced with Perkin-Elmer Clarus 600T mass spectrometer (software version 5.4.2.1617, Perkin Elmer, Shelton, CT, USA).

Injector and oven temperatures were as described in 2.3.1; transfer line temperature, 280°C; ion trap temperature, 220°C; carrier gas, helium, adjusted to a linear velocity of 30cm/s; split ratio, 1:40; ionization energy, 70eV; ionization current, 60µA; scan range, 40-300u; scan time, 1s.

The retention indices (RI) were calculated in-lab relatively to *n*-alkanes on the DB-1 column. The identity of the components was assigned by comparison of their RI, to C_9-C_{17} *n*-alkane indices and GC-MS spectra from a lab-made library, constructed based on the analyses of reference oils, laboratory-synthesised components and commercial available standards.

2.4. Formulation Development

The emulsions were developed in pharmaceutical technology laboratory of Faculdade de Farmácia da Universidade de Lisboa.

Three EO (*Thymus caespititius*, *Thymbra capitata* and *Myrtus communis*) were applied due to their antimicrobial activity, described in previous studies, in O/W emulsions. Among different kinds of possible formulations, it was chosen an O/W emulsion type formulation for different reasons:

- Solubilizing capacity for both lipophilic and hydrophilic ingredients;
- Heterogeneous systems (water and oil phases);
- Easier to test efficacy of antimicrobial preservation than other formulation.

Two different series of O/W emulsions were formulated, the first one with 1% of EO from *Thymbra capitata* and *Thymus caespititius* and the second with 0.5% of EO from *Thymbra capitata*, *Thymus caespititius* and *Myrtus communis*. For both series, a placebo (without EO) was prepared. The excipients used in formulations are described in Table 2.4.1.

Table 2.3 Excipients used in the emulsions with their chemical structure.

Trade name	INCI*	Main functions/ Additional functions
Emulgin B1®	Ceteareth-12	Non-ionic O/W emulsifier
Emulgin B2®	Ceteareth-20	Non-ionic O/W emulsifier
	Cetyl alcohol	Viscosity controlling ; emollient; emulsions stabilizing
	Paraffinum liquidum	Occlusive emollient
Tegosoft DO®	Decyl oleate	Lipophilic emollient
	Glycerin	Humectant
	Purified Water	Solvent

*INCI – International nomenclature of cosmetics ingredients.

All the above excipients are recognized as safe materials cosmetic products.

O/W emulsions composition for both series are shown in the Table 2.4.

Table 2.4 Percentages of ingredients in formulation of O/W emulsions for 1st and 2nd series.

1st Series		2nd Series	
Ingredients	**Percentages (w/w)**	**Ingredients**	**Percentages (w/w)**
Ceteareth-12	1.5	Ceteareth-12	1.5
Ceteareth-20	1.5	Ceteareth-20	1.5
Cetyl alcohol	2.0	Cetyl alcohol	2.0
Paraffinum liquidum	2.5	Paraffinum liquidum	2.5
Decyl oleate	4.5	Decyl oleate	4.5
Glycerin	5.0	Glycerin	5.0
Purified Water	82.0	Purified Water	82.5
Essential Oil	1.0	Essential Oil	0.5

In summary:

- Thc EO emulsion 1% or 0.5% represented the O/W emulsion with 1% or 0.5% *Thymus caespititius* EO, respectively;
- Tc EO emulsion 1% or 0.5% represented the O/W emulsion with 1% or 0.5% *Thymbra capitata* EO, respectively;
- Mc EO emulsion 0.5% represented the O/W emulsion with 0.5% *Myrtus communis* EO.

And the preparations of the O/W emulsions are described in the flow chart is represented in Fig. 2.2.

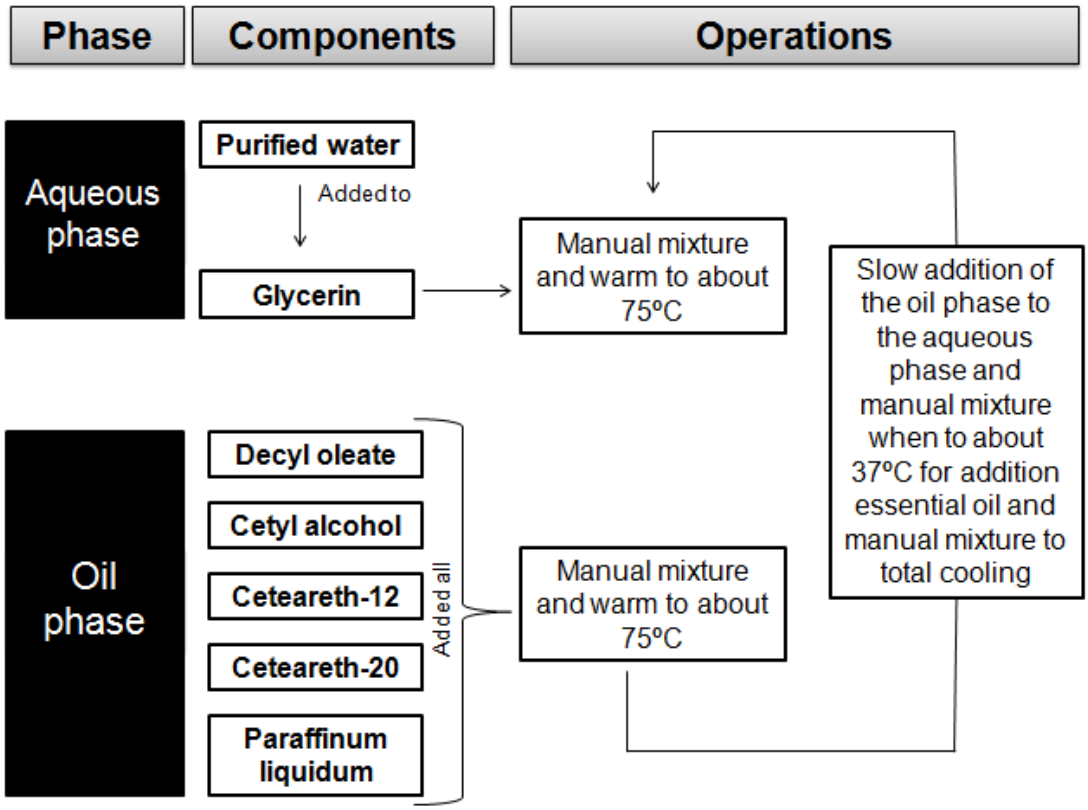

Fig. 2.2 Flow chart of the preparation of the emulsions.

2.5. Physicochemical characterization

Physicochemical characterization is very important to understand the interactions among ingredients and for stability studies. Thus all emulsions with EO and placebo were subdued to the determination of the pH values, viscosity, phase separation and the size distribution of the droplets.

2.5.1. Viscosity

Viscosity was measured through a rotational digital viscometer Brookfield DV II and small sample adapter (SSA) – Brookfield Engineering Laboratories, EUA. A small amount of sample, approximately 25 mL, was placed in the recipient of the viscometer, and it was subdued to a shear rate sweep (from 0.6 to 122), keeping each shear rate during 30 s. It was used a spindle nº 21 for all emulsions and placebo. Measurements were performed just after the preparation.

2.5.2. Determination of the pH

The pH measurements were performed with the pH-meter S20 Seven easy pH, Mettler Toledo and the data collected after 5 minutes at room temperature. Three replicates of measurement were performed for each emulsion.

2.5.3. Phase separation

Phase separation was measured through a centrifuga Heraus Sepatech, Medifuge and the data obtained after 10 min at 4x1000 RPM/min. Measurements were done for each emulsion and were performed just after the preparation.

2.5.4. Droplet size analysis

The size distribution of the emulsions was measured by light scattering using a Malvern Mastersizer 2000 (Malvern Instruments, Worcestershire, UK) coupled with a Hydro S accessory. Measurements were performed just after the preparation. For a correct turbidity, about 0.5 g of each formulation (Thc EO emulsion, Tc EO emulsion, Mc EO emulsion and placebo), corresponding to an obscuration between 5-10%, was added in the sample chamber containing 150 ml of water using a stirrer at 500 rpm. Data was expressed in terms of relative distribution of volume of particles and given as diameters values corresponding to percentiles of 10, 50 and T90% (mean± SD; n=5). The span value is a statistical parameter useful for characterizing the wideness of particle size distribution, calculated according to the following equation):

$$\text{Span} = \frac{d(90) - d(10)}{d(50)} \qquad \text{Eg. 1}$$

2.6. Antimicrobial activity

The evaluation of antimicrobial activity of three EO was included the determination of minimum inhibition concentration (MIC), minimum bactericidal concentration (MBC) and efficacy of antimicrobial preservation against Gram-positive and Gram-negative bacteria, yeast and fungi.

