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Chemical characterization and bioactivity of commercial essential oils obtained from Portuguese logging residues and thinnings

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Abstract

Nowadays, the demand of cosmetics with sustainable and natural origin ingredients is a common practice in the cosmetic industry. Essential oils (EOs) and hydrolates are natural sources of biologically active ingredients because of their chemical composition and broad application. In this study, national producers (mainland Portugal and Azores archipelago) EOs (11) and hydrolates (7) obtained from forest logging and thinning of *Eucalyptus globulus*, *Pinus pinaster*, *Pinus pinea* and *Cryptomeria japonica*, were chemically evaluated, and their bioactivity and sensorial properties were assessed.

EOs and hydrolates volatiles (HVs) were analyzed by gas chromatography and gas chromatography-mass spectrometry. 1,8-Cineole was *E. globulus* EOs and HVs dominant compound, while α- and β-pinene dominated *P. pinaster* EOs. Limonene and α-pinene were predominant in *P. pinea* and *C. japonica* EOs, respectively. *P. pinaster* and *C. japonica* HVs were dominated by α-terpineol and terpinene-4-ol, respectively.

Antioxidant activity was determined by radical scavenging ability of 1,1-diphenyl-2-picrylhydrazyl (DPPH), oxygen radical absorbance capacity (ORAC) and intracellular ROS measurement. *C. japonica* EO showed the highest antioxidant activity, whereas one of *E. globulus* EOs showed the lowest activity.

Antimicrobial activity was evaluated by the microdilution plate method determining the minimum inhibitory concentration for *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 9027, *Bacillus subtilis* ATCC 6633, *Escherichia coli* TCC 8739, *Candida albicans ATCC* 10231 and *Aspergillus brasiliensis* ATCC 16404. *Eucalyptus* EOs showed the greatest efficacy against the selected strains while *C. japonica* EO had no antimicrobial activity against these strains. *P. pinea* EO had similar efficacy against *B. subtilis* ATCC 6633 as two of *E. globulus* EOs.

Sensory double-blind evaluation, approved by the local Ethical Committee, was performed in human volunteers. A structured questionnaire was used to collect data about the perception and applicability of different emulsions perfumed with 0.5% of EOs. *C. japonica* emulsion, which has a fresh and earthy odour, was chosen as the most pleasant by 60% of the volunteers, followed by *P. pinea* emulsion odour with 53%. *C. japonica* emulsion was also the one chosen for improving the sense of well-being. Overall, 19% of the volunteers selected *C. japonica* and *P. pinea* emulsions as their favorite ones.

The studied EOs and Hds showed relevant antioxidant activity and promising antimicrobial activity. Moreover, they address the demand for sustainable and responsibly sourced odour accepted by consumers.

Keywords: Essential Oils; Hydrolates; Antioxidant; Antimicrobial; Sensory evaluation.

Resumo

Atualmente, o interesse por cosméticos com ingredientes de origem sustentável e natural é uma realidade na indústria cosmética. Os óleos essenciais (OEs) e hidrolatos (Hds) são fontes naturais de ingredientes biologicamente ativos devido à sua composição química e ampla aplicação. Neste trabalho, os onze OEs e sete Hds de produção nacional (Portugal Continental e do arquipélago dos Açores), obtidos de sobrantes de operações de desbaste e corte de *Eucalyptus globulus* Labill, *Pinus pinaster* Aiton, *Pinus pinea* L. e *Cryptomeria japonica* D. Don, foram analisados quimicamente e avaliados quanto a bioatividade e propriedades sensoriais.

Os OEs, e os respetivos voláteis dos Hds (VHs), obtidos de *E. globulus*, *P. pinaster*, *P. pinea* e *C. japonica* foram isolados das folhas, agulhas e folhagem das plantas por destilação por arrastamento de vapor, analisados por cromatografia gasosa para a quantificação dos seus componentes, e por cromatografia gasosa acoplada a espectrometria de massa para a identificação dos compostos. Os OEs isolados são complexas misturas, nas quais foram identificados entre 44 a 49 constituintes no *E. globulus*, entre 63 a 68 no *P. pinaster*, 50 no *P. pinea* e 79 na *C. japonica*. Nos VHs das amostras de *E. globulus* foram identificados entre 46 a 58 constituintes, 38 e 42 no *P. pinaster* e 44 no *C. japonica*. Monoterpenos oxigenados foi a classe dominante de compostos no OE e nos VHs de *E. globulus*, 56-72% e 84-92%, respetivamente. Os hidrocarbonetos monoterpénicos variaram entre 24-43% neste OE e 2-14% nos VHs enquanto que os sesquiterpenos variaram de 0.2 a 6% no OE e os sesquiterpenos oxigenados entre vestigial a 1% nos VHs. Além disto, os monoterpenos oxigenados também foram a classe predominante nos VHs de *P. pinaster* e *C. japonica*, 95 e 79%, respetivamente. Os hidrocarbonetos monoterpénicos variaram de vestigial a 4% para os VHs de *P. pinaster* e nos VHs de *C. japonica* estiveram presentes em 2%. Sesquiterpenos oxigenados foram identificados nos VHs de *P. pinaster* e *C. japonica* em valores vestigiais e de 10%, respetivamente. Além disto, fenilpropanóides estavam presentes em valores vestigiais nos VHs de *P. pinaster*. Hidrocarbonetos diterpénicos, foram identificados nos VHs de *C. japonica* (5%). Os OEs de *P. pinaster*, *P. pinea* e *C. japonica* foram, predominantemente, compostos por hidrocarbonetos monoterpénicos (70 a 82%, 88% e 66%, respetivamente). Monoterpenos oxigenados foram identificados em menor concentração, 1-3%, 7% e 6%, respetivamente. Os sesquiterpenos variaram de 0.1 a 23% para o OE de *P. pinaster*, de 1 a 4% no OE de *P. pinea* e de 2-8% no OE *C. japonica*. Diterpenos e fenilpropanóides foram identificados no OE de *C. japonica* em 17% e em valores vestigiais, respetivamente. 1,8-Cineole, foi o composto dominante no OE e nos VHs de *E. globulus*, enquanto α- e β-pineno dominaram no OE de *P. pinaster*. Limoneno e α-pineno foram os principais compostos presentes nos OEs de *P. pinea* e *C. japonica*, respetivamente. Nos VHs de *P. pinaster* e *C. japonica*, os compostos dominantes foram α-terpineol e terpinen-4-ol, respetivamente.

A atividade antioxidante dos OEs e Hds foi determinada utilizando-se a capacidade de eliminação do radical 1,1-difenil-2-picrilhidrazil (DPPH), a capacidade de absorção do radical de oxigénio (ORAC) e a medição intracelular de espécies reativas de Oxigénio (ROS). No ensaio DPPH os OEs apresentaram fraca atividade antioxidante, exceto o de *C. japonica* que apresentou o menor valor de IC50, seguindo-se de uma das amostra de OE de *P. pinaster*. A atividade antioxidante demonstrada pelo OE de *C. japonica* pode ser justificada pela presença, em menores quantidades, de compostos antioxidantes como o sabineno, β-mirceno e γ-terpineno. Por outro lado, a atividade antioxidante contra o radical DPPH, apresentada pelo OE de *C. japonica*, pode dever-se à presença de compostos como o terpinen-4-ol, β-pineno e limoneno, conhecidos pelas suas propriedades antioxidantes. Os Hds da *C. japonica* não apresentaram atividade contra o radical DPPH. A atividade antioxidante contra o radical DPPH, apresentada pela amostra de OE de *P. pinaster* pode dever-se a uma elevada concentração de β-mirceno,

anteriormente descrito como tendo atividade antioxidante. No método do ORAC os OEs apresentaram maior capacidade antioxidante, com ênfase para uma amostra de *P. pinaster* e para o OE de *C. japonica*. A atividade antioxidante determinada por esta metodologia foi demonstrada por uma das amostras de OE de *P. pinaster* o que poderá ser justificado pela presença de compostos antioxidantes, em menores quantidades, como o β-cariofileno. Por outro lado, os Hds demonstraram ter atividade antioxidante, embora menor. Uma das amostras de Hd de *E. globulus* foi a que apresentou maior capacidade antioxidante, devido provavelmente à presença do limoneno, presente numa maior percentagem quando comparado com as restantes amostras. No ensaio de medição intracelular de ROS, os OEs apresentaram baixa capacidade de redução da percentagem de ROS quando comparados com as amostras de Hds. Contudo, o OE de *C. japonica* apresentou uma maior atividade antioxidante de redução de % de ROS, comparativamente aos restantes e, por outro lado, uma das amostras de OE de *E. globulus* foi a que teve a menor atividade. Relativamente aos hidrolatos, o Hd de *C. japonica* apresentou a melhor capacidade antioxidante provavelmente devido ao seu principal componente, o terpinen-4-ol, que revelou capacidades antioxidantes noutros estudos.

Os valores da concentração inibitória mínima (MIC) para os OEs e respetivos Hds permitiu determinar a atividade antimicrobiana pelo método da microdiluição em placa. Utilizaram-se as estirpes de *Staphylococcus aureus* ATCC 6538, *Bacillus subtilis* ATCC 6633, *Pseudomonas aeruginosa* ATCC 9027, *Escherichia coli* ATCC 8739, *Candida albicans* ATCC 10231 *e Aspergillus brasiliensis* ATCC 16404. Em relação às bactérias gram-positivas e negativas, observou-se que o *Staphylococcus aureus* apresentou uma maior resistência contra a maioria dos OEs; *Bacillus subtilis* mostrou-se bastante suscetível aos OEs, sobretudo para uma das amostras de *E. globulus*; *Pseudomonas aeruginosa* foi extremamente resistente aos OEs e, finalmente, *Escherichia coli* foi a mais suscetível sobretudo às amostras de OE de *E. globulus*. As amostras de OE de *E. globulus* foram mais eficazes contra leveduras, apresentando melhor atividade anti-levedura que *P. pinaster* e *P. pinea*. Os OEs não demonstraram atividade antifúngica contra a estirpe *Aspergillus brasiliensis*. O OE de *C. japonica*, e uma amostra de OE de *P. pinaster*, não apresentaram propriedades antimicrobianas, anti-leveduras e antifúngicas contra nenhuma das estirpes selecionadas. Por outro lado, nenhum dos Hds apresentou atividade antimicrobiana. Assim, os OEs de eucalipto apresentaram maior eficácia contra as estirpes selecionadas, enquanto o OE de *C. japonica* não apresentou atividade antimicrobiana contra essas estirpes. O OE de *P. pinea* teve eficácia idêntica a dois OEs de *E. globulus* contra a *B. subtilis* o que pode estar relacionado com o conteúdo em 1,8-cineol.

A avaliação sensorial de emulsões de aplicação cutânea com 0.5% de cada um dos OEs foi realizada em 100 voluntários após aprovação do estudo pelo Comité de Ética local. Um questionário estruturado foi usado para avaliar a perceção e a aplicabilidade de diferentes emulsões perfumadas. A emulsão de *C. japonica*, caracterizada por apresentar um odor fresco e terroso, foi escolhida como a mais agradável por 60% dos voluntários, seguida da emulsão de *P. pinea* responsável por 53% das respostas. Por outro lado, a emulsão de *E. globulus* foi considerada como tendo o odor mais intenso, em comparação com as restantes, e tendo sido identificada como sendo a menos agradável (36%). A emulsão perfumada com *C. japonica* também foi a escolhida por dar a melhor a sensação de bem-estar. No geral, 19% dos voluntários selecionaram as emulsões contendo OEs de *C. japonica* e *P. pinea* como as suas favoritas. Estes resultados estão relacionados com a composição química dos OEs.

De uma forma resumida, e perante os resultados obtidos, pode-se concluir que os OEs e Hds avaliados possuem atividade antioxidante relevante e atividade antimicrobiana promissora. Além disto, foram bem aceites pelo painel de voluntários o que permite a sua utilização como ingredientes naturais e sustentáveis em produtos cosméticos.

Palavras-chave: Óleos Essenciais; Hidrolatos; Antioxidante; Antimicrobiano; Avaliação sensorial.