2.6.1. Microbial strains

In this study, the EO were tested against a large panel of microorganisms. Bacteria were purchased from international culture collections ATCC and obtained from collection of Faculdade de Farmácia da Universidade de Lisboa (FFUL). They included Gram-positive bacteria and Gram-negative bacteria, the yeast *Candida albicans* ATCC 10231 and fungi *Aspergillus niger* ATCC 16404 (Reclassification as *Aspergillus brasiliensis* ATCC 16404, (Houseknecht *et al*., 2008, Varga *et al*., 2007)) (Table 2.5).

S. aureus ATCC 6538 and *P. aeruginosa* ATCC 9027 were indicated as type strain for efficacy test sensitive to antibiotic, while *S. aureus* ATCC 43866, *P. aeruginosa* FFUL 1401, *E. coli* FFUL 3889 and *K. pneumoniae* FFUL 2320 were multidrug- resistant strains.

All strains were stored at −80°C in the appropriate medium containing 15% glycerol and regenerated twice before use in bioassays.

Table 2.5 Microorganisms strains used.

Gram-positive bacteria	**Gram-negative bacteria**	**Yeast**	**Fungi**
Staphylococcus aureus ATCC 6538	*Pseudomonas aeruginosa* ATCC 9027	*Candida albicans* ATCC 10231	*Aspergillus brasiliensis* ATCC 16404
Staphylococcus aureus ATCC 43866	*Pseudomonas aeruginosa* FFUL 1401		
	Escherichia coli FFUL 3889		
	Klebsiella pneumoniae FFUL 2320		

2.7. Determination of antimicrobial activity by the disk diffusion method

The susceptibility testing of different EO were performed by disk diffusion method, in Müeller-Hinton (MH) agar based on the Kirby-Bauer method (Bauer *et al.*, 1966) and according to procedures adapted from Clinical and Laboratory Standards Institute (CLSI, 2012). The disk diffusion method performed by Kirby-Bauer technique is a qualitative method. Briefly, a suspension of the test microorganisms, *P. aeruginosa* ATCC 9027 and *S. aureus* ATCC 6538 (10^6 CFU/mL) were spread on surface MH agar plates. Thc, Tc and Mc EO were tested at 1:100 dilution DMSO (show as: Thc EO

(1:100); Tc EO (1:100); Mc EO (1:100), respectively), and also without dilution. From each compound and DMSO (negative control) were soaked (15 µL) on sterile filter paper disks (6 mm in diameter) placed on agar surface after inoculation of bacteria. Then they were incubated at 37°C for 24 h. The test was performed in duplicate. The diameters of inhibition zones were measured in millimetres.

2.8 Evaluation of antimicrobial activity

2.8.1 Determination of minimum inhibitory concentration by the microdilution method

The microdilution method in MH broth with a sterile 96-wells microplate according to the standards CLSI (CLSI, 2006) was used to determine the minimum inhibitory concentration (MIC) to EO from Tc (100µL), Thc (100µL) and Mc (100µL) were tested at 1:100 dilution DMSO and also without dilution. Dermosoft ®OMP (INCI: Methylpropanediol, Caprylyl Glycol, Phenylpropanol) and benzyl alcohol were used as control.

Six different bacterial strains were used: Gram-positive (*Staphylococcus aureus* ATCC 6538; *Staphylococcus aureus* ATCC 43866) and Gram-negative (*Pseudomonas aeruginosa* FFUL 1401; *Pseudomona aeruginosa* ATCC 9027; *Escherichia coli* 5ECX CTX-M-35 FFUL 3889; *Klebsiella pneumonia* FFUL 2320). The yeast, *Candida albicans* ATCC 10231 and the fungi *Aspergillus brasiliensis* ATCC 16404.

First, it was prepared a suspension of each strain at concentration of $1,5x10^8$ the colony forming units (CFU)/mL (0.5 McFarland standard scale). Dilutions were made in MH broth, initially being pipette 100µL of whole broth MH plate and then 100µL of the different compounds tested only in the first well. Next, it performed dilutions of 1:2, ranging of 500µL/mL to 0.4µL/mL, and then were added 10µL bacterial suspension prepared above. Bacterial growth and solvent DMSO controls were used in parallel with assay in each microplate.

After incubation at 37°C for 24h the plates were read according to the presence or absence of turbidity that is indicative of growth or not, respectively. The lowest concentration of compound which was observed no turbidity corresponds to the MIC. All tests were performed in duplicate.

2.8.2 Determination of minimum bactericidal concentration

The determination of the minimum bactericidal concentration (MBC) indicated whether the EO have bactericidal or bacteriostatic activity. It was determined from each well without turbidity, with loop were discharged on surface of MH agar plate. The plates were left to incubate at 36°C for 24h to confirm if there are or not bacterial growth. Bactericidal or bacteriostatic activity occurs when the values of MIC and MBC were equal or when MIC was lower than MBC, respectively.

2.9 Efficacy of antimicrobial preservation

The test of efficacy of antimicrobial preservation was the contamination of the test emulsions prepared (described in 2.4) with specific microorganisms (Table 2.6). The bioassay is according to the Portuguese Pharmacopoeia 9 (Portuguese Pharmacopoeia, 9.8).

Table 2.6 Test microorganisms used in this study.

Microorganisms	Reference
Pseudomonas aeruginosa	ATCC 9027
Staphylococcus aureus	ATCC 6538
Candida albicans	ATCC 10231
Aspergillus brasiliensis	ATCC 16404

Each emulsion with a volume of 20 mL was contaminated with 200µL of microorganism suspension at 10^8 CFU/mL. It was determined the number of colony forming units per mL by membrane filtration at 0 days, 2 days, 7 days, 14 days and 28 days, following the acceptance criterion for local and topical preparations described in Portuguese Pharmacopoeia 9.8 (Table 2.7).

Table 2.7 Acceptance criterion for local and topical preparations (Portuguese Pharmacopoeia 9.8).

		Log_{10} reduction			
		2 d	7 d	14 d	28 d
Bacteria	A	2	3	-	NI
	B	-	-	3	NI
Fungi	A	-	-	2	NI
	B	-	-	1	NI

NI, No increase.

Chapter 3. Results and Discussion

3.1 Composition of the essential oils from *Thymus caespititius* Brot., *Thymbra capitata* (L.) Cav and *Myrtus communis* L.

Thymus caespititius (Thc), *Thymbra capitata* (Tc) and *Myrtus communis* (Mc) essential oils (EO) showed faint yellow colours. The isolated EO were complex mixtures, in which 64 constituents were identified in *T. caespititius*, 30 in *T. capitata* and 34 in *M. communis*, Table 3.1, representing 96-99% of the total volatiles. For each studied species the identified volatile components are listed in Table 3.1, in order of their elution on the DB-1 column.

Table 3.1 Percentage composition of the essential oils isolated from *Thymbra capitata, Thymus caespititius* and *Myrtus communis*.

Components	**RI**	***Thymbra capitata***	***Thymus caespititius***	***Myrtus communis***
Isobutyl isobutyrate	909			0.2
Tricyclene	921		t	
α-Thujene	924	2.0	2.0	0.2
α-Pinene	930	0.8	1.2	17.3
α-Fenchene	938		t	
Camphene	938	0.1	1.2	t
Sabinene	958	0.3	1.1	t
1-Octen-3-ol	961	0.3	1.1	
β-Pinene	963	t	1.1	0.4
Dehydro-1,8-cineole	973		0.1	
β-Myrcene	975	2.1	2.0	0.2
α-Phellandrene	995	0.3	0.2	0.3
δ-3-Carene	1000	0.1	0.1	0.1
α-Terpinene	1002	1.7	0.1	0.1
p-Cymene	1003	5.8	13.9	0.1
1,8-Cineole	1005		0.4	36.7
β-Phellandrene	1005	0.3	0.4	
Limonene	1009	0.3	1.7	9.8
cis-β-Ocimene	1017		t	t
trans-β-Ocimene	1027	t	0.2	0.5
γ-Terpinene	1035	7.5	6.5	0.2
trans-Sabinene hydrate	1037	t	0.1	
Fenchone	1050		0.1	
2,5-Dimethyl styrene	1059		0.1	

Components	RI	*Thymbra capitata*	*Thymus caespititius*	*Myrtus communis*
Terpinolene	1064	0.2	0.4	0.2
Phenyl ethyl alcohol	1064			1.1
cis-Sabinene hydrate	1066		0.1	
Linalool	1074	0.8	0.2	1.1
2-Methyl butyric acid, isoamyl ester	1074			0.3
1-Octen-3-yl acetate	1086		0.3	
trans-p-2-Menthen-1-ol	1099		0.1	
Borneol	1134	0.2	1.6	
Terpinen-4-ol	1148	0.9	1.6	0.3
α-Terpineol	1159	0.1	27.4	2.8
Methyl Chavicol (= Estragole)	1163			1.4
Myrtenol	1168			1.4
trans-Carveol	1189		0.2	
cis-Myrcenol	1205		0.1	
Citronellol	1207	0.1		
Methyl thymol	1210		0.1	
Carvone	1210	0.1		
Carvenone	1211		0.3	
Geraniol	1236			0.8
Linalyl acetate	1245			t
trans-Anethole	1254		0.1	
Cumin alcohol	1260		0.2	
Bornyl acetate	1265		0.6	
Thymol	1275	0.4	0.2	
Carvacrol	1286	73.1	10.3	
Myrtenyl acetate	1290			17.1
Eugenol	1327	t		t
α-Terpenyl acetate	1334			0.1
cis-Myrcenyl acetate	1334		0.1	
Carvacrol acetate	1348	t	0.5	
Geranyl acetate	1370			1.7
α-Copaene	1375		0.1	
Methyl eugenol	1377			1.7
β-Bourbonene	1379		0.2	
β-Elemene	1388		0.1	
β-Caryophyllene	1414	2.1	1.3	0.9
α-Humulene	1447	t	0.2	0.6
γ-Muurolene	1469		0.1	
Germacrene-D	1474		0.5	
3,3,5,5,8,8- Hexamethyl-7-Oxabicyclo [4.3.0] non-1(6)ene-2,4-dione	1488			0.7

Components	RI	*Thymbra capitata*	*Thymus caespititius*	*Myrtus communis*
β-Dihydroagarofuran	1489		1.0	
γ-Cadinene	1500		1.9	
trans-Calamenene	1505		0.2	
δ-Cadinene	1505		0.7	
Kessane	1517		0.4	
Elemol	1530		0.7	
β-Caryophyllene Oxide	1561	0.2		
Viridiflorol	1569		0.7	
UI A	1596		0.6	
epi-Cubenol	1600		1.0	
UI B	1609		1.0	
T-Cadinol	1616		4.6	
β-Eudesmol	1620		0.9	
α-Eudesmol	1634		1.3	
UI C	1648		0.5	
UI D	1662		t	
% of Identification		99.8	93.9	98.3
Group components				
Monoterpene hydrocarbons		21.5	32.1	29.4
Oxygen-containing monoterpenes		75.7	44.3	62.0
Sesquiterpene hydrocarbons		2.1	5.3	1.5
Oxygen-containing sesquiterpenes		0.2	10.6	
Phenylpropanoids		t	0.1	3.1
Others		0.3	1.5	2.3

RI: In-lab calculated retention indices relative to C_9-C_{17} *n*-alkanes on the DB-1 column, t: trace (<0.05%), UI (A to D): Unidentified compounds.

Monoterpenes dominated the EO isolated from the Lamiaceae species, *Thymbra capitata* (97%) and *Thymus caespititius* (76%), as well as the Myrtaceae species, *Myrtus communis* (91%). All the EO were dominated by oxygen-containing monoterpenes (76%, 44% and 62%, respectively), while sesquiterpenes ranged from 2 to 16%, and phenylpropanoids from traces to 3%. A fraction designated by others, Table 3.1, since components were neither terpenes nor phenylpropanoids, occurred in all EO from 0.3-2%.

α–Terpineol was the main component in *T. caespititius* (27%) EO. This result is in accordance with previous studies (Table 3.2) where some quantitative differences were observed in the amounts of α–terpineol (t-68%) present in EO obtained from different populations, collected at different locations in

Portugal. *p*-Cymene (14%) and carvacrol (10%) were other important constituents in this EO with some variations when compared to the percentages obtained from the wild Portuguese plants in other studies (Table 3.2). This chemical variability is common in wild aromatic plants (Oliveira *et al.*, 2012). The Ibero-Macaronesian Thc is characterized by a high chemical polymorphism mainly between the EO isolated from individuals collected in mainland Portugal and in the Azores archipelago. Whereas in the mainland the EO were of the α-terpineol chemotype, in the Azores archipelago, carvacrol, thymol, α-terpineol, sabinene, carvacrol/α-terpineol, α-terpineol/T-cadinol and carvacrol/thymol chemotypes were recorded (references in Figueiredo *et al.*, 2010).

The reason for such chemical polymorphism may reflect a genetic diversity probably relating to the heterogeneity of Portuguese environmental conditions such as humidity degree, thermic amplitude and soil type (Figueiredo *et al.*, 2008). This chemical polymorphism is mainly due to genetic variability of populations (Salgueiro, 1994).

On the opposite of *Thymus caespititius* EO, the previous studies on *Thymbra capitata* EO isolated from plants collected in Portugal, Table 3.2, showed a great chemical homogeneity characterized by high carvacrol relative amounts (60-75%), thus being typical terpene phenolic EO. In this study, carvacrol was also the main component in Tc (73%) EO, which is in accordance with those previous studies (Table 3.2). Other components with percentages higher than 5% are biogenetic precursors of carvacrol, such as γ-terpinene, the major component of monoterpene hydrocarbons with 8% and *p*-cymene with 6% being in accordance with previous studies (Table 3.2).

T. capitata is growing wild in other Mediterranean areas where EO with higher percentages of carvacrol (51-82%) were recorded (Benbelaid *et al.*, 2014; Bounatirou *et al.*, 2007; Casiglia *et al.*, 2015; Costa *et al.*, 2013; in Figueiredo *et al.*, 2008; in Nabavi *et al.*, 2015; Passinio *et al.*, 1999). However, some EO isolated from populations of Tc from Apulia, Puglia and Sardinia in Italy, were dominated by thymol (29-72%) (Consentino *et al.*, 1999; Miceli *et al.*, 2006; Tommasi *et al.*, 2007).

Table 3.2 Studies on the essential oils of *Thymus caespititius, Thymbra capitata* and *Myrtus communis* grown in Portugal.

Genus / Species	Occurrence	Plant part	Isolation method	Main components (≥5%)	References
Genus Thymus					
Thymus caespititius Brot.	Azores archipelago Madeira archipelago Mainland of Portugal	FF V	DE H	α–Terpineol t-68% Carvacrol t-66% Thymol t-58% *p*-Cymene t-19% Thymol acetate t-19% T-Cadinol t-16% γ-Terpinene t-13%	Barbosa *et al.*, 2010; Dandlen *et al.*, 2010; Faria *et al.*, 2013; in Figueiredo *et al.*, 2010; Lima *et al.*, 2010; Miguel *et al.*, 2004; Pereira *et al.*, 2000; Pinto *et al.*, 2014; Salgueiro *et al.*, 1997; Santos *et al.*, 2005; Trindade *et al.*, 2008; Trindade *et al.*, 2009.
Genus Thymbra					
Thymbra capitata (L.) Cav.	Azores archipelago and mainland of Portugal - Algarve	FF DF	H	Carvacrol 60-79% *p*-Cymene 5-7% γ-Terpinene 5-7%	Barbosa *et al.*, 2010; Faleiro *et al.*, 2005; Faria *et al.*, 2013; in Figueiredo *et al.*, 2010; in Figueiredo *et al.*, 2008; Miguel *et al.*, 2010; Miguel *et al.*, 2003; Miguel *et al.*, 2003; Oliveira *et al*., 2012; Salgueiro *et al.*, 2004
Genus Myrtus					
Myrtus communis L.	Mainland of Portugal	FF DF	H	1,8-Cineole 11-37% α-Pinene 7-24% Limonene 5-13% Myrtenyl acetate 21-34%	Faria *et al.*, 2013; Pereira *et al.*, 2009

t: traces (<0.05%), DE: Distillation–extraction using a Likens–Nickerson type apparatus, H: Hydrodistillation using a Clevenger-type apparatus, FF: Fresh aerial parts in flowering phase, DF: Dried aerial parts in flowering phase, V: Vegetative phase.

M. communis EO was predominantly composed of oxygen-containing monoterpenes (62%) being 1,8-cineole (37%) the major compound. Myrtenyl acetate (17%) was other important constituent. The monoterpene hydrocarbons were dominated by α-pinene (17%) and limonene (10%). These results are in accordance with previous reports for this species grown in Portugal (Table 3.2).