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List of Abbreviations

AAPH - 2,2'Azobis (2-methylpropionamidine) dihydrochloride

ANOVA – Analysis of variance

AUC – Net area under the curve

BHA - Butylated hydroxyanisole

BHT - Butylhydroxytoluene

BTW – Buffered peptone water

CFU - Colony-forming nits

DCF - Dichlorodihydrofluorescein

DCFH 2-DA – 2 ′,7 ′ - Dichlorodihydrofluorescein diacetate

DCFH 2 - 2',7' - Dichlorodihydrofluorescein

DF - Disodium fluorescein

DMC – Dimethyl carbonate

DPPH - 1,1-diphenyl-2-picrylhydrazyl

ET – Electron transfer

EO – Essential oil

EU – European Union

FRAP - Ferric Reducing Antioxidant PPower

GC - Gas chromatography

GC-FID - Gas chromatography with a flame ionization detector

GC-MS - Gas chromatography coupled to mass spectrometry

HAT - Hydrogen atom transfer

Hd - Hydrolate

IBET – Instituto de Biologia Experimental e Tecnológica

IC50 – Antioxidant concentration required to reduce by 50% the initial DPPH radical absorbance

INCI - International Nomenclature of Cosmetics Ingredients

ISO - International Organization for Standardization

MAC – Medicinal, aromatic, and cosmetic plant

MAP - Medicinal and aromatic plant

MH – Müeller-Hinton

MIC - Minimum Inhibitory Concentration

ORAC - Oxygen Radical Absorbance Capacity

PBS - Phosphate-Buffered Saline

RFU – Relative fluorescence units

RI - Retention indices

ROS – Reactive oxygen species

SD – Standard deviation

TEAC - Trolox Equivalent Antioxidant Capacity

Trolox - 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid

UV-Vis - Ultraviolet-visible

Chapter 1. Introduction

Nowadays, consumers have a growing interest in acquiring substances of natural origin. Furthermore, there is a concern with potentially harmful synthetic additives, leading to the use of extracts and essential oils from aromatic and medicinal plants in the pharmaceutical, cosmetic and food industries [1].

1.1 Aromatic and medicinal plants

Medicinal and aromatic plants (MAPs) are defined as the plants used firstly for their medicinal or aromatic properties in pharmacy or perfumery [2]. Due to their medicinal properties MAPs are commonly used, for example, as natural preservatives, promoting human health [3,4]. Furthermore, they can also be used for cosmetic purposes and are called aromatic, and cosmetic plants, with the acronym MAC [2]. The MAPs have several biological properties conferred by the presence of metabolites. These metabolites are obtained as an end product of the metabolic processes of plants and are called secondary metabolites. The physical environment such as light, temperature, precipitation, and soil properties can influence the growth and development of MAPs, and consequently their metabolites [5].

In developing countries, MAPs play an essential role in people's healthcare. Until the appearance of modern medicine, the treatment of human and livestock diseases depended on plants uses. Over centuries, human societies have accumulated vast indigenous knowledge about medicinal uses of plants, such as poison for fish and hunting, purifying water, and controlling pests and diseases of crops and livestock [6]. It is estimated that about 80% of the developing countries population still uses traditional medicines from plants to treat human diseases [7].

MAPs are distributed worldwide, including South and Southeast Asia, America, Europe, Africa, and Australia. More than 7500 species of plants are used in ethnomedicine in Asia. This value is equivalent to about half of the native plant species in the Asian country [8]. In China, 6000 species are used due to their medicinal properties [9], and in the African continent, more than 5000 species of plants are used for the same purposes [10]. Finally, on the European continent, at least about 2000 species of plants are being used, and two-thirds of them are native [5]. Since MAPs are widely distributed throughout the world, they can be classified according to the different climatic zones in which they were cultivated. For this reason, the same plant species may have different chemical properties/active ingredients considering different climates [5].

Solomou *et al* [11] mentioned that based on the way they are used, MAPs could be separated into five distinct groups, namely:

1. *Raw materials for essential oils* (EOs) *extraction*. Raw materials are the primary source of use of MAPs around the world.
2. *Culinary spices*. The non-leaf part of the plant is used as a condiment or seasoning.
3. *Culinary herbs*. Plants leafy or soft flowering parts are used as a condiment or seasoning.
4. *Medicinal group*. Natural or semi-synthetic medicine.
5. *Miscellaneous group*. MAPs used for various purposes such as cosmetics, dyes, disinfectants, etc.

In Portugal, MAPs have gained increasing interest and in a Barata *et al* [12] study with some MAPs producers, in the country, about thirteen taxa were identified with marketing levels above one ton, as shown in **Table 1.1**. Among these MAP producers some were also identified as being EOs producers and it was reported that about five taxa were used for this purpose, with the main dominance for eucalyptus (*Eucalyptus globulus*) (**Table 1.2**). Since 2010, the production of EOs, in Portugal (12968 Kg), has been increasing and from 2017 to 2018 there was a considerable rise of 13467 Kg and 15532 Kg, respectively. The production of *E. globulus* EO, in Portugal, has increased considerably from 2010 to 2018 since in 2010 the recorded production was 12010 Kg and in 2018 it slightly exceeded 15200 Kg [13].

Table 1.1 Representation of the most commercialized cultivated and wild aromatic and medicinal plants (MAPs) in Portugal, in 2010 (>1ton) (Barata *et al* [12])

Plant Species	Quantity commercialized (Kg)
Cultivated	
Lippia triphylla (L'Hérit.) O. Kuntze	13340
Origanum vulgare L.	5160
Lavandula stoechas L.	4200
Melissa officinalis L.	2960
Rosmarinus officinalis L.	2960
Olea europaea L.	1850
Mentha piperita L.	1560
Thymus x citriodorus Schreb.	1480
Cymbopogon citratus Stapf.	1020
Wild	
Eucalyptus globulus Labill	48300
Equisetum telmateia Ehrh.	4450
Pterospartum tridentatum L. Willk.	3300
Centaurium erythraea Rafn	1070

Table 1.2 Representation of the main essential oils (>50 kg) produced in Portugal, in 2010 (Barata *et al* [12])

Plant Species	Essential oil production (Kg)
Eucalyptus globulus Labill	12010
Rosmarinus officinalis L.	715
Lavandula stoechas L.	105
Cistus ladanifer L.	60
Pinus pinaster Ait.	60

1.2 Essential Oils

1.2.1 The Meaning of Essential Oils

The aromatic plants include odorous, volatiles, hydrophobic, and highly concentrated compounds designated by Essential oils (EOs) [14]. Generally, EOs are extracted from plants located in warm and temperate zones as well as in tropical and Mediterranean countries where they form a fundamental part of the traditional pharmacopoeia [3]. The word *essential* derives from oils that are naturally very aromatic and therefore can capture the "*essence*" of the plant from which it was extracted [14]. The EOs consist of a complex mixture of volatile compounds obtained from various parts of aromatic plants as secondary metabolites, such as flowers, buds, seeds, leaves, twigs, bark, wood, fruits and roots [15]. They are volatile, liquid, limpid and rarely coloured, lipid-soluble and soluble in organic solvents with a usually lower density than water [3].

The plants designated as "*essential oil plants*" can obtain EO of commercial interest [16]. A plant is used as an "*essential oil plant*" when it has:

1. A unique mix of volatiles. For example, the rose (*Rosa* spp.) and jasmine (*Jasminum sambac*) are plants that have flower scents. The flowers mentioned can produce and instantly emit the volatiles by the epidermal layers of their petals [17];
2. The capacity of secreting and accumulating volatiles in their specialized anatomical structures. The anatomical storage structures for EOs can be the secretory idioblasts (secretory cells), cavities/ducts, or glandular trichomes [18].

The volatile compounds of the EOs individually, and the EOs as a whole, are fundamental for biomedical or pharmaceutical purposes, considering their antiseptic properties such as bactericide, fungicide, as they can be used as analgesics, sedatives, and anti-inflammatories in medicines [19].

1.2.2 Extraction of Essential Oils

The EOs can be extracted from dry and fresh plant materials and partially dehydrated [20]. EOs extraction occurs from secretory structures that can be located in different parts of the plants, such as in plant leaves (eucalyptus), berries (juniper), roots (vetiver), fruit (orange), wood (cedar), petals (rose) among others [21]. The EOs are generally extracted using conventional techniques such as hydrodistillation using a Clevenger-type extractor which is globally considered the most used technique to isolate the volatiles present in plant oils, being, usually, a laboratory process, and steam distillation method which is considered the most common industrial process used [22]. Together with the EO, two more fractions are obtained, namely, the decoction water and the hydrolate (Hd). The EO obtained from the steam distillation method has been commonly used in pharmacological and food activities [20].

The International Organization for Standardization (ISO) has defined an EO as "the product obtained from a natural raw material of plant origin, by steam-distillation, dry-distillation, or a mechanical process without heating. Usually, EOs are separated from the aqueous phase by a physical process which does not significantly affect their composition" [23].

The EO quality which will be present or not is directly dependent on several factors such as the age of the plants, the parts of the plants used for extraction, the stage of the vegetative cycle, climatic effects and others [20]. The type of extraction is selected according to the intended completion. Several factors can influence the quality, quantity and composition of the products obtained from the extraction process, namely, climate, soil composition, plant organ, age and stage of the vegetative cycle [24]. For this reason and to obtain EOs that have a constant composition, they must be extracted under the same conditions as the organ of the plant that developed in the same soil, under the same climatic conditions, being harvested in the same season of the year [3].

1.2.2.1 Hydrolate: A co-product from the distillation of essential oils

Hydrolates (Hds), also called aromatic distilled waters and hydrosols, result from obtaining EOs by steam distillation and hydrodistillation. The distillation process is based on the evaporation of water together with the EO [25]. In contact with cold pipes, the vapours condense and, consequently, the liquefied components are divided into two distinct phases inside the Florentine collecting vessel, namely, EO and Hd [25]. Hds present small amounts of the constituents of EOs in their constitution, which provides them with specific organoleptic properties and flavour [26].

Hd is a co-product that has a very similar odour to its corresponding EO, although it is much more diluted. Unlike EOs and as they are water-soluble extracts, they can be added to formulas with a high-water content and for this reason it is not necessary to use emulsifying agents or surfactants. Since they are more dilute than EOs, they need to be used in higher amounts, not having the same odour intensity as EOs. Therefore, they can be used to flavour a certain water-based product without affecting its stability. Furthermore, as, unlike EOs, they are co-products that are neither affected by the hot procedure nor oxidized, they do not need an antioxidant substance in their formulations to maintain their odour over time [27].

Hds characteristics make them widely used in various industries such as cosmetics and food, mainly due to their biological properties. Several types of Hds are commercially used, mainly as cosmetic and food ingredients. They have become increasingly popular, especially in the area of aromatherapy [26]. These compounds are promising natural raw materials in many different products [28]. According to their chemical composition of Hds, they usually contain less than 1 g/L (i.e., 0.10%) of EO water-soluble aromatic compounds, which get dissolved in the water phase. Furthermore, Hds that include 0.17% of aromatic compounds that remained after the water fractions are very aromatic, and their composition can be very different from that presented by the corresponding EO [29]. Hds have an acidic pH ranging from 4.5 to 5.5 and may have a pleasant or unpleasant odour. Additionally, there may or may not be a similar odour to the corresponding EO [30].

The amount of water-soluble compounds that the Hds present will influence their quality. Consequently, absolute quantification must be carried out to have quality control and, consequently, avoid the possible occurrence of adulterations that quickly occur in the dilution Hds in water. Hds are mainly composed of volatile compounds belonging to monoterpene alcohols, aldehydes, ketones and sesquiterpene alcohols. Furthermore, they are highly polar (hydrophilic) compounds [31]. The similarities between Hds volatiles and the corresponding EOs are related and mainly dependent on the relationship between hydrocarbons and oxygenated compounds present in EOs. If oxygenated compounds are dominant in the EO, the similarity between the EO and the Hd will be very high. On the other hand, if hydrocarbons are dominant in the EO, the composition of the Hd volatiles will be very different from that of its EO [31]. Usually, in their constitution Hds have some of the water-soluble compounds present in the EO, with other water-soluble plant secondary metabolites [32].

Antimicrobial and antioxidant activities are the main biological activities involved in studies related to Hds. The effective performance of Hds as natural antimicrobial agents is primarily dependent on the absolute amount of their dominant soluble aromatic compounds [25] Furthermore, the antimicrobial properties of Hds may depend on the microbial strain selected and the dilutions made. Usually, a higher sample concentration is required for Hds to have the same inhibitory efficacy as EOs. The antioxidant activity that Hds can have may depend on the methods used for its evaluation. However, several studies have shown a lower antioxidant activity presented by Hds when compared to EOs [31].

1.2.3 Chemical Composition of Essential Oils

The chemical composition of the EOs plant is mainly represented by mono- and sesquiterpene hydrocarbons and their oxygenated derivatives, as well as aliphatic aldehydes, alcohols and esters [33]. The chemical profile of EOs products differs in the number of molecules and the stereochemical types of the molecules extracted considering the type of extraction [24]. The chemical composition of EOs may vary within the same plant species. The variations that EOs present may be related to distinct chemotypes, the adaptation of the plant species to the environment, and their state of development.

The EOs' composition gives EOs their properties and economic value. The considerable advance in studying the chemical composition of EOs was mainly due to the development of chromatography techniques. Gas chromatography (GC) is considered the best chromatography technique in quantifying EOs components, primarily due to its simplicity and efficiency [34]. Several detection systems can be coupled to GC to provide a higher quantitative and qualitative analysis. An example of this is the flame ionization detector (FID) and the mass spectrometer (MS) [35]. The GC-FID can quantitatively determine materials present in low concentrations from the use of the flame ionization detector (FID) [36]. While this technique is traditionally used to quantify EOs and the volatiles of Hds, GC-MS is the most used analytical method for identifying its components [34]. Recently, GC-MS techniques have been increasingly applied in studies related to the analysis of medicinal plants. The GC-MS techniques have proved to be an asset for analysing non-polar components and volatile EOs [36]. When the GC is coupled to the MS, it has a higher resolution power than other traditional detection methods such as the FID. GC-MS has numerous advantages such as, analysing an increased number of analytes, identifying components separated from mass spectra, high sensitivity and a low detection limit [37].

1.2.4 Current Applications of Essential Oils

Nowadays, EOs have been widely used for various purposes. There has been a growing interest from various industries such as pharmaceuticals, cosmetics, and food in using EOs, mainly due to their biological properties such as antifungal, antibacterial and antioxidant [38]. In the European Union (EU), they have been mainly used as flavouring agents in the food industry, in perfumes and aftershaves, in the cosmetic industry and, finally, as functional ingredients in the pharmaceutical industry [39]. About 3000 EOs are known, and approximately 300 have a commercial importance, mainly for the industries mentioned above and for the agronomic, sanitary and perfume industry [3]. **Figure 1.1** demonstrates some more examples of the uses and activities performed by EOs. It is possible to observe that the food industry uses EOs to produce soft drinks and food confectionary. The cosmetic industry uses them in perfumes, skin products and finally, the pharmaceutical industry uses them for their functional properties [14].

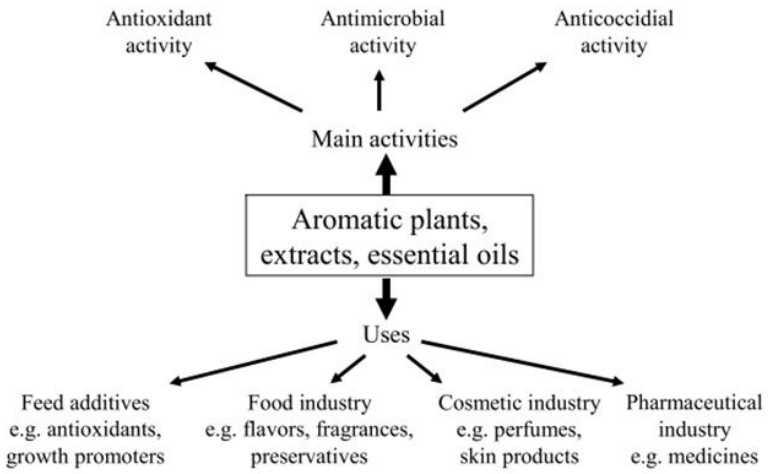

Figure 1.1 Illustration of the activities and uses of aromatic plants, extracts and their essential oils (Christaki e*t al* [40])

1.2.4.1 Therapeutic benefits of Essential oils in health

For centuries, EOs plants and their constituents have been used for medical purposes and have shown several health benefits. **Table 1.3** summarizes some of the potential therapeutic benefits of EOs in human health.

Argania spinosa EO showed in several studies beneficial effects against various diseases, for example, heart disease, cancer and diabetes [41–43]. *Melaleuca alternifolia* EO has been shown to have medical benefits in accelerating the healing of abscessed wounds and cellulitis [44]. Calcabrini *et al.* [45] conducted an *in vitro* study to assess the potential antitumor activity of the *Melaleuca alternifolia* EO as well as its dominant compound, terpinen-4-ol, and found that both showed the ability to impair the growth of human melanoma cells. *Syzygium aromaticum* EO as well as its main compound (eugenol) have been reported to have potential cytotoxic and anticancer properties, for example against colon, gastric, breast, prostate, skin cancer and melanoma and leukaemia [46,47]. Furthermore, clove EO has also been described as having potential anti-diabetic activities [48]. For the first time, in the study of Han *et al.* [49] the biological activity in pre-inflamed human dermal fibroblast of a commercially obtained *Origanum vulgare* EO was evaluated and its potential as anti-inflammatory, wound healing and anticancer was perceived.

Table 1.3 Potential therapeutic benefits of some EOs in human health

Essential Oil	Plant	Active compounds	Properties	References
Argan	*Argania spinosa*	1,10-di-epi-cubenol; viridiflorol and selina-3,7(11)-diene	antiproliferative, antidiabetic, cardiovascular and cancer chemopreventive effects	[41–43]
Tea tree	*Melaleuca alternifolia*	terpinen-4-ol	wound healing and anti-tumoral activity	[44,50]
Clove	*Syzygium aromaticum*	eugenol and eugenyle acetate	cytotoxicity, anticancer, and anti-diabetic activity	[46,51,47]
Oregano	*Origanum vulgare*	carvacrol	antiproliferative, anti-inflammatory, wound healing, and anticancer activity	[49,52]
Cinnamon	*Cinnamomum cassia*	cinnamaldehyde and trans-cinnamaldehyde	antiproliferative, anti-inflammatory, anti-cancer activity	[53,54]
Cumin	*Cuminum cyminum*	α-pinene	anti-diabetic activity	[48]
Lemongrass	*Cymbopogon citratus*	citral	anti-fungal and anti-inflammatory activity	[55,56]
Geranium	*Pelargonium asperum*	geraniol, β-*citronellol*	anti-inflammatory activity	[55]
Spearmint	*Mentha spicata*	carvone	antinociceptive and anti-inflammatory activity	[55,57]
Thyme	*Thymus bovei*	geraniol	cytotoxicity antiherpetic, and antihypertensive activities	[58]

In addition, Avola *et al.* [52] also evaluated the anti-inflammatory capacity of the EO from aforementioned species, as well as its wound healing capacity, concluding that it was effective in treating inflammation and wound healing. *Cinnamomum cassia* EO was investigated for its anti-inflammatory effects as well as its active compound (cinnamaldehyde) and it was demonstrated that its anti-inflammatory capacity is justified by the presence of this compound [54]. On the other hand, Yang *et al.*

[53] evaluated the anticancer potential of the same EO and revealed *in vitro* an anticancer capacity.

Tahir *et al.* [48] reported for the first time the antidiabetic potential of *Cuminum cyminum* EO. *Cymbopogon citratus*, *Pelargonium asperum* and *Mentha spicata* EOs revealed *in vitro* anti-inflammatory capabilities against neutrophil activation [55]. Boukhatem *et al.* [56] analysed *in vivo* and *in vitro*, respectively, the anti-inflammatory and anti-fungal capacity of the EO from *Cymbopogon citratus.* Its potential in the treatment of fungal infections as well as skin inflammations was demonstrated. In a study of Mogosan *et al.* [57], it was demonstrated that *Mentha spicata* EO has anti-inflammatory capabilities and antinociceptive properties. Both anti-inflammatory and antinociceptive properties have been associated with a high carvone content. Finally, it was demonstrated that *Thymus bovei* EO have several cytotoxic properties against human cervical carcinoma cells, colon cancer cells and lung adenocarcinoma cells. Furthermore, its main active compound, geraniol, has shown potential inhibitory capabilities and is therefore considered to be promising in the treatment of herpes and hypertension. Both *Thymus bovei* EO and geraniol were considered as possible cytotoxic, antiherpetic and antihypertensive agents [58].

1.2.4.2 Essential oils applications in Cosmetics

In the last decade, there has been a growing interest in consumer demand for cosmetic products obtained from natural sources [59]. The Regulation (EC) No 1223/2009 from the European Parliament defines cosmetics as "any substance or mixture intended to be placed in contact with the various external parts of the human body (epidermis, hair system, nails, lips and external genital organs) or with the teeth and the mucous membranes of the oral cavity with a view exclusively or mainly to cleaning them, perfuming them, changing their appearance, protecting them, keeping them in good condition or correcting body odours" [60]. EOs as well as their individual compounds have a variety of benefits, such as, fragrance, antioxidant, antimicrobial, and because of that they have been widely used in cosmetic products [61,62]. Generally, fatty acids, fatty oils and surfactants that are used in the elaboration process of cosmetic products have an unpleasant odour. Thus, the main interest in using EOs in cosmetics is their pleasant odour [61]. Cosmetics can be grouped by product use into some distinct classes, namely: 1) skin care and maintenance; 2) cleansing; 3) odour improvement; 4) hair removal; 5) haircare and maintenance; 6) care and maintenance of mucous membranes and 7) decorative cosmetics [63]. Generally, EOs are present as ingredients in the aforementioned different cosmetics groups and therefore, the cosmetic characteristics presented by aromatic plants, especially the fragrance, are associated with EOs. On the other hand, there is an increasing trend in using natural compounds in order to improve human health and beauty since there is a greater concern with the possibility of human risks related to the use of synthetic ingredients [59].

As EOs as well as their individual compounds have been recognized for their health and beauty benefits, they are increasingly being used in the production of a wide range of cosmetic formulations [21] and **Table 1.4** shows some of the potential applications of EOs in cosmetics. *Helichrysum italicum* EO, known by the common name of immortelle, is considered one of the most famous EOs in cosmetics and has skin care benefits such as stimulating blood circulation in the skin, which helps to regenerate the skin as well as minimize appearance of fine lines and wrinkles. In cosmetic products, it is mainly used in soaps as aroma and in perfumes [61,64]. Furthermore, Han *et al.* [65] demonstrated the tissue-remodelling and the anti-proliferative effects of immortelle EO which may contribute to the wound healing process. *Citrus aurantium* EO better, known as neroli, is considered one of the most relevant EOs, especially in the soap and perfume industry, due to its very fine aroma. Citral is the compound, as one of its main constituents, that has several benefits in terms of cell regeneration. Thus, it is widely used to prevent

and heal stretch skin marks and to treat wrinkles as it promotes skin elasticity [66].

Rose x. damascene EO, commonly known as rose, has antibacterial properties that act against the bacteria which is responsible for acne. It also has the ability to make skin more hydrated, being therefore widely used in dry and acneic skin treatments [61]. *Santalum spicatum* EO, commonly known as sandalwood, is known for its cell renewal effects, minimizing the accumulation of dead cells. Adding to this, it has the ability to naturally make skin more luminous and to reduce the occurrence of blackheads and blemishes [66]. Kim *et al.* [67] showed the healing and hydrating effects of *Oenothera biennis* EO on the skin, known as evening primrose, in which it can become a relevant ingredient in anti-aging products as well as skin care moisturizers.