A large variability is found in the composition of previous studies on myrtle EO, mainly depending on the plant part evaluated and methodology of extraction used. Comparatively to other studies on the EO isolated from Mc flowering aerial parts, collected in different geographical locations, and extracted

by hydrodistillation, 1,8-cineole was also the main component found in the EO isolated from plants growing in Montenegro (24-26%) (Dukié *et al.*, 2010). However, α-pinene dominated the EO isolated from plants collected in Italy (16-42%), and Algeria (34-51%) (Senatore *et al.*, 2006; Flamini *et al.*, 2004; Bouzabata *et al.*, 2015, respectively). The Algerian myrtle EO were characterized by the absence of myrtenyl acetate (Bouzabata *et al.*, 2015). Recent reports showed that Turkish myrtle EO was linalool (37%) and linalyl acetate (16%) rich (Senatore *et al.*, 2006).

3.2 Formulation Development

Based on chemical characterization of EO, two EO (*Thymus caespititius* –Thc, and *Thymbra capitata* -Tc) out of three were chosen to be incorporated 1% as bioactive ingredients as topical formulations and the three EO (Thc, Tc and *Myrtus communis* -MC) were chosen to be incorporated 0.5% as bioactive ingredients and the same based emulsion was used in formulations. All the process and formulation details are fully described in Materials and Methods Chapter.

Chemical characterization of formulations described a percentage of allergens present in different EO and the maximum percentage acceptable for its allergens without indicated in list of ingredients in accordance with Cosmetics Regulation nº 1223/2009, annex III (Table 3.3). Mc EO though incorporated into formulation with the lowest percentage in study, does not meet the criteria of the regulation, since the Mc EO has high levels of methyl eugenol (Table 3.3). However assays were performed in order to determine its antimicrobial activity.

The ingredients: limonene, linalool, citronellol and eugenol must be indicated in the list of ingredients referred to in subparagraph g) of nº1 of article 19º to Cosmetics Regulation 1223/2009, annex III, if its concentration exceeds: 0.001% in the products leave-on and just for methyl eugenol its maximum concentration in the ready to use product: 0.0002%.

Table 3.3. Qualitative, quantitative composition of the allergens presented in EO of *Thymus caespititius* (Thc), *Thymbra capitata* (Tc) and *Myrtus communis* (Mc).

Essential Oil	Ingredient (INCI)	CAS Number	Concentration in EO (g/100g)	Concentration in the emulsions with 1% EO (g/100g)	Concentration in the emulsions with 0.5% EO (g/100g)*
Thymus caespititius (Thc)	Limonene	5989-27-5	1.7	0.0170*	0.0085*
	Linalool	78-70-6	0.2	0.0020*	0.0010*
Thymbra capitata (Tc)	Limonene	5989-27-5	0.3	0.0300*	0.0015*
	Linalool	78-70-6	0.8	0.0800*	0.0040*
	Citronellol	106-22-9	0.1	0.0100*	0.0005*
	Eugenol	97-53-0	t	t	t
Myrtus communis (Mc)	Limonene	5989-27-5	9.8	0.0980*	0.0490*
	Linalool	78-70-6	1.1	0.0110*	0.0055*
	Geraniol	106-24-1	0.8	0.0080*	0.0040*
	Eugenol	97-53-0	t	t	t
	Methyl eugenol	93-15-2	1.7	0.017*	0.0085*

t- traces (<0.0001%); * Must be indicated in the list of ingredients. The presence of the substance must be indicated in the list of ingredients referred to in subparagraph g) of nº1 of article 19º to Cosmetics Regulation 1223/2009, annex III, if its concentration exceeds:**0.001%** in the products leave-on and just for methyl eugenol its maximum concentration in the ready to use product: **0.0002%**.

3.3 Physicochemical characterization

Physicochemical characterization could be used as a tool to assess parameters that help understanding the emulsions' structure. In this study, the emulsions were analyzed macroscopically (organoleptic and phase separation) and pH, viscosity and droplet size were assessed.

3.3.1 Determination of the pH

The pH values of topical products should respect the acidic physiologic pH of the skin.

The common range of the pH of healthy skin is often around 5. Most of topical application products have pH values between 4 and 8, between 4-6 for healthy skin (Schmid-Wendtner *et al.*, 2007). The pH of the emulsions produced with1% and 0.5% of EO, ranging between 4.4-4.7 and 4.3-4.4, respectively, fits well within these limits (Table 3.4).

Table 3.4. pH values for the emulsions at 25ºC (n=3; mean ±SD) and apparent viscosity values calculated at the appex of the loops (122.36 s^{-1}).

Formulations	pH	Apparent Viscosity (Pa.s) at 122.36 s-1
Thc EO emulsion 1%	4.44±0.02	188.00
Tc EO emulsion 1%	4.50±0.04	80.00
Placebo emulsion	4.68±0.04	302.00
Thc EO emulsion 0.5%	4.33±0.02	58.00
Tc EO emulsion 0.5%	4.39±0.01	28.00
Mc EO emulsion 0.5%	4.31±0.01	51.00
Placebo emulsion	4.39±0.05	36.50

3.3.2 Viscosity

Continuous shear experiments measure the ability of topical formulations to resist structural breakdown during the application procedure. Representative flow curves are shown in Figures 3.1 and 3.2 for first series (Thc and Tc EO emulsions 1% and placebo emulsion) and second series (Thc, Tc and MC EO emulsions 0.5% and placebo emulsion), respectively.

Apparent viscosity values provide a comparison of the resistance to structural breakdown between the formulations and the loop areas compare the amount of structure that fractures in the standardized cycle and the values calculated at the apex of the loop are also resumed in Table 3.4.

The flow curves (Fig. 3.1 and 3.2) showed that the emulsions were non-Newtonian fluids, which means that their viscosity varies with the shear rate variation. This characteristic, the non-linear relation between the shear stress and the shear rate, is typical of non-Newtonian behaviour (Schramm, 2006).

Same emulsions (Thc EO emulsion 1%; Tc EO emulsion 1%; Thc EO emulsion 0.5%; Mc EO emulsion 0.5%) were also characterized as thixotropic, exhibiting a decrease in viscosity with increasing shear rate that is not completely recovered when the shear rate was ceased.

As it is possible to see also in Figure 3.1 and 3.2, that emulsions with Thc and Mc EO (0.5%) need a higher shear stress to flow than Tc EO emulsion or placebo emulsion, showing that Thc and Mc EO emulsions are more structured than Tc EO emulsion or placebo emulsion, since it needs more shear stress per unit of area to be disrupted and flow.

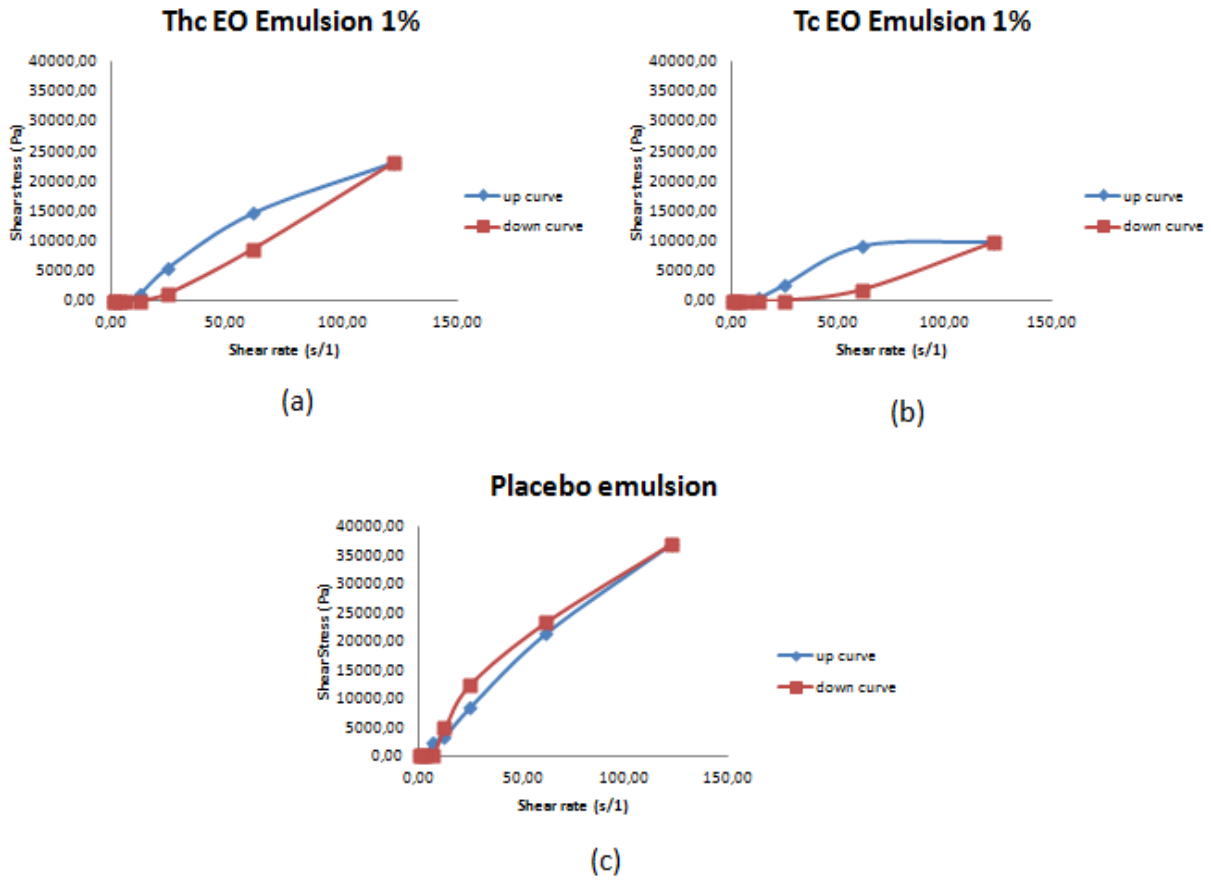

Fig. 3.1 Flow curves. Shear Stress as function of Shear Rate of Thc EO emulsion 1% (a), Tc EO emulsion 1% (b) and placebo emulsion (c).