Table 1.4 Potential applications of some EOs in cosmetics

Essential Oil	Plant	Main compounds	Application	Properties	References
Immortelle	*Helichrysum italicum*	α-pinene**;** *cis*- α -santalol; limonene; linalyl acetate; linalool; γ-curcumene; β-selinene; β- caryophyllene	Skin care	skin stimulant and regenerator; anti-aging and antiproliferative effects	[61,64,65]
Neroli	*Citrus aurantium*	β-pinene; α-terpineol; limonene; sabinense; nerol; nerolidol; lynalyl acetate; α-pinene; citral	Skin care	skin regeneration and elasticity; healing skin marks	[66]
Rose	*Rosa x. damascene*	citronellol; geraniol; nerol; farnesol; androse oxide	Skin care	skin hydration and anti-acne effect	[61]
Sandalwood	*Spantalum spicatum*	α-bisabolol; (*E*)-farnesol; nuciferol; α-santalol; β-santalol	Skin care	hydration, refresh, and plumper skin; decrease appearance of skin marks	[66]
Evening primrose	*Oenothera biennis*	β-amyrin; 1-hexacosanol; linoleic acid; γ-linolenic acid; 1-tetracosanol; squalene	Skin care	skin hydration anti-wrinkle effect	[67]
Sweet orange	*Citrus sinensis*	Limonene; myrcene; α-pinene; β-pinene; sabinene	Skin care	anti-acne	[68]
Lavender	*Lavandula angustifolia*	linalyl acetate; linalool; terpinene-4-ol; camphor; limonene; 1,8-cineole	Hair care	hair growth effects	[69,70]
Peppermint	*Mentha piperita*	menthol; menthone; carveone; 1,8-cineole; limonene; methyl acetate; neomenthol	Hair care	hair growth effects	[71]
German Chamomile	*Matricaria chamomilla*	β-farnesene; farnesol; chamazulene; α-bisabolol; bisabolol oxide; 1,8-cineole; α-terpineol	Skin and hair care	anti-inflammatory and healing skin lesions effects; hair growth	[72,73]
Rosemary	*Rosmarinus officinalis*	1,8-cineole; α-pinene; borneol; camphene; camphor; β-caryophyllene; p-cymene; limonene; linalool; myrcene; β-pinene; α-terpineol	Skin and hair care	hair loss and growth; antidandruff; skin hydration and elasticity	[61,74]
Tea tree	*Melaleuca alternifolia*	α-pinene; β-pinene; sabinense; myrcene; α-phellandrene; α-terpinene; limonene; 1,8-cineole; p-cymene; linalool; terpinen-4-ol; α-terpineol	Skin and hair care	anti-acne effects and antidandruff	[73,75]

According to a reported study by Sun *et al.* [68], *Citrus sinensis* EO, or sweet orange, has the ability to

alleviate acne lesions caused by the bacterium *Cutibacterium acnes.* A study conducted by Hay *et al.* [70] revealed that *Lavandula angustifolia* EO, known as lavender, has hair growth benefits in patients with alopecia areata. Peppermint Eos, extracted from *Mentha piperita,* also has hair growth effects and can therefore be used as a natural medicinal alternative against human hair loss [71]. *Matricaria chamomilla* EO, known as german chamomile, is considered one of the most widely used EO in cosmetics and has been shown to have the ability to relieve itching and inflammation, facilitating the healing of peristomal skin lesions [72]. Furthermore, Aburjai and Natsheh [73] demonstrated that a few drops of german chamomile EO during hair washing or directly placed in shampoo can facilitate healthy hair growth. *Rosmarinus officinalis* EO, or rosemary, is well-known and used in cosmetics, such as cologne, fragrance, and soaps. It has also been widely used in hair treatments as it helps nourishing and promoting hair growth and works as an anti-dandruff. In addition, it has the ability to strengthen damaged hair follicles and is used in hair loss treatments [61]. On the other hand, Montenegro *et al.* [74] showed the skin care efficacy of rosemary EO in which its potential use in the treatment of skin disorders involving loss of skin hydration and elasticity was reported. In a study by Enshaien *et al.* [75], it is shown that *Melaleuca alternifolia* EO, also commonly known as tea tree, is efficient in the treatment of mild to moderate acne vulgaris, when 5% topically of the EO is used. In addition, Aburjai and Natsheh [73] also reported the capillary efficacy of tea tree EO in the treatment of dandruff.

1.2.4.2.1 Essential oil as sensorial enhancers

Nowadays, fragrances play an increasingly important role in the attractiveness for cosmetic products. Cosmetics that have pleasant smells have a significant positive impact on consumers.[76] There is a bigger concern about "green consumerism" and consequently in acquiring natural products, especially in the cosmetics industries [76]. Due to this, there is an increased interest in using EOs as aroma and flavour ingredients, once they are natural fragrances that present a complexity of active compounds and intense fragrance properties. Thus, they are widely used in the incorporation of existing cosmetic products [77]. The different fragrances that EOs can present are grouped into families based on their predominant odoriferous characteristics. So, in 1983, Michael Edwards, a fragrance specialist, developed his own fragrance classification scheme, called the fragrance wheel [78].

The fragrance wheel is represented as a fragrance classification chart that was developed with the aim of simplifying the classification of different scents. The wheel is divided into four main family groups, namely, floral, fresh, woody and oriental that lead to fourteen different sub-families establishing the relationships between them, as shown in the **Figure 1.2** [79]. The floral family group is characterized by fragrances with a sweet and floral odour, with notes of roses, jasmine, lilies and peonies. This group can be subdivided into floral, which is represented as one of the most appreciated and used fragrances with a typically floral aroma, like fresh-cut flowers (e.g., rose and lily) and into soft floral, characterized by soft, powdery, sweet and creamy scent [78,80,81]. On the other hand, the fresh family group includes fruity, citrus, water, green and aromatic notes which means they are sweet, refreshing, spicy and vibrant scents. Usually, fruity notes present a sweet, edible, and tropical aroma like peach, apple, and pear. Citrus notes include lemon, tangerine, and bergamot with a refreshing and tangy character. In addition, water notes are made with aquatic odours that smell of sea spray or rain combined with oceanic notes [78,80,81]. The green notes embrace scents of freshly cut grass and crushed green leaves aroma and finally, the aromatic notes that have clean, fresh herbal scents combined with lavender or woody scents [78,80,81].

Furthermore, the oriental family group includes typically warmer, sweeter and spicy fragrances that can range from oriental scent, characteristic of presenting warm and sweet notes such as cinnamon, vanilla and musk; floral oriental, which includes spices, orange blossom and aldehydes giving a

characteristic oriental floral aroma; soft oriental, characterized by being a soft oriental scent of flowers mixed with spices, amber and incense being slightly less sweet and heavy than the oriental scent itself and finally, woody oriental, distinguished by its deep and sensual scent with sandalwood or patchouli [78,80,81].

Finally, the wood family group is characterized by having a mysterious, warm and captivating odour, being widely used in after-shaves. It includes wood-based aromas such as cedar, sandalwood, vetiver and amber; mossy woods odours, also recognized by perfumers as Cyprus, which have an earthy, sweet, and smooth scents like for example oakmoss and amber and lastly, dry woods characteristic of having a smouldering and smoky scent with leather odours [78,80,81]. Furthermore, the industry of fragrances tends to classify the EOs in three different levels, according to their odours characteristics, volatility and diffusion rate in air, designated by top, middle and base notes [21]. Top notes are defined as being the first perceptible odours as well as the most volatile such as bergamot, juniper, cinnamon, and gardenia. Middle notes are usually related to more floral or spicy aromas, constituting the body of the product such for example ylang-ylang, geranium, lavender, jasmine, and clove. Base notes provide a scent with depth and durability. They are less volatile EOs which means they remain for longer hours like for example, myrrh, vanilla, sandalwood and frankincense [20].

Figure 1.2. Michael Edwards' perfume fragrance wheel [78]

1.2.4.2.2 Odour profile of essential oils and their main constituents

In cosmetics industry, EOs as well as their isolated constituents are widely used mainly because of their pleasant scents. Substances such as fatty acids and fatty oils have an unpleasant odour and are commonly used in cosmetic product formulations [61]. Therefore, EOs are increasingly used as a source

of natural products that present a wide range of natural pleasant aromas with unique notes that can mask unwanted odours [76]. There are compounds that have been described in the literature as being directly associated with the odour of a given EO, as shown by the **Table 1.5** where the odour profile of some EOs, usually used in cosmetics and perfume industries, are represented with the main active compounds responsible for its scents. In addition, the odour description of individual EOs main constituents is described in **Table 1.6**.

Eucalyptus globulus EO has a characteristic camphor odour mainly due to the high presence of the 1,8-cineole compound, which by itself already has the same type of aroma [82,83,84]. *Citrus sinensis* and *Citrus reticulata* EOs have a characteristic orange and mandarin aroma, respectively, that is due to the presence of compounds such as ethyl butanoate, ethyl 2-methylbutanoate, octanal, decanal and acetaldehyde, in the case of *Citrus sinensis* EO and linalool, octanal, α-pinene, limonene, and (*E,E*)-2,4-decadienal for *Citrus reticulata* EO [85]. *Cryptomeria japonica* EO has a typical woody cedar odour due to the presence of compounds such as a-pinene and sabinene [90,93,95,86,87]. Lavender EO (*Lavandula anfustifolia*) was described by Xiao *et al.* [88] as having a characteristic floral and sweet aroma derived from the presence of compounds such as limonene, linalool, linalyl acetate and camphor, which by themselves already have a strong, floral, sweet and pleasant odorous character.

Table 1.5 Odour profile of some essential oils used as scents in cosmetic and/or perfume industries

Plant derived essential oils	Plant part	Active compounds	Odour description	References
Eucalyptus globulus	Leaves	1,8-cineole and α-pinene	Camphor-like balsamic fresh odour	[82]
Citrus reticulata	Fruits	linalool, octanal, α-pinene, limonene, and (*E,E*)-2,4-decadiena	Mandarin-like odour	[85]
Citrus sinensis	Fruits	ethyl butanoate, ethyl 2-methylbutanoate, octanal, decanal, and acetaldehyde	Orange-like odour	[85]
Cryptomeria japonica	Foliage	α and β-pinene and sabinene	Woody cedar peppery odour	[86,87]
Feniculum Vulgare	Seeds	trans-anethole, estragole, fenchone and 1-octen-3-ol	Strong, anise and camphoric odour	[89]
Lavandula angustifolia	Flowering tops	linalool, linalyl acetate and camphor, limonene	Sweet floral aroma	[88]
Pinus pinaster	Needles	α and β-pinene	Fresh and strong pine odour, resinous, woody	[86,90]
Pinus pinea	Needles	limonene, α and β-pinene	Fresh and strong pine odour, resinous, woody	[86,90]
Thymus vulgaris	Leaves	thymol and carvacrol	Herbaceous, slightly sweet, and mint notes	[89]

Another example is the fennel (*Foeniculum vulgare*) EO that has been shown that its strong, anise and camphoric odour has been associated with the presence of compounds such as trans-anethole, trazodone, fenchone and 1-octen-3-ol that show odoriferous characteristics of anise, licorice, and camphor. On the other hand, the thyme (*Thymus vulgaris)* EO showed an herbaceous, slightly sweet, and mint notes odour, which was associated with the presence of compounds such as thymol and carvacrol, characteristic of having an oregano, thyme and spicy notes aroma [89]. Furthermore, the EOs of *Pinus pinaster* and *Pinus pinea* present fresh, strong pine odours characterized by the presence of compounds such as α and β-pinene [90,93,95,86,90].

Table 1.6 Odour descriptions and cosmetic restrictions from some of the main individual constituents of essential oils

Essential oils main constituents	Odour description	Cosmetic Restrictions*	References
α-Pinene	Fresh, earthy, pine, woody, resinous	peroxide value less than 10 mmoles/L	[90,93,95]
β-Pinene	Fresh, earthy, woody, turpentine	peroxide value less than 10 mmoles/L	[76, 95]
1,8-Cineole	Camphor-like odour	0,1%	[83,84]
Limonene	Citrus fruits aroma, strong orange odour	0,001% - leave-on products; 0.01% - rinse-off products	[61]
trans-Anethole	Anise and liquorice odour		[89]
Sabinene	Woody, citrus, pine, spice		[84]
Estragole	Anise, liquorice, sweet		[89]
Fenchone	Mint, camphor, warm		[89]
1-Octen-3-ol	Mushroom		[89]
(*E*,*E*)-2,4-Decadiena	Deep fried, oily notes		[85]
Linalool	Floral, grassy, pleasant, citrus	0,001% - leave-on products; 0.01% - rinse-off products	[85]
Linalyl acetate	Floral, sweet citrus		[91]
Camphor	Camphoraceous, rancid, oily aroma		[89]
Thymol	Oregano, thyme, spicy notes		[89]
Decanal	Green, citrus like odour		[85]
Octanal	Citrus-like, soapy		[85]
Ethyl butanoate	Fruity odour		[85]
Acetaldehyde	Fresh odour	prohibited	[85]
Ethyl 2-methylbutanoate	Fruity odour		[85]
Carvacrol	Oregano, thyme, spicy notes		[89]

*Maximum concentration in ready for use preparation, according to Regulation (UE) nº 12223/2009.

Nowadays, the evaluation of fragrances is a key parameter to confirm the safety, good performance and quality of a certain cosmetic product. The evaluation of the quality of a given EO can be done by examining the organoleptic properties of the product by sensory analyses performed by a panel [92].

1.2.4.2.3 Sensory analysis

Sensory analysis is considered a multidisciplinary science that includes the measurement, interpretation and understanding of human responses regarding the properties of a particular product obtained, considering the senses [93]. In the cosmetics industry, the data obtained from sensory evaluation has been an asset since it has helped the development of cosmetic products according to the sensory/olfactory preferences of consumers [94]. Thus, sensory evaluation has been widely used to determine the acceptability and optimization of a given cosmetic product [93]. Sensory analysis can be a very important tool to assess the identity, quality, safety and efficiency of a given EO [95]. These analyses can be performed by a sensory analysis panel that will assess the selected EO. The main advantage of using sensory analysis is to avoid costly investments in analytical materials [92].