The inclusion of EO significantly increased the resistance to structural breakdown: as a result, all formulations prepared with 1% of Thc and Tc EO (Fig. 3.1) showed lower values of apparent viscosity at the appex of the curve viscosity compared to placebo; and Thc, Tc and Mc EO emulsion 0.5% (Fig. 3.2) showed a higher values of viscosity compared to placebo. However, Tc EO emulsion 0.5% and placebo had similar values of apparent viscosity (Table 3.4).

Zillich *et al.,* (2015) reported that the incorporation of polyphenols into emulsions can influence their rheological properties as well as their stability, particularly the decrease of viscosity could be observed. But the reasons of this effect are not completely understood up to now. Besides the obvious dilution effect, one reason could be based on interactions of polyphenols with emulsifiers. Although the Thc, Tc and Mc EOs do not present polyphenols, the importance of phenylpropanoids in the values of apparent viscosity, remains to be elucidated.

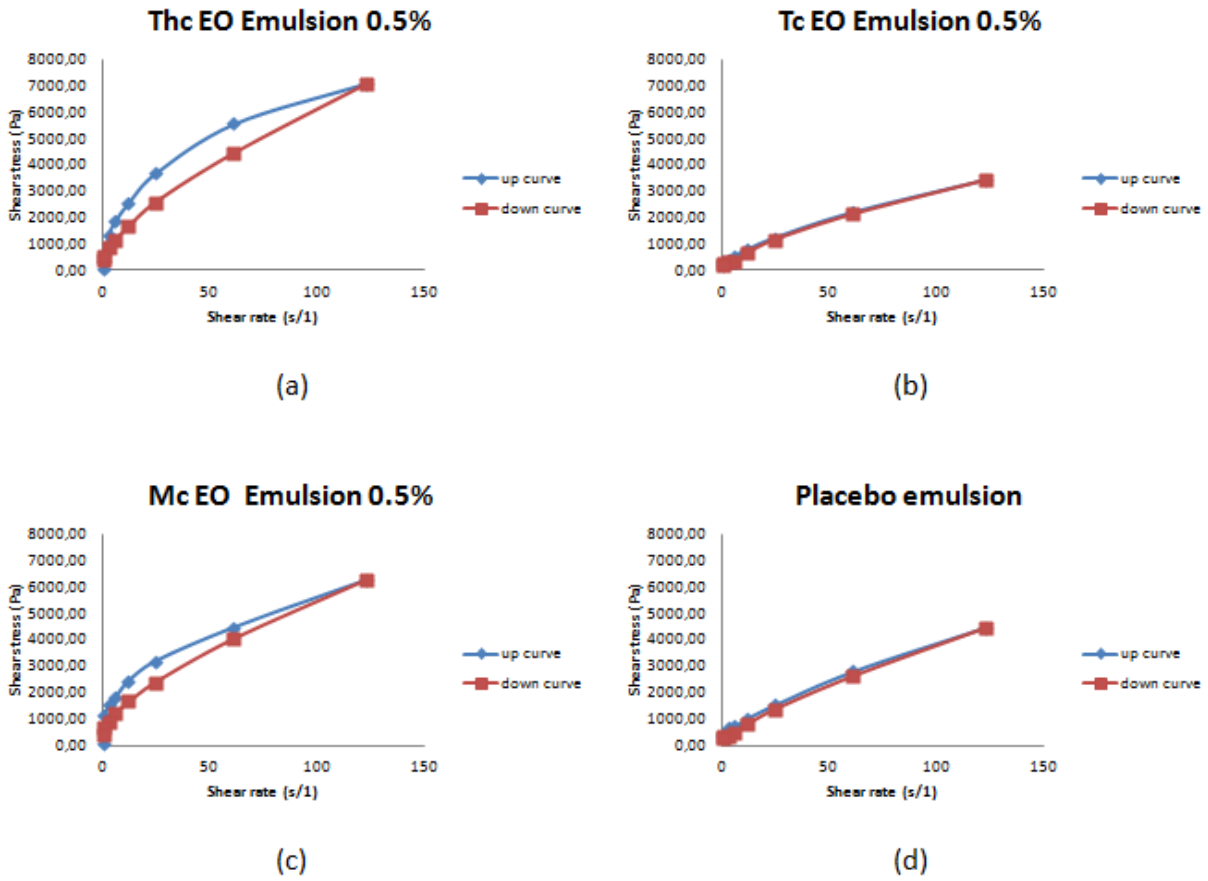

Fig. 3.2 Flow curves. Shear Stress as function of Shear Rate of Thc EO emulsion 0.5% (a), Tc EO emulsion 0.5% (b) Mc EO emulsion 0.5% and placebo emulsion (d).

3.3.3 Phase separation

The seven formulations: placebo emulsions, Thc, Tc and Mc EO emulsion 0.5%, (Fig.3.3) and Thc, Tc EO emulsion 1% (Fig.3.4) were centrifuged for 10 min at 4000 rpm and it was observed phase separation for all formulation with 0.5% of EO and respectively placebo emulsion. However, the formulation with 0.5% of Mc EO presented a less pronounced phase separation. The emulsions containing 1% of EO (Thc, Tc) and respectively placebo emulsion did not separate. For all emulsions, the results are in accordance with physicochemical studies.

However, the two series of emulsions were prepared in different days and, they exhibited different separation behaviours since the changes as the emulsion can be influenced by chemicals, solvents, mixing conditions, size distributions of the droplets, heat, and the vessel used for separation (Sjöblom, 2001).

These results are in accordance with the data obtained from the rheological studies.

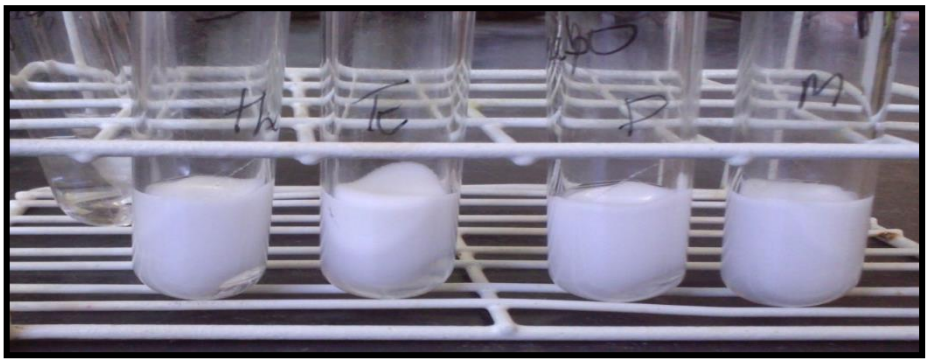

Fig. 3.3 Phase separation for Thc, Tc EO emulsion 0.5%, placebo emulsion and Mc EO emulsion 0.5%, by order.

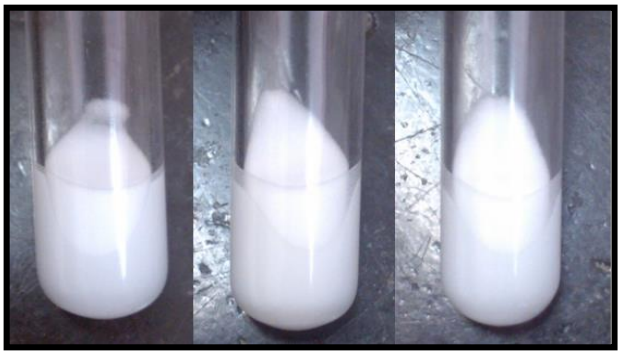

Fig. 3.4 Phase separation fot Thc, Tc EO emulsions 1% and placebo emulsion, by order.