1.2.4.2.4 Allergenic Character of Essential Oils

EOs as well as their main constituents are considered to be the most relevant substances that can be included in perfumes, since there is a wide range of possibilities to obtain different fragrances or formulations [96]. However, the use of EOs and their individual compounds, in formulations, don't include

only advantages. They may constitute a potential source of allergenic reactions [21]. Since there are several examples of adverse reactions derived from the use of EOs, it is necessary to ensure a safe cosmetics production with certain precautions, such as regarding the dose, composition, dilution, frequency of use and application of a certain product [76]. There are certain EOs that have a greater tendency to cause adverse reactions, compared to others, and the adulteration of EOs can also influence their allergenic action [21,76]. In cosmetics, adverse reactions that occur due to the use of EOs are, usually, related to direct contact with the skin, such as dermal reactions, contact dermatitis and phototoxicity/photosensitivity reactions by sun exposure [97].

Generally, these topical reactions are related to the existence of certain chemical compounds in their composition and some examples of this are cinnamic alcohol, aldehyde, eugenol and baptapene [98]. Thus, it is extremely relevant to be aware of the allergenic compounds described in cosmetics as well as the EO associated with adverse reactions. Twenty-six possible allergenic fragrances were defined, eighteen of which may be included as ingredients of EOs, as shown in the **Table 1.7** [61].

Table 1.7 List of 26 allergenic fragrances according to EU Directive

INCI Name	CAS Number	Origin	Can be found in
Amylcinnamal	122-40-7	Synthetic	N/A
Amylcinnamyl alcohol	101-85-9	Synthetic	N/A
Anise alcohol	105-13-5	Synthetic or Natural	honey, essential oils of anise, tomatoes, tahiti vanilla
Benzyl alcohol	100-51-6	Synthetic or Natural	peru balsam, tolu balsam, essential oils of jasmin, apricot, almond, apple, asparagus, banana, black currant, blackberry
Benzyl benzoate	120-51-4	Synthetic or Natural	peru balsam, tolu balsam, essential oils of jasmin, ylang-ylang
Benzyl cinnamate	103-41-3	Synthetic or Natural	peru balsam, tolu balsam, copahu
Benzyl salicylate	118-58-1	Synthetic or Natural	propolis
Cinnamyl alcohol	104-54-1	Synthetic or Natural	*Hyacinth*
Cinnamal	104-55-2	Synthetic or Natural	essential oils of cinnamon, *Hyacinth*, patchouli, nutmeg
Citral	5392-40-5	Synthetic or Natural	essential oils of lemon, orange peel and eucalyptus, grapefruit, orange, celeris, apricot, blackcurrant, grape, kiwi, mango, ginger, melon, plum, raspberry, rose
Citronellol	106-22-9	Synthetic or Natural	essential oils of lemon grass and ceylon , apple, apricot, cassis, blackberry, blueberry, orange, passion fruit, peach, rose
Coumarin	91-64-5	Synthetic or Natural	woodruff, flowers, sweet clover, angelique, berce
Eugenol	97-53-0	Synthetic or Natural	essential oils of clove, allspice, bay (*Myrcia acris*), avens, ceylon cinnamon, laurel, *Cistus ladanifer*, basil sassafras, basil java, cassie, sweet flag, carnation, boldo, cascarille, galangal, bay leaves, nutmeg, pale rose, ylang-ylang, marjoram, calamus, camphor, lemongrass, patchouli

INCI Name	CAS Number	Origin	Can be found in
Farnesol	4602-84-0	Synthetic or Natural	essential oils of rose, neroli, ylang-ylang, lime tree, tolu balsam
Geraniol	106-24-1	Synthetic or Natural	rose oil, orange, palmarosa, thyme, verbena, neroli, lemongrass, geranium, hyssop, laurel, lavender, mandarine, melissa, nutmeg, myrtle, apple, apricot, black cranberries, blackcurrant, blackberry, coriander, ginger, nutmeg, thyme, geranium, rose, palmarosa, ylang-ylang
Hexyl cinnamicaldehyde	101-86-0	Synthetic	N/A
Hydroxy-citronellal	107-75-5	Synthetic	N/A
Hydroxy-methylpentylcyclohexenecarboxaldehyde	31906-04-4	Synthetic	N/A
Isoeugenol	97-54-1	Synthetic or Natural	essential oils of citronella, ceylon and ylang ylang
D-Limonene	5989-27-5	Synthetic or Natural	essential oils of lemon, common juniper, orange, verbena, neroli, niaouli, melaleuca, lemon balsam, pepper mint, nutmeg, myrrh, angelique, aspic, badiane, bergamot, mandarin, bigaradier, caraway, celery, lavender, lime
Linalool	78-70-6	Synthetic or Natural	essential oils of: thyme, lavender, pine, laurel, sour orange, marjoram; peppermint, lemon, orange, thyme, ylang ylang, verbena, myrtle, neroli, coriander, geranium, lime, lemon balsam, nutmeg, lemongrass, basil, bergamot, rosewood, banana, blackberry, bean, blueberry, apple, apricot, artichoke, thyme, rose, palmarosa
Methyl heptin carbonate	111-12-6	Synthetic	N/A
3-Methyl-4-(2,6,6-tri-methyl-2-cyclohexen-1-yl)-3-buten-2-one	127-51-5	Synthetic	N/A
Evernia prunastri (Oak moss)	90028-68-5	Natural	Oak moss extract
Evernia furfuracea (Tree moss)	90028-67-4	Natural	Tree moss extract
2-(4-tert-Butylbenzyl) propionaldehyde	80-54-6	Synthetic	N/A

For this reason, rules were established for the cosmetic products labelling of containers for EOs to enable the identification of the contents, which are regulated by ISO/TS 211:2014 standard [99]. Therefore, it is extremely important that the information on the concentration of these allergenic fragrances is labelled on the packaging so that, for example, the permitted concentration of 0.01% for shower gels and baths and 0.01% for body oils, massage oils and creams, is not exceeded [61].

Several examples of allergenic reactions associated with EOs in cosmetics have been reported, such as the case of tea tree EO (*Melaleuca alternifolia*) that was described to have caused about 1% of allergic reactions by the study population and this susceptibility to EO was related to the high percentage value of the eucalyptol compound (1,8-cineole) [100]. Eucalyptus EO is also associated with adverse reactions, due to the high content of the aforementioned compound, especially related to allergic contact dermatitis [101]. Peppermint EO (*Mentha piperita*) is an example of another allergenic EO. In this case, its adverse reactions (e.g., inflammation) are associated with high concentrations of menthol, menthone, carvone, pulegone and limonene [102]. On the other hand, lavender EO is also reported to have adverse reactions, in more than 10% of the population studied, related to allergic contact dermatitis, mainly due to its dominant compounds namely, linalyl acetate and linalool [103,104]. In ylang-ylang EO, the sensitization associated with this EO use is due to the presence of isoeugenol [105] in its constitution.

1.2.5 Essential oils as antioxidants

Antioxidant substances are molecules capable of reacting with radicals or with a reducing power capable of neutralizing the oxidative stress caused by free radicals. In other words, they are compounds capable of retarding the oxidation of an oxidizable material [106]. The aromatic plants and their EOs are good sources of natural antioxidants, and their properties are related to the phenolic compounds present in the oil or other phytochemical fractions [107]. Phenolic compounds may have the ability to interrupt or slow down the aerobic oxidation of organic matter [108]. The antioxidant activity of phenolic compounds is related to their high redox properties and chemical structure that can neutralize free radicals, chelate transitional metals, quench singlet and triplet oxygen by delocalization or decomposing peroxides [109]. Due to the mentioned properties, phenolic compounds provide benefits linked to human health as they help delay many diseases related to oxidative stress, such as cardiovascular disease, cancer, diabetes and Alzheimer's [110].

Oxidative stress results from an uncontrolled production of reactive oxygen species (ROS). ROS represents several small active molecules that result from the metabolism of molecular oxygen. They are highly reactive molecules such as superoxide (O_2), hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$) [111]. The harmful effects of ROS are minimized by antioxidant defence mechanisms that eliminate these reactive species, preventing them from accumulating excessively. In addition, they also help to repair possible damage caused by these species. Nevertheless, if imbalances that favour pro-oxidants (chemicals that facilitate the induction of oxidative stress from ROS production) occur, it will lead to a deleterious state previously referred to as oxidative stress [112].

EOs and their volatile compounds, acting as natural antioxidants, can neutralize the action of free radicals, minimizing the cell damage they can cause, preventing oxidative stress [113].There is a growing interest in searching for natural antioxidants that are not toxic to human health. For this same reason, there are many studies about the antioxidant potential that EOs can present [114]. This is extremely important as most common synthetic antioxidants such as butylated hydroxyanisole (BHA) or butylhydroxytoluene (BHT) can cause severe problems to human health [114], for example their consumption can lead to oxidative stress, carcinogenicity, reproductive toxicity, apoptosis and DNA fragmentation [115,116]. Two distinct parameters describe the ability of a compound to inhibit the spontaneous oxidative degradation of a substrate, namely, the stoichiometric factor or also called antioxidant capacity. This factor represents the number of radicals that an antioxidant molecule can capture. The other parameter is the reactivity which is considered the most relevant in determining the antioxidant activity depending on the reaction rate constant between the antioxidants and the chain-carrying radicals [117].

1.2.5.1 Methods for assessing antioxidant capacity

Several methods have been suggested to estimate the antioxidant activity, such as direct competition methods and indirect methods [106]. The direct competition methods are based on the competition of antioxidants for the peroxyl radical using a radical reference scavenger that is effectively detected by UV-Vis or fluorescence spectrophotometry. Nevertheless, this method has a standard main limitation that relates to calculating the antioxidant activity of the area under the fluorescence or absorbance curve (AUC) versus time or after a fixed time-lapse [106]. However, using the AUC approach can be very useful as it can be used in the same way for antioxidants that have different latency phases as well as those that do not have latency phases. Therefore, it is very effective for food samples as it usually includes several

ingredients with complex reaction kinetics [118]. The main advantage of direct methods is their sensitivity although, on the other hand, they mostly include time-consuming assays and experience in chemical kinetics is required. Therefore, they are not the ideal methods to be used in routine trials with natural products [119]. An example included in the mentioned methods is the β-carotene bleaching test which measures, in the presence/absence of antioxidants, the decay of absorption, at 470 nm, due to β-carotene, considering a flux of free radicals [120]. Another method is the *Oxygen-Radical Antioxidant Capacity* (ORAC) assay, which will be discussed later.

In the indirect methods, probes in coloured persistent radicals or metal cations are used. The change in colour in the solution results from the reaction and is measured by UV-Vis spectrophotometry. This method has limitations mainly related to the chemically different probes. This will result in radical scavenging power rather than actual antioxidant activity, which means that molecules that can reduce these probes cannot always stop the oxidative chain [106]. However, the indirect methods are used more often than direct methods. They are methods that provide information about the ability to eliminate free radicals from natural products and, in addition, they are easy to handle and more productive[119]. *Ferric Reducing Antioxidant Power* (FRAP) is an example of the aforementioned method where Fe3+ is reduced to Fe2+ by the antioxidant, forming a coloured complex with 2,4,6-tripyridyl-s-triazine [121]. Another example is the *2,2-diphenyl-1-picrylhydrazyl radical scavenging* (DPPH) test which will be discussed later. Several direct and indirect analytical methods are used nowadays to quantify and monitor ROS generation biologically. An example of this is the fluorescent probes that are very effective, mainly due to their high sensitivity, simplicity, and reproducibility. For these same reasons, they have been widely used in measuring ROS [122]. One of the most commonly used probes is 2 ′,7 ′ - dichlorodihydrofluorescein diacetate (DCFH 2 - DA), which was first developed in the 1960s to detect hydrogen peroxide in cell-free systems [123]. This method will be discussed later on.

1.2.5.1.1 DPPH assay

The DPPH (*1,1-diphenyl-2-picrylhydrazyl*) assay assesses the potential of an antioxidant molecule in scavenging free radicals. It is one of the most used methods since it is considered an easy standard colorimetric method to analyse the antioxidant properties that compounds may have [124]. This assay is commonly used to measure the antioxidant content of edible seed oils, herbs, vegetables, wheat grain and bran, linoleic acids, using different solvent systems including ethanol, aqueous acetone, methanol alcohol and benzene [125]. DPPH is a stable radical that exhibits a characteristic purple colour in a solution and absorbs at 517 nm. This method assumes that DPPH accepts a hydrogen atom (H) from the antioxidant molecule or also called scavenger, which causes a reduction of DPPH to DPPH2. Posteriorly, it results in a change of the characteristic purple colour of the radical to yellow (represented in **Figure 1.3**) [106]. This colour change will be monitored by spectrophotometry and consequently used to determine the antioxidant properties of the extracts.