3.3.4 Droplet size analysis

The presence of 1% EO ingredient influences the droplet size distribution. The emulsion series with 1% of EO present a bimodal population (Fig. 3.5).

The droplet size (90% of the droplets) immediately after preparation showed different for three emulsions, 127.57 ± 0.62 µm, 106.30 ± 20.52 µm and 88.79 ± 2.06 µm, for placebo emulsion, Tc and Thc EO emulsion, respectively (Table 3.5). Thc and Tc Eo emulsion 1% had lower droplet size than placebo emulsion and consequently more structure and stability. This data is in accordance with previous results.

Table 3.5 Droplet size distribution of emulsions with 1% of EO (*Th. caespititius* and *T. capitata*) and placebo emulsions, immediately after preparation (n=5, mean ± SD).

Droplet size (µm)	*d*(10)	*d*(50)	*d*(90)	Span
Placebo Emulsion	5.23±0.03	53.78±0.61	127.57±0.62	2.28±0.03
Thc EO emulsion 1%	4.16±0.42	35.38±0.73	88.79±2.06	2.39±0.01
Tc EO Emulsion 1%	3.74±0.57	21.90±1.34	106.30±20.52	4.65±0.62

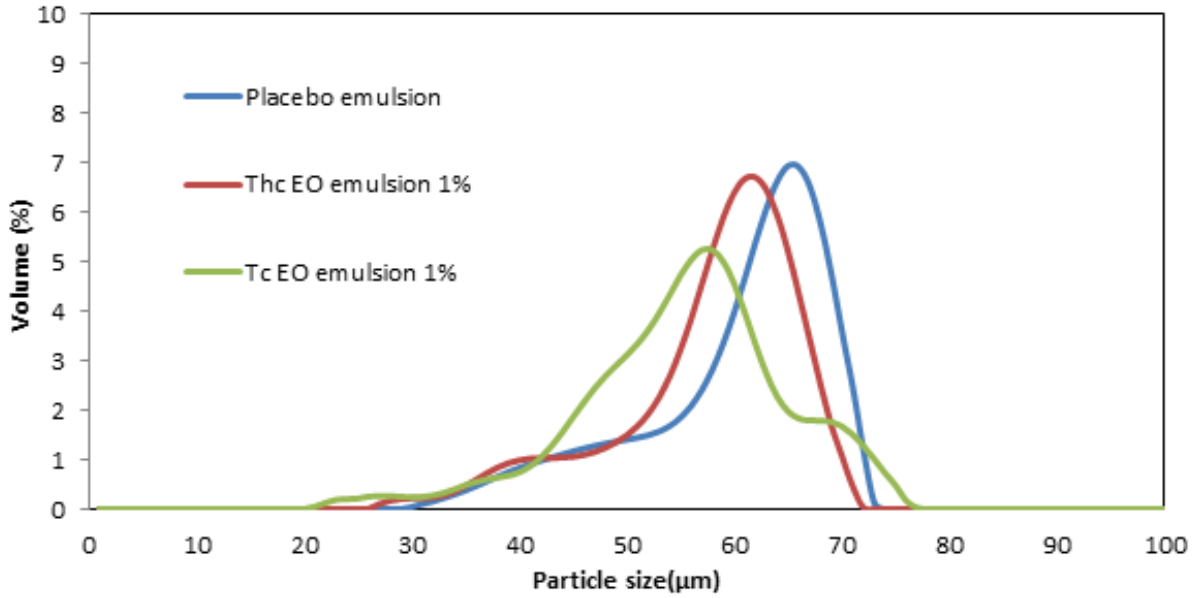

Fig. 3.5 Droplet size distribution of Thc and Tc EO emulsion 1% and placebo emulsion storage at room temperature.

Nonetheless, the reduction in droplet size results can be responsible for the increase in the viscosity and storage modulus of the emulsions, which suggested an enhancement in emulsion stability.

Also, the presence of 0.5% EO ingredient influences the droplet size distribution. The emulsions series with 0.5% of EO present a bimodal population (Fig. 3.6).

90% the droplet size of the placebo emulsion (*d*(90)= 103.12±2.08 µm) had higher than Thc and Mc EO emulsion 0.5% (*d*(90)= 94.72±4.71 µm; 99.38±7.67µm, respectively) (Table 3.6). Revealing that Mc and Thc EO emulsion 0.5% had the better structure than placebo emulsion. However, 90% the droplet size of Tc EO emulsions 0.5% (*d*(90)= 119.41±2.49µm) had higher than placebo emulsion. These results are in accordance with viscosity results.

In general, the three emulsions (Thc, Tc and Mc EO emulsions 0.5%), Tc EO emulsions 0.5% showed the nearest droplet size values of the placebo emulsion. Therefore, this EO had that less influenced the droplet size.

Table 3.6 Droplet size distribution of emulsions with 0.5% of EO (*Th. caespititius*, *T. capitata* and *M. communis*) and placebo emulsions, immediately after preparation (n=5, mean ± SD).

Droplet size (µm)	*d*(10)	*d*(50)	*d*(90)	Span
Placebo Emulsion	9.25±0.15	49.21±0.81	103.12±2.08	1.91±0.01
Thc EO Emulsion 0.5%	7.44±0.97	41.34±2.71	94.72±4.71	2.11±0.05
Tc EO Emulsion 0.5%	11.69±0.33	56.31±1.10	119.41±2.49	1.91±0.00
Mc EO Emulsion 0.5%	5.31±0.58	37.38±3.08	99.38±7.67	2.52±0.03

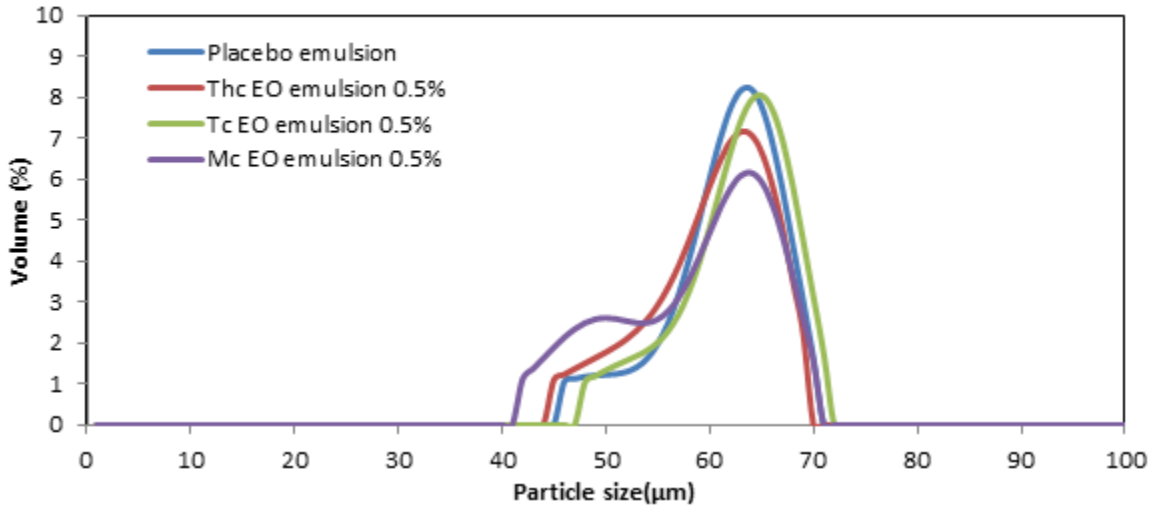

Fig. 3.6 Droplet size distribution of Thc, Tc and Mc EO emulsion 0.5% and placebo emulsion storage at room temperature.

3.4 Activity antimicrobial

3.4.1 Characterization of bacterial susceptibility profile to study essential oils

The disk diffusion method was used as a screening method and it is considered a standard method to evaluate the bacterial susceptibility to antimicrobial compounds. This method allowed verifying the presence or absence of inhibition zone that is an indirect measure of the EO to inhibit or not the

bacterial growth. The antibacterial activity of the Tc, Thc and Mc EO were used at 1:100 dilution DMSO and also without dilution in selected strains (*P. aeruginosa* ATCC 9027 and *S. aureus* ATCC 6538) (Fig. 3.7).

The values of the inhibition zones (mm) obtained for *P. aeruginosa* and *S. aureus* are shown in Table 3.7.

Table 3.7 Bacterial susceptibility profile to Portuguese *Th caespititius, T. capitata* and *M. communis* essential oils.

Test microorganisms	***Pseudomonas aeruginosa***	***Staphylococcus aureus***
Thc EO	-	+++
Tc EO	++	+++
Mc EO	-	++
Thc EO (1:100)	-	+
Tc EO (1:100)	-	+
Mc EO (1:100)	-	-
DMSO*	-	-

*DMSO was used as a negative control. Inhibition zone diameter in mm. Inhibition area: (-) < 7mm, (+) 7-10mm, (++) 11-16mm, (+++) >16mm. Assay done in duplicate for each test compound and the results shown are the average of these values.

EO of *T. capitata*, *Th. caespititius* and *M. communis* showed antibacterial activity against *S. aureus.* The inhibition diameters were 29, 17, 13 mm, respectively. Concerning EO diluted in DMSO, just Tc EO presented an inhibitor zone: 11mm.

Disks with DMSO did not observed inhibition zones on two bacteria under study, so there is no inhibition of bacterial growth. *P. aeruginosa* only was susceptible to the EO of Tc (12mm) (Fig 3.7).

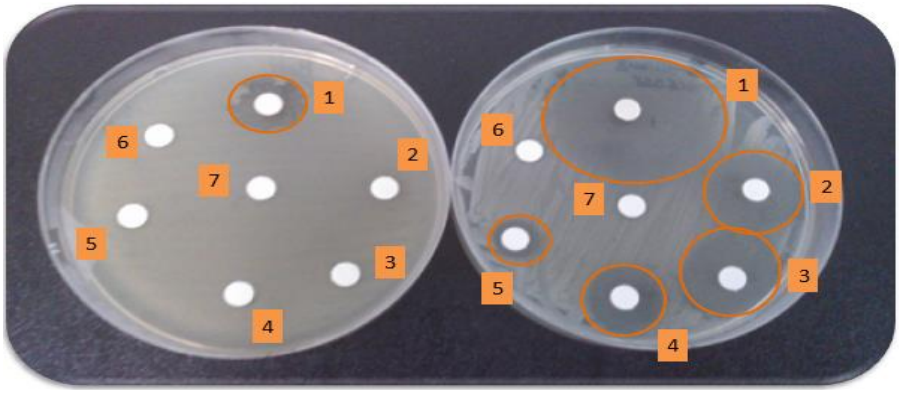

Fig. 3.7 Disk diffusion method. *P. aeruginosa* plate in left and right of the plate *S. aureus.* 1- Tc EO; 2- Thc EO; 3- Mc EO; 4- Tc EO (1:100); 5- Thc EO (1:100); 6- Mc EO(1:100); 7- DMSO.

3.4.2 Evaluation of antimicrobial activity

The method of microdilution plate (quantitative method) was used to determine the MIC all EO under study. It was also tested the bactericidal or bacteriostatic activity of the different EO.

The test was performed at an initial concentration of 100μg/mL for Thc, Tc and Mc EO; and after it was tested the activity Thc and Tc EO diluted in DMSO (1:100). Dermosoft® OMP, benzyl alcohol and DMSO were used as controls, without dilution.

Dermosoft® OMP (INCI: Methylpropanediol, Caprylyl Glycol, Phenylpropano) is a combination of alcohols to improve the spreadability of ingredients in cosmetics and to regulate the viscosity of transparent products. Currently, it is a widely used ingredient provides for many applications, including a strong antimicrobial activity. Benzyl alcohol may be a natural constituent of some EO or it can be produced chemically. Now, benzyl alcohol is defined as preservative and simultaneously appears in the list of ingredients with restriction in the Regulation 1223/2009, annex III. These characteristics, present in Dermosoft® OMP and benzyl alcohol, were the reason for their use as controls in this study.

Table 3.8 Antimicrobial activity (MIC) of the essential oils from *Thymus caespititius, Thymbra capitata* and Dermosoft® OMP and benzyl alcohol against yeast, fungi and bacteria Gram-positive and Gram-negative.

	Strains	**C.a[1]**	**A.b[2]**	**S.a[3]**	**S.a[4]**	**P.a[5]**	**P.a[6]**	**E.c[7]**	**K.p[8]**
Thc EO	MIC[a]	<0.4	<0.4	<0.4	<0.4	>500.0	>500.0	3.3	7.5
Tc EO	MIC[a]	<0.4	<0.4	<0.4	<0.4	7.5	61.3	<0.4	0.8
Dermosoft® OMP	MIC[a]	>500.0	125.0	30.7	30.7	61.3	61.3-125.0	30.7	61.3
Benzyl alcohol	MIC[a]	<0.4	125.0	30.7	30.7	30.7	15.0-30.7	30.7	15.0

Thc EO –*Th. caespititius* essential oil; Tc EO –*T. capitata* essential oil.

C.a[1] *Candida albicans* ATCC 10231; A.b[2] *Aspergillus brasiliensis* ATCC 16404; S.a[3] *Staphylococcus aureus* ATCC 6538; S.a[4] *Staphylococcus aureus* ATCC 43866; P.a[5] *Pseudomonas aeruginosa* FFUL 1401; P.a[6] *Pseudomonas aeruginosa* ATCC 9027; E.c[7] *Escherichia coli* 5ECX CTX-M-35 FFUL 3889; K.p[8] *Klebsiella pneumoniae* FFUL 2320. [a] MIC were determined by microdilution method and expressed in μg/mL (w/v).

Thc EO had better activity for Gram-positive than Gram-negative bacteria. *S. aureus* ATCC 6538 and *S. aureus* ATCC 43866 showed MIC values were equal for the MBC values (<0.4 μg/mL) showing the bactericidal activity. Among the Gram-negative bacteria, *E.coli* was the most susceptible with

same values for MIC and MBC (3.3 µg/mL), indicating bactericidal activity. For *K. pneumoniae* the MIC values (7.5 µg/mL) were lower than MBC values (15 µg/mL), indicating a bacteriostatic activity. *P. aeruginosa* FFUL 1401 and *P. aeruginosa* ATCC 9027 were the less susceptible (Table 3.8). *C. albicans* and *A. niger brasiliensis* were susceptible to the Thc EO with MIC values of <0.4 µg/mL for both. When the Thc EO was diluted in DMSO (1:100) and tested in *S. aureus* ATCC 6538 and *P. aeruginosa* FFUL 1401 and the best result was for first one (Table 3.9). These results are in accordance with previous studies: the Thc EO exhibited a broad-spectrum antifungical activity, including: *Candida* spp., *Aspergillus* spp. and dermotophytes (Pinto *et al.*, 2014).

Tc EO was showed better activity for Gram-positive than Gram-negative bacteria. For the Gram-positive, MIC values were equivalent to the MBC values (<0.4 µg/mL), indicating a bactericidal activity. Among the Gram-negative, *E.coli* was the bacteria more susceptible (Table 3.8), while *K. pneumoniae* and the *P. aeruginosa* FFUL 1401 the MIC and MBC values were equal (0.8 and 7.5 µg/mL, respectively), indicating a bactericidal activity. The MIC for *P. aeruginosa* ATCC 9027 was 61.3 µg/mL and the MBC values was 250 µg/mL, indicating bacteriostatic activity. Tc EO showed efficacy against *C. albicans* and *A. brasiliensis* even when diluted in DMSO (1:100) (Table 3.9). These results from the activity of the Tc EO upon *C. albicans* ATCC 10231 and *A. brasiliensis* ATCC 16404 are in accordance with previous reports for these species grown in Portugal (Salgueiro *et al.*, 2004; Figueiredo *et al.*, 2008; Oliveira *et al.*, 2013). Comparatively to other studies on the EO from *T. capitata* collected in different geographical locations of Mediterranean area, were more active against Gram-positive strains than Gram-negative strains, and had a strong effect against yeast, *C. albicans* ATCC 10231 (Casiglia *et al.*, 2015).

Dermosoft® OMP showed better results against Gram-positive than Gram-negative bacteria. Concerning *S. aureus* ATCC 6538 it was observed the same MIC and MBC indicating bactericidal activity. While for *S. aureus* ATCC 43866, 30.7 µg/mL and 125 µg/mL were the values obtained for MIC and MBC, respectively, indicating bacteriostatic activity. Among Gram-negative, *E.coli* and *K. pneumoniae* showed the same values from MIC and MBC (30.7; 61.3 µg/mL, respectively). *P. aeruginosa* FFUL 1401 and *P. aeruginosa* ATCC 9027 had MIC values of (61.3-125 µg/mL) that were lower than MBC values (125-250 µg/mL), indicating the bacteriostatic activity. For the *A. brasiliensis* and *C. albicans* the MIC values of (125; >500 µg/mL, respectively) were high, showing a weak effect

against fungi.