Blois first demonstrated the DPPH assay in 1958, in which he described the ability of the DPPH free radical to accept an H atom from the cysteine molecule [124]. Usually, the results are expressed as IC50, which is defined as the concentration of the antioxidant required to reduce by 50% the initial absorbance of the DPPH radical. It is a method which depends on the reaction time. Therefore, if the parameter is considered by itself, it does not provide relevant information about the natural reactivity of the antioxidant. Additionally, data can only be compared when they result from similar configurations [106].

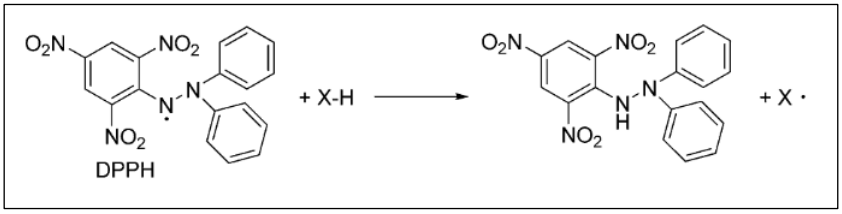

Figure 1.3 Scheme of the reaction process of an antioxidant with the DPPH radical (Amorati, Foti and Valgimigli [106])

1.2.5.1.2 ORAC assay

The ORAC (*Oxygen Radical Absorbance Capacity*) method is often used to assess pure compounds or mixtures antioxidant capacity, such as biological fluids, foods, dietary supplements, or cosmetic products [126]. The ORAC method was described by Glazer [127] and developed by Cao, Alessio, and Cutler [128] and Cao *et al* [129]. This assay assumes that the antioxidant substance competes with a fluorescent probe, such as phycoerythrin and fluorescein (30,60-dihydroxy-3H-spiro [2- benzofuran-1,90-xanthen]-3-one), to eliminate peroxyl radicals that were produced by 2,20-azobis (2-ami-dinepropane) dihydrochloride (AAPH), which is a soluble thermal azoinitiator in water (represented in **Figure 1.4**). The addition of an antioxidant compound will slow down fluorescence decay [119]. We observed the protective effect of the antioxidant compound used from the evaluation of the area under the fluorescence decay curve (AUC) of the sample compared to the blank in which no antioxidant is present [126]. Antioxidant capacity is quantified from the integrated liquid areas under the fluorescence decay curves, accounting for the lag time, the initial rate, and the total extent of inhibition as a single value. The results obtained are usually compared to those of a standard antioxidant, generally being Trolox [130].

Cao *et al* [129] developed the ORAC procedure to simulate the oxidative processes that occur in human cell degeneration and, therefore, the assay is usually performed at 37ºC.

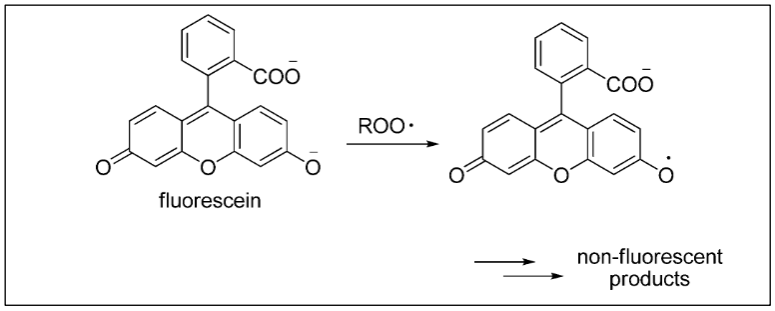

Figure 1.4 Scheme of the reaction process of an antioxidant with the ORAC radical (Amorati, Foti and Valgimigli [106])

1.2.5.2 Intracellular ROS measurement

In biological systems, the intracellular detection of ROS species requires probes capable of reacting very quickly with these species so they can compete with the antioxidants, producing stable products that are quantified [111].

Recently, several probes became capable of detecting ROS, such as, 2′,7′ -

dichlorodihydrofluorescein diacetate (DCFH 2 - DA) that is commonly used to detect intracellular H_2O_2. DCFH 2 - DA, 2′,7′ - dichlorodihydrofluorescein (DCFH 2), and dichlorodihydrofluorescein (DCF) are structurally similar to fluorescent probes such as fluorescein. DCFH 2-DA and DCFH 2 are non-fluorescent and colourless, while DCF has a yellowish colour and intense fluorescence under excitation [112]. This method is based on the use of this cell-permeable nonfluorescent probe (DCFH 2–DA) that once inside the cell undergoes a biological "catalysis" process in which the cell is hydrolysed by intracellular esterase's forming DCFH 2 [131]. Subsequently, DCF will be formed from DCFH 2 resulting from an oxidation process. This process involves two steps of single-electron oxidation in which first, DCFH 2 loses one electron to form the intermediate DCF while the last one loses another electron to form DCF [112].

1.2.6 Essential oils as antimicrobial agents

Plant molecules have been recognized for their antimicrobial properties, especially plant EOs, which have been shown to inhibit a broad spectrum of bacterial pathogens such as gram-positive and negative bacteria [132]. The action process of EO against bacteria has been demonstrated in several studies and is related to the chemical composition of EOs. The chemical composition of EOs is constituted by a great diversity of molecules in which each one acts on a specific target [38]. Usually, EOs that present high percentages of phenolic compounds in their constitution, such as carvacrol, eugenol and thymol, demonstrate significant antimicrobial capabilities [133,134] Several studies have demonstrated antimicrobial activities of these compounds against a wide range of bacterial strains, such as *Escherichia coli*, *Bacillus cereus*, *Listeria monocytogenes*, *Staphylococcus aureus*, among others [135]. Certain alcohols, aldehydes, ketones as well as monoterpenes (e.g., geraniol, linalool, menthol, terpineol, thujanol, γ-terpinene, p-cymene, among others) also showed antimicrobial capabilities although, within these, carvacrol has been reported in previous studies to be the strongest antimicrobial [136]. Furthermore, it is thought that the hydrophobicity presented by EOs and their components is a characteristic that allows them to accumulate in the lipid bilayer of the cell membrane of bacteria and mitochondria, causing a disturbance at the level of cell structures and making them more permeable [137].

Some EOs can interrupt cell homeostasis as an antimicrobial mechanism, which leads to an inhibition of growth and, consequently, cell death [138]. It has also been proposed that the functional hydroxyl group (-OH) and the aromaticity presented by EOs may be responsible for the presence of antibacterial activity [139]. Nowadays, EOs are recognized as a source of natural antimicrobial substances, making them widely used in various industries such as the food industry, where they are commonly used as bio-preservatives to prevent the deterioration of food and consequently increase the shelf life of a given product. Furthermore, the continuous use of chemical preservatives has several side effects on human health that can be minimized using EOs as natural preservatives [140].

1.2.6.1 Methods for evaluating antimicrobial activity

Several methods assess or screen the *in vitro* antimicrobial activity that a particular extract or pure compound may have. The dilution methods are the most used in determining the minimum inhibitory concentration (MIC) values because they allow the estimation of the concentration of the tested antimicrobial in the agar or broth medium (macrodilution and microdilution). The methods mentioned allow to quantitatively evaluate the *in vitro* antimicrobial activity against bacteria and fungi [141]. The MIC evaluation obtained from dilution methods is defined as the lowest concentration of the tested antimicrobial agent that can inhibit the visible growth of the microorganism used [141].

1.2.7 Essential oils resulting from forest waste

Thirty five percent of the mainland Portugal is occupied by forest, that is why there is a great potential in the use of residues from forest biomass such as roots, trunks, and branches that can be removed during thinning as well as shrubs, trees sick or killed by fire and others [142].

The forest biomass obtained from forest resources must be recovered and maintained since it is from this that is possible to manufacture renewable bioenergy, biofuels and other biological products that allow replacing products based on fossil fuels [143]. The recovery of forest biomass will be an alternative to open agricultural burning which will bring countless economic, environmental and social benefits [143]. EOs and their co-products such as Hds are an example of products that can result from forest biomass and are included in the concept of biorefinery. Biorefinery uses renewable energy sources, such as forest biomass, to obtain value-added products, namely, biochemicals, biofuels, heat and electricity. From forest resources obtained from Mediterranean forest species, it is possible to obtain valuable EOs [144]. The growing interest of EOs in the market value makes the exploitation of these forest resources economically viable [145]. The *Eucalyptus globulus*, *Pinus pinaster*, *Pinus pinea* and *Cryptomeria japonica* EOs are examples of what can be obtained from forest waste.

1.2.8 Eucalyptus globulus Labill.

The *Eucalyptus* genus is the second-largest genus after acacia and includes about 800 species that have been identified around the world.[146] *Eucalyptus* genus belonged to the *Myrtaceae* family and in 1788, it was named for the first time and described by Charles-Louis L'Héritier de Brutelle, a French botanist [147]. *Eucalyptus* are evergreen woody trees that can range from shrubs to tall trees and can reach gigantic size, as they are fast-growing trees.

The vast majority of the species belonging to this genus are endemic to Australia and Tasmania. Nevertheless, they are distributed worldwide, grown in most subtropical and temperate regions and cultivated in many other climates [146].

1.2.8.1 Geographical distribution

The *Eucalyptus* genus is native from Australia, Tasmania, Africa and tropical to southern temperate America. The *Eucalyptus globulus* Labill is naturally distributed in Tasmania and South-eastern Australia. Furthermore, as it is a widely cultivated and naturalized species, it is distributed in subtropical regions worldwide as this genus can adapt to different types of climates and environments. But it is in countries with warmer climates that they find the optimal conditions for their development [146]. In Portugal, the *E. globulus* species is spread throughout the country, from North to South, from coastal areas to the interior where edaphic conditions or altitude mitigate the aridity of the environment. There are factors that condition their survival such as dryness and low temperatures [148].

1.2.8.2 Botanical description

E. globulus has broad juvenile leaves carried in opposite pairs on square stems. The length of the leaves is around 6 to 15 cm, and they are greyish, which is why the common name used to designate the

species is “blue gum”. Furthermore, the mature leaves of the species have a bright dark green colour, a sickle shape and they are narrow. The buds are ribbed and verrucous, top-shaped, with a flattened operculum with a central bud hidden inside [149]. The last-mentioned feature is related to the origin and meaning of the genus *Eucalyptus* as it is of Greek origin and means “well covered” or “well hidden” [150]. In the axils of the leaves, individual cream-coloured flowers are born, producing nectar with intensely flavoured honey. The fruits have dimensions between 1.5 and 2.5 cm in diameter, being woody [149].

1.2.8.3 Economic and forest importance of Eucalyptus globulus species in Portugal

In Portugal, *Eucalyptus* (*E. globulus*) plantations have been experiencing a systematic increase in the last 50 years and represent about 812 thousand hectares, which corresponds to 26% of the continental forest [151]. It is considered as one of the most economically important forest species nationally, and its wood has various uses such as in the pulp and paper industry, wood panels, biomass pellets, civil construction, parquets floors and carpentry. In addition to the economic interest in eucalyptus wood, its leaves, bark, and flowers can have several uses, such as for the extraction of EO that is widely used, especially in the pharmaceutical and perfume industries [152,153]. Barata *et al* [12] described the leaves of the *E. globulus* species as being the most commercialized in Portugal, with about 48300 kg of dry weight. On the other hand, the EO obtained from the aforementioned species was also indicated as being one of the most produced in Portugal, with 12010 kg annually.

1.2.8.4 Eucalyptus globulus Essential Oil

1.2.8.4.1 Phytochemistry

The main chemical classes of the compounds obtained from *E. globulus* EO are oxygenated monoterpenes, monoterpenes hydrocarbons and oxygenated sesquiterpenes [150]. The *E. globulus* EO obtained from different plant parts such as leaves, mature fruits, branches, and flower buds has many valuable compounds in its chemical composition. The main characteristic is the richness in 1,8-cineole (eucalyptol). *E. globulus* EO obtained from the leaves has 1.8 cineole as its main chemical constituent, with percentage variations between 4-50%. It may have higher or lower percentages according to the maturity and origin of the plant species place of harvest [154]. α-Pinene [traces (t, ≤ 0.05%)-18%)], *p*-cymene (t-27%), cryptone (0-18%) and spathulenol (0.1-17%) were other main components found in the EO of the leaves [154]. In addition, the *E. globulus* EO obtained from the fruits, buds and branches is known to contain α-thujene (0%, 12% and traces respectively), 1,8-cineole (15%, 37% and 57% respectively) and aromadendrene (23%, 17% and 8%, respectively) [154].

1.2.8.4.2 Ethnobotany

Eucalyptus is a primarily used medicinal plant. In ancient times, the aboriginal Australians used it to heal wounds, cure fungal infections, and reduce fever. Later, *E. globulus* EO began to be used as a disinfectant and expectorant by Chinese, Greek, European and Ayurvedic medicine. Nowadays, the medicinal applications of this EO are related to its use in steam massages on the chest, cough and cold medicines, sore throat sprays, topical analgesics, among others [154].

1.2.8.4.3 Pharmacological uses

In recent years, the pharmacological activity of *E. globulus* EO has been studied for many purposes, among which: antioxidant, antimicrobial and antifungal. *E. globulus* EO has long been used to treat bronchitis, asthma and respiratory problems as an antiseptic and antispasmodic stimulating agent. As it can be used externally, it has increasing effects on blood flow and skin temperature. Thus, it has been commonly used in the treatment of cough, in promoting the healing of burns or skin lesions from semi-solid pharmaceutical products [155]. Eucalyptol is recognized as a medicinally relevant component that causes a sensation of cold and, consequently, ease breathing. For this reason, *E. globulus* EO is also widely used as an inhalant due to its antimicrobial properties [154].

Luís *et al* [156] evaluated the antioxidant activity of EOs from two species of *Eucalyptus*: *E. globulus* and *E. radiata*. The *E. globulus* EO showed a more significant antioxidant activity because it had a value of IC 50% lower. So, a small concentration of the *E. globulus* EO was enough to inhibit the DPPH radical. The EO antifungal activity from *E. globulus* was evaluated against twelve yeast-like fungi and filamentous fungi, and it proved to be effective, obtaining MIC values between 0.025 and 1% (v/v).

Furthermore, *E. globulus* EO was tested against two distinct *Candida albicans* strains. A concentration of only 0.05% was enough for the EO to have the capacity to inhibit the growth of the strains, MIC values of 2-8 mg/ml were obtained [154]. Bachir *et al* [157] evaluated the antimicrobial activity of the *E. globulus* EO from the leaves against *Staphylococcus aureus* and *Escherichia coli* strains. The results demonstrated an inhibitory effect of *E. globulus* EO against gram-positive and negative bacteria, respectively.

1.2.9 Pinus pinaster Aiton and Pinus pinea L.

The genus *Pinus* represents the largest genus of conifers and constitutes more than 100 distinct species. It belongs to the *Pinaceae* family due to morphological characteristics such as sprout dimorphism, which includes short sprouts, called fascicles, which have one to eight narrow needles surrounded by bud scales at the base. EOs obtained from pine trees contain over 50 ingredients. Several factors influence its EO concentrations, such as the variety of the plant, crop, method of distillation and the plant part. Pine EOs have long been used mainly as perfumes and repellent ingredients [158]. *Pinus pinaster* Aiton, also known as maritime pine, is considered one of the most relevant species in the Mediterranean area, mainly due to its ecology and wood production [159]. The *Pinus pinea* L., also known as stone pine or umbrella pine, is widely distributed all over the Mediterranean Basin. It is cultivated mainly for its edible pine nuts. As an ornamental tree, it is often planted in gardens and parks [160].

1.2.9.1 Geographical distribution

Middle Europe and the Mediterranean region are considered the main areas of origin of the genus *Pinus*. *P. pinaster* is mainly located in the western Mediterranean area that reaches the High Atlas and the northern part of Africa, Tunisia. This plant species can develop in different types of substrates and different regimes of the Mediterranean climate, such as semi-arid to humid climates [159]. The *P. pinea* grows naturally in the coastal areas of the northern Mediterranean, in the Aegean Sea, southern Europe, North Africa and from Spain to Turkey [160].

In Portugal, the *P. pinaster* species mainly grow in the North and Centre of the country. Furthermore,

it is located in regions of the central and southern interior, such as Penamacor, Idanha-a-Nova, Serra de S. Mamede and Santo Aleixo da Restauração (Moura). In Portugal, the species of *P. pinea* is found from North to South, mostly occurring in the south of Tagus, in the region of Setúbal [148].

1.2.9.2 Botanical description

P. pinaster is a medium-sized tree about 20-35 cm in height. It has a reddish-orange bark with considerable thickness and is highly fissured at the base of the trunk. The needles are bluish green to yellowish green in colour, always in pairs and with a length between 12 and 22 cm. The cones are initially greenish, maturing to a bright reddish-brown colour around 24 months of age. They are about 10-20 cm long and 4-6 cm wide at the base when closed [161]. *P. pinea* is characterized by being a perennial coniferous tree that can reach 25-30 m high. It is considered a medium-sized tree whose trunks have a diameter greater than 2 m. The crown of the tree varies according to its age. When it is young, it presents a globose and shrubby crown [162].

In middle age it has the shape of an umbrella and when it is mature, it is flat and wide. The trunk usually has several angular branches with foliage at the ends, being short in size. The bark is reddish-brown, covered with cracks, and orange-purple plaques flattened at the top. The needles are bluish green, with an average age of 2-4 years, separated into two fascicles, about 8-15 cm long, and have a characteristic onion smell. The seed cones vary according to age and are green when young and reddish-brown when mature. They have an ovoid-globular shape about 8-12 cm long [162].

1.2.9.3 Economic and forest importance of Pinus pinaster and Pinus pinea species in Portugal

In Portugal, the pine forests (*P. pinaster* and *P. pinea*) represent the second forest formation with an area of about 1 million hectares. Pine forests, especially maritime pine, have been suffering reductions due to fires and forest pests [163]. *P. pinaster* is considered the most abundant conifer in Portugal and is one of the forest species with high economic importance nationally, being predominantly used in industrial applications such as paper and pulp, in the production of resin (e.g. turpentine), as well as in the wood industry, especially in the production of wood for civil construction, joinery, furniture, wood panels, biomass pellets, among others [153,164]. *P. pinea* is the second most abundant pine species in Portugal. Although both species produce pine nuts, only *P. pinea* are edible, representing a high national economic importance. *P. pinea* wood is mainly used in carpentry, joinery and shipbuilding and is also used for the production of resin [164]. Barata *et al* [12] also referred the EO of *P. pinaster* as being one of the most produced nationally, with an annual production of about 60 kg.

1.2.9.4 Pinus pinaster and Pinus pinea Essential Oil

1.2.9.4.1 Phytochemistry

The main chemical classes of the compounds obtained from pine needle EOs are, usually, monoterpenes and sesquiterpenes [159]. Several studies described in the literature have shown different chemical compositions of *P. pinaster* and *P. pinea* EO. Macchioni *et al* [165] analysed the chemical composition of the needles, branches and cones of the EO of *P. pinaster*, from Italy, in which the main constituents were the α-pinene and β-pinene. In Iran, Zolfaghari and Iravani [161] also proved that the α-pinene was the main compound, but in this case, in the *P. pinaster* EO obtained from bark. On the other hand, Petrakis *et al* [166] evaluated the chemical composition of needles of several pine species, including

P. pinaster, growing in Greece and α-pinene, β-pinene and germacrene D were the major compounds, in distinct concentrations. Furthermore, Mimoune *et al* [159] demonstrated that the β-caryophyllene and β-selinene were the dominant compounds of *P. pinaster* EO needles from Algeria. Meullemiestre *et al* [167] also reported β-caryophyllene as the major compound in *P. pinaster* EO obtained from sawdust waste in Algeria and France. The chemical composition of *P. pinea* EO from needles, fruits, and bark was analysed and presented different compositions in Jordan [168]. The *P. pinea* EO needles had guaiol, limonene and β-caryophyllene as main constituents. The *P. pinea* EO fruits presented the limonene and α-pinene as dominant compounds. Furthermore, the *P. pinea* EO from bark had the β-caryophyllene as the major compound [168]. Amri *et al* [169] reported that the main constituents in the *P. pinea* EO needles growing in Tunisia were the limonene and β-caryophyllene.

Tümen *et al* [170] demonstrated that *P. pinea* EO from cones contained mainly limonene and β-caryophyllene in Turkey. The chemical composition of EOs can show a high variability that can be explained by different geographical locations, climatic conditions, harvesting times, genotype, drying procedure, the plant part used for the distillation process and the method itself [168,171,172].

1.2.9.4.2 Ethnobotany

The maritime and stone pines have been used for medicinal purposes, with emphasis on traditional medicine. The needles, bark, juvenile shoots, pine branches, resin as well as the EO isolated from the needles and resin (the essence of turpentine) are the parts of the plant that are mostly used [164].

Small cones, young sprouts and needles have been widely used in infusions, which have antiseptic, expectorant, diuretic, and tonic functions. Infusion is widely used against asthma, bronchitis, lung problems and coughs [164]. The infusion can also have an external action acting as an aerosol, relieving rheumatic pain in the treatment of abscesses and washings as a hair colour-fortifying agent. Furthermore, tiny pinecones, young sprouts and needles have other medicinal uses, such as making syrups. The EO obtained from maritime pine needles and turpentine essence is widely used in treating catarrh. It externally acts as rubefacients in treating sprains and rheumatic pain. Pine nuts are also widely used in meat dishes, salads and bread as they are a good source of nutrients for health [164].

The *P. pinea* EO has also been widely used in folk medicine mainly due to its medicinal properties as an analgesic and anti-inflammatory agent in treating skin conditions, namely, eczema and psoriasis. On the other hand, the EO of *P. pinea* obtained from the cones and needles showed a notorious healing effect and rheumatic pain [168]. There are also reports in the literature according to which *P. pinea* EO is used against respiratory diseases, as an inhaler in herbal steam baths [169].

1.2.9.4.3 Pharmacological uses

Some studies published in the last years involve *P. pinaster* EO evaluated for antioxidant [167,170], anti-inflammatory[170] and antimicrobial[97]. On the other hand, several authors have assessed the antimicrobial, anti-inflammatory, antifungal and herbicidal activity of *P. pinea* EO [169,173–175]. The EO of *P. pinaster* showed a significant antioxidant activity because it could reduce the iron-tripyridyl-triazine complex in its ferrous form [167]. Tümen *et al* [170] assessed EOs antioxidant and anti-inflammatory activity, and the cones revealed the highest activities. Kim *et al* [176] proved that α-pinene monoterpene had an anti-inflammatory, elevating effect. The presence of the α-pinene compound in *P. pinaster* EO may favour the healing effect by having an anti-inflammatory effect. Hmamouchi *et al* [173] evaluated the antimicrobial activity of four species of EOs from the *Pinus* genus needles. The authors conclude that

the EO from *P. pinaster* and *P. pinea* were the only ones to exhibit antimicrobial activity against all strains studied.

Ulukanli *et al* [174] demonstrated the antimicrobial efficacy of *Pinus pinea* essential oil, against Gram-positive and negative bacteria as well as yeasts. Süntar *et al* [175] studied the wound healing activity of *P. pinea* EO from cones and needles and realized that the EO obtained from the cones was the one that showed the highest effects. Furthermore, Amri *et al* [169] analysed the antifungal and herbicidal capacity of *P. pinea* EO against ten plant pathogenic fungi and three weeds, respectively, in Tunisia. The results showed that *P. pinea* EO demonstrated an antifungal and phytotoxic activity, which means that the EO of *P. pinea* could become a natural alternative as a fungicide and herbicide.

1.2.10 Cryptomeria japonica D. Don

Cryptomeria japonica D. Don is the only existing species and has three distinct varieties, two of which are var. *japonica* and var. *radians.* They are located in the humid temperate region of the Japanese archipelago, from Aomori Prefecture to Yakushima Island [177]. *C. japonica*, also known as Japanese cedar or Sugi, is a monotypic genus of conifer included in the cypress family *Cupressaceae*, widely distributed throughout the world and one of the most commercialized conifers in the Asian country [178–180].

1.2.10.1 Geographical distribution

C. japonica is a widely distributed and abundant conifer species, occupying about 18% of the forest area of the Japanese archipelago [181]. Around the 19th century, it was introduced in the Azores archipelago and currently represents about 57% of the total forest area of the Azores. It is mainly used for wood production [182]. *C. japonica* is a significant tree in forestry and, therefore, it has been widely used in forest plantations in Japan, China, and the Azores. Furthermore, it is used as an ornamental tree in other temperate areas, such as in the United Kingdom, Europe, North America and some parts of the eastern Himalayas, such as Nepal and India [180].