Benzyl alcohol effective against *K. pneumoniae* and *E.coli* with MIC and MBC values equal (15; 30.7 µg/mL, respectively), indicating a bactericidal activity. *P. aeruginosa* ATCC 9027 and *P. aeruginosa* FFUL 1401 the MIC values (15-30.7 µg/mL) was lower than MBC (61.3 µg/mL), indicating a bacteriostatic activity. For Gram-positive bacteria, *S. aureus* ATCC 6538, the MIC and MBC were the same values (30.7 µg/mL); and the *S. aureus* ATCC 43866 the MIC was lower than MBC values (30.7; 125 µg/mL, respectively). The benzyl alcohol was effective for the *A. brasiliensis* than *C. albicans* (Table 3.8).

Table 3.9 Antimicrobial activity (minimum inhibitory concentration) of the EO from *Th. caespititius*, *T. capitata* diluted in DMSO (1:100) and *M. communis* EO against *S. aureus* ATCC 6538 and *P. aeruginosa* ATCC 9027.

Strains	*Staphylococcus aureus* ATCC 6538	*Pseudomonas aeruginosa* ATCC 9027
Thc EO (1:100)	1.6	125.0
Tc EO (1:100)	<0.4	61.3
Mc EO	30.7	125.0

Thc EO (1:100) – Essential oil of *Th. caespititius* diluted in DMSO (1:100); Tc EO (1:100) – Essential oil of *T. capitata* diluted in DMSO (1:100); Mc EO - *M. communis* essential oil.

[a] MIC were determined by microdilution method and expressed in µL/mL (v/v).

M. communis EO was tested against *S. aureus* ATCC 6538 and *P. aeruginosa* ATCC 9027. The results showed better activity for Gram-positive than Gram-negative bacteria (Table 3.9). In other studies, for example the *Algerian myrtle* EO present higher activity against dermatophytes than *Candida* spp. and *Aspergillus* spp. (Bouzabata *et al.*, 2015).

3.5 Efficacy of antimicrobial preservation

The evaluation of preservatives efficacy for EO to include in a pharmaceutical formulation was performed according to challenge test described in Portuguese Pharmacopoeia 9. It was expected that the EO will decrease over testing the number of colony forming units (CFU), meeting the acceptance criteria described in Portuguese Pharmacopoeia 9 (Table 2.7). Initially, the test of efficacy of antimicrobial preservation was performed in O/W emulsion with 1% of EO from Thc and Tc (Table

3.10).

Table 3.10 Efficacy of antimicrobial preservation of Thc EO emulsion 1% and Tc EO emulsion 1% as preservatives.

		***P. aeruginosa*[1]**	***S. aureus*[2]**	***C. albicans*[3]**
0 days	Thc EO emulsion 1%	10^6	10^6	10^6
	Tc EO emulsion 1%	10^6	10^6	10^6
	Placebo	10^6	10^6	10^6
2 days	Thc EO emulsion 1%	0	0	0
	Tc EO emulsion 1%	0	0	10^2
	Placebo	10^6	10^6	10^6
7 days	Thc EO emulsion 1%	0	0	0
	Tc EO emulsion 1%	0	0	0
	Placebo	10^6	10^6	10^6
14 days	Thc EO emulsion 1%	0	0	0
	Tc EO emulsion 1%	0	0	0
	Placebo	10^6	10^6	10^6
28 days	Thc EO emulsion 1%	0	0	0
	Tc EO emulsion 1%	0	0	0
	Placebo	10^6	10^6	10^6

P.aeruginosa[1] – *Pseudomonas aeruginosa* ATCC 9027; *S. aureus*[2] - *Staphylococcus aureus* ATCC 6538; *C. albicans*[3] – *Candida albicans* ATCC 10231. * The efficacy of antimicrobial preservatives was expressed in CFU/mL. 0 - no growth.

The Gram-positive and Gram-negative bacteria were the most susceptible for both preservatives, with microbial growth to reduce to 6 Log as early as 48h. For the yeast, *C. albicans* was agreed with acceptance criteria (Table 2.7). The placebo had no anti microbial activity against bacteria and fungi. These results (Table 3.10.) were satisfactory and were in accordance with the acceptance criteria, so the tests were performed with Thc, Tc and Mc EO emulsion 0.5% (Table 3.11).

In general, the fungi were more sensitive to Mc EO emulsion 0.5% than the Thc or Tc EO emulsion 0.5%. For the *C. albicans* the Mc EO was in accordance with acceptance criteria (Table 2.7). The placebo had no anti microbial activity against bacteria and fungi. The Gram-negative and Gram-positive bacteria were susceptible ranging of 2 days to 28 days for all EO in study and the results for bacteria (Table 3.11) were in accordance with acceptance criteria. The present study demonstrates that only Mc EO meets the acceptance criteria of challenge test for all microorganisms tested (Gram-

positive, Gram-negative bacteria and fungi).

Table 3.11 Efficacy of antimicrobial preservation of Thc EO emulsion 0.5% and Tc EO emulsion 0.5% and Mc EO emulsion 0.5% as preservatives.

		***P. aeruginosa*[1]**	***S. aureus*[2]**	***C. albicans*[3]**	***A. brasiliensis*[4]**
0 days	Thc EO emulsion 0.5%	10^6	10^6	10^6	10^6
	Tc EO emulsion 0.5%	10^6	10^6	10^6	10^6
	Mc EO emulsion 0.5%	10^6	10^6	10^6	10^6
	Placebo	10^6	10^6	10^6	10^6
2 days	Thc EO emulsion 0.5%	0	0	10^6	10^4
	Tc EO emulsion 0.5%	0	0	10^6	10^4
	Mc EO emulsion 0.5%	0	0	10^4	10^4
	Placebo	10^6	10^6	10^6	10^6
7 days	Thc EO emulsion 0.5%	0	0	10^4	10^2
	Tc EO emulsion 0.5%	0	0	10^4	10^2
	Mc EO emulsion 0.5%	0	0	0	0
	Placebo	10^6	10^6	10^6	10^6
14 days	Thc EO emulsion 0.5%	0	0	10^4	10^2
	Tc EO emulsion 0.5%	0	0	10^4	10^2
	Mc EO emulsion 0.5%	0	0	0	0
	Placebo	10^6	10^6	10^6	10^6
28 days	Thc EO emulsion 0.5%	0	0	0	10^2
	Tc EO emulsion 0.5%	0	0	10^2	10^2
	Mc EO emulsion 0.5%	0	0	0	0
	Placebo	10^6	10^6	10^6	10^6

P. aeruginosa[1] – *Pseudomonas aeruginosa* ATCC 9027; *S. aureus*[2] - *Staphylococcus aureus* ATCC 6538; *C. albicans*[3] – *Candida albicans* ATCC 10231; *A. brasiliensis* [4] – *Aspergillus brasiliensis* ATCC 16404. 0 - no growth.

* The efficacy of antimicrobial preservatives was expressed in CFU/mL.

Chapter 4. CONCLUSION

The increasing need for alternatives to current preservatives, as well as the need created by the consumer of cosmetic products with natural ingredients, lead to look for alternatives that guarantee also a long shelf life to the finished cosmetic product. The present study meets this reality and evaluated the EOs of three Portuguese plants: *Thymus caespititius* (Thc), *Thymbra capitata* (Tc) and *Myrtus communis* (Mc), as an alternative to current preservatives.

For the three plants study, the extraction and analysis of the EO are performed successfully. The main constituents are: α–terpineol, carvacrol and 1,8-cineole, for Thc, Tc and Mc EO, respectively. These results are in agreement with other studies performed in plants with the same occurrence and with the same extraction and analysis conditions.

Thc, Tc and Mc EO are used as preservatives of the O/W emulsions. Same emulsions are prepared with 1% of Thc or Tc EO and another emulsions are prepared with 0.5% of Thc, Tc and Mc EO. For all, the results of pH are within limits pH values for topical application to healthy skin products. The results of viscosity , droplet size and phase separation are in accordance.

According to law (Regulation 1223/2009, annex III), the allergens (linalool, limonene, eugenol and citonellol) present in Thc and Tc EO must be indicated in the list of ingredients of the cosmetic products.

All EOs (Thc, Tc and Mc) show efficacy against Gram-positive and Gram-negative bacteria and fungi. The challenge test results show the Thc and Tc EO emulsion 1% and Thc, Tc and Mc EO emulsion 0.5% are in accordance with acceptance criteria A (reduction 2 Log_{10}) for fungi and bacteria.

In conclusion, it is possible to use the Thc and Tc EO as preservatives in topical cosmetic formulations. Although, Mc EO show efficacy against microorganisms, the percentage of the allergen, methyl eugenol, exceed the maximum concentration allowed for law. Thus, it would be interesting to study the mix of Thc or Tc EO with Mc EO, as well as study different concentrations of EO.

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