1.2.10.2 Botanical description

C. japonica is an evergreen tree with a reddish-brown bark that can reach 70 m (230 feet) in height and a trunk diameter of up to 4 m (13 feet) [180]. The leaves are long, arranged in a spiral-resembling needle about 0.5-1 cm. The seed cones have a globular shape about 1-2cm in diameter and have scales ranging from 20-40 cm. *C. japonica* has a slow to an average growth rate of about 20 feet in 20 years [180]. It usually has a heartwood with a reddish colour. However, in some situations, a dark or even black colour may occur, which results in a reduction in its commercial value [183]. The plant species has a long life. There are specimens in Japan which are about 650 years old. When it grows in warm, moist conditions and in deep, well-draining soils, it has a faster growth rate. Therefore, it does not grow well in poor soils and harsh climates [184].

1.2.10.3 Economic and forest importance of Cryptomeria japonica species in Portugal

In the Azores, *C. japonica* occupies about 12500 hectares of production forest. The production of *C.*

japonica wood represents a high economic importance in the Azores archipelago [185]. The characteristics of its wood, such as the production of a soft wood, easy to handle, light and durable, make it highly valuable as a building material, being widely used in civil construction, carpentry and furniture [185].

1.2.10.4 Cryptomeria japonica Essential Oil

1.2.10.4.1 Phytochemistry

Several studies described in the literature have demonstrated the variability in the chemical composition of the EO of *C. japonica*. Cheng *et al* [186] analysed the chemical composition of *C. japonica* EO leaves at different ages, in which the main constituents were the 16-kaurene and elemol. Wang *et al* [187] also evaluated the chemical composition of the leaves from *C. japonica* EO and the elemol was the dominant compound following the 16-kaurene. Mahdian *et al* [179] demonstrated that elemol and terpinen-4-ol were the dominant compounds of *C. japonica* EO from aerial parts. The chemical composition of *C. japonica* EO from foliage, heartwood, and bark was analysed and presented with different compositions [182]. The *C. japonica* EO foliage had α-pinene and phyllocladene as main constituents. The *C. japonica* EO heartwood presented the cubebol and *epi*-cubebol as dominant compounds. Furthermore, the *C. japonica* EO from bark had the δ- Cadinene, α-muurolene, and *epi*-zonarene as the major compounds [186].

The variability of the chemical composition of *C. japonica* EO may be due to differences in the seasons in which the plant parts were collected, the ages of the plant species and other factors [186].

1.2.10.4.2 Ethnobotany

In Asian traditional medicines, *C. japonica* has demonstrated several uses, such as its bactericidal and pesticide activity. Nowadays, it is widely used in aromatherapy mainly due to its aromatic properties. It is also commonly used to renew the smell of furniture made from natural cedar. Furthermore, the *C. japonica* EO has insecticidal properties and is often used directly on the skin or added to sprays and candles acting as an insect repellent [180].

1.2.10.4.3 Pharmacological uses

Over the years, interest in *C. japonica* EO has increased, especially in China, Japan and Korea, due to numerous biological properties, such as antifungal [178], antiulcer [188], antimicrobial [189], repellent and insecticide [187], among others. Cheng *et al* [178] evaluated the antifungal activity of the *C. japonica* EO from the heartwood, sapwood and leaves. The heartwood oil was the one that showed the highest antifungal activity against several fungal species. Takayuki *et al* [188] analysed the antiulcer properties of *C. japonica* EO from leaves, and both EO and its main isolated constituents revealed an antiulcer activity as they were influential in the formation of gastric ulcers. Cha *et al* [189] assessed the antimicrobial activity of *C. japonica* EO aerial parts against several species of bacteria. The results demonstrated an inhibitory effect of *C. japonica* EO against all the bacteria tested. The high percentages of caryophyllene oxide, α- and β-pinene and 1,8-cineole present in the EO are related to its antimicrobial activity.

Finally, Wang *et al* [187] investigated the repellent and insecticide activities of the *C. japonica* EO from leaves. They concluded that it would be a potential option to be used as a reagent to minimize the damage caused by silverfish, acting as a repellent and insecticide.

Objectives

The main objective of this dissertation is to evaluate the chemical composition, antioxidant, and antimicrobial activities of *Eucalyptus globulus*, *Pinus pinaster*, *Pinus pinea* and *Cryptomeria japonica* essential oils and hydrolates obtained from biomass wastes resulting from Portuguese forest maintenance. The sensorial evaluation of *Eucalyptus globulus*, *Pinus pinaster*, *Pinus pinea* and *Cryptomeria japonica* EOs in skin care emulsions is also one of the objectives of this thesis.

According to sustainability and reuse of forest biomass, the specific aims of the present study was:

1) characterize the chemical composition of essential oils from forest species: *Eucalyptus globulus* Labill., *Pinus pinaster* Aiton, *Pinus pinea* L. and *Cryptomeria japonica* D. Don, obtained by national producers from the mainland Portugal and Azores archipelago;

2) chemically characterize the volatiles of the hydrolates of the same essential oils;

3) evaluate the antioxidant and antimicrobial capacity of these essential oils and hydrolates;

4) conduct a sensory evaluation of the essential oils, in order to perceive which formulation(s) presented the most pleasant odour for the majority of selected participants and assess the acceptability of the essential oils formulations.

Chapter 2. Material and Methods

2.1 Essential oils and Hydrolates from national producers

The essential oils (EOs) and hydrolates (Hds) of four plant species were obtained from local producers from mainland Portugal and Azores archipelago. The national producers provided eleven samples of EOs from *Eucalyptus globulus*, *Pinus pinaster*, *Pinus pinea* and *Cryptomeria japonica*, and seven samples of Hds for the same species except for *P. pinea* (**Table 2.1**). The EOs and Hds volatiles samples were stored at -20ºC until analysis.

Table 2.1 List the studied essential oils (EOs) and hydrolates (Hds) and their respective codes and lots

Plant species	EOs code*	EOs Lot	Hds code	Hds Lot
Eucalyptus globulus	Eg_OE_1_G	AV202017	Eg_Hd_1_G	AV202017
	Eg_OE_2_B	OEGA	-	-
	Eg_OE_3_O	-	Eg_Hd_2_O	-
	Eg_OE_4_E	10EG17	Eg_Hd_3_E	AV202017
	Eg_OE_5_P	L26_3_2019	Eg_Hd_4_P	PRT02
	Eg_OE_6_S	200428	-	-
Pinus pinaster	Pp_OE_1_G	22	Pp_Hd_1_G	22
	Pp_OE_2_P	L10_3_2019	Pp_Hd_2_P	PRT04
	Pp_OE_3_S	190705	-	-
Pinus pinea	Ppi_OE_1_B	Fev2020	-	-
Cryptomeria japonica	Cj_OE_1_M	22_05_20	Cj_Hd_1_M	22_05_20

* To ensure data protection each producer was assigned with an arbitrary code letter.

2.2 Hydrolate volatiles extraction

The hydrolate volatiles (HVs) were obtained after liquid-liquid extraction of each *E. globulus*, *P. pinaster* and *C. japonica* hydrolate, using distilled *n-pentane*, in a proportion of 3 volumes of *n-pentane* per volume of hydrolate. Pentane extracts were concentrated, without drying, at room temperature under reduced pressure on a rotary evaporator (Rotary Evaporator RE-51). Each extract was collected in a flask and concentrated, at room temperature, under nitrogen flux.

2.3 Essential Oils and Hydrolates volatiles Chemical Analysis: Identification and Quantification of EOs and Hds Volatiles Constituents

The EOs and HVs studied were analysed by gas chromatography with flame ionization detector (GC-FID) for component quantification and gas chromatography coupled to mass spectrometry (GC-MS) for component identification as described by Neves *et al* [190].

2.3.1 Gas chromatography - flame ionization detector (GC-FID)

The analyses of gas chromatography (GC) were accomplished using a Perkin Elmer Claus 400 gas chromatograph (Perkin Elmer, Shelton, CT, USA) equipped with two flame ionization detectors (FIDs),

a data processing program, and an injector with two columns of distinct 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.).

The oven temperature was programmed from 45 to 175° C, at 3° C/min, then at 15 ° C/min to a 300°C. Subsequently, the temperature was maintained isothermal for 10 min. The injector and detector temperatures were 280° C and 300° C, respectively. The hydrogen carrier gas was adjusted for a linear velocity of 30 cm/s. The EOs and Hds volatiles were injected using a split sampling technique (ratio 1:50). The percentage of EOs and Hds components was calculated with the normalization method of the GC peak areas, computed as the average value of two injections for each of the samples of EOs.

Routine quantification of the EO components was performed by calculating the percentage composition from the normalization method using the GC percentage peak areas obtained as mean values of two injections of each EO, not using correction factors.

2.3.2 Gas chromatography-Mass Spectrometry (GC-MS)

The identification of the constituents of the EOs and HVs was performed with a gas-liquid chromatograph coupled to mass spectrometry (GC-MS) using a Perkin Elmer 600 gas chromatograph equipped with 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 a Perkin Elmer Clarus 600-T mass spectrometer (software version 5.4.2.1617, Perkin Elmer, Shelton, CT, USA).

The temperatures of the injector and oven were the same as previously mentioned; transfer line temperature, 280°C; ion source 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 evaluated in-lab comparatively to n-alkanes on the DB-1 column. The compounds of the EOs were identified by comparing their RI relative to n-alkanes C_9-C_{17} indices and GC-MS spectra from a laboratory-made library based on the analyses of reference oils, laboratory-synthesized components, and commercially available standards.

2.4 Determination of Antioxidant activity

The antioxidant activity was analysed through two different chemical methods: 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity and oxygen radical absorbance capacity (ORAC), as follows:

2.4.1 1,1-diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Activity

The following method assesses the antioxidant activity through the capacity of the compounds present in the samples to scavenge DPPH radicals.

To assess the antioxidant activity, an ethanolic solution containing DPPH radicals with a concentration of 1.60 x 10^{-3}mol/L was prepared. Several dilutions in absolute ethanol were prepared from each analysed samples' stock solution of EOs (1:10, 1:20, 1:30, 1:40, 1:50, 1:60, 1:70 e 1:80 μL/ μL). The same dilutions were repeated for the hydrolates but in phosphate-buffered saline (PBS).

The ascorbic acid stock solution was prepared by dissolving 1.76 mg of ascorbic acid in 10 ml of water (final concentration of 1 x 10^{-3} mol/L). The samples with a final volume higher than 1000 µL were removed 950 µL and added to an empty Eppendorf. For the remaining samples with the last volumes of 1000 µL, it was only necessary to remove 50 µL from the sample. Posteriorly, 50 µL of the DPPH stock solution was added to the EOs samples' dilutions to make the same final volume of 1000 µL for each sample.

The negative control was prepared with 50 µL of DPPH stock solution and 950 µL of absolute ethanol. 200 µL were pipetted from EOs solutions with DPPH stock solution to a 96-well plate. The selected samples and the ascorbic acid (positive control) were pipetted on the plate for the same dilutions. Lastly, the negative control (DPPH and absolute ethanol) and the blank (absolute ethanol) was pipetted in triplicate on the plate. In the hydrolates the negative control was DPPH and PBS. The plate was covered with aluminium foil to be protected from light for 25/30 min.

The absorbance was measured at 517 nm in the presence of different concentrations of the samples EOs, using a fluorescence microplate reader (FLUOstar BMGLabtech, Ortenberg, Germany).

The antioxidant activity was calculated as percentage inhibition of DPPH, using the following equation:

$$\textbf{\% Inhibition} = \left(\frac{A_{DPPH-A_S}}{A_{DPPH}}\right) \textbf{x 100}$$

Where, A_{DPPH} is the absorbance of DPPH solution and A_S is the absorbance of the solution when the EOs samples were added. Each experiment was carried out in triplicate, and results are expressed as mean ± SD. The half-maximal inhibitory concentration (IC_{50}), which consists of the concentration of the antioxidant required to reduce by 50% the initial absorbance of the DPPH radical, present in the solution, was calculated using the GraphPad Prism 5.0 *software*.

2.4.2 Oxygen Radical Absorbance Capacity (ORAC)

The ORAC method was used to measure the antioxidant activity of the samples of EOs and Hds towards peroxyl radicals. The samples of the EOs and Hds were analysed by the group of Food Functionality & Bioactives Lab-Food and Health Division of the Institute of Experimental and Technological Biology (IBET) in Oeiras, followed the protocol described by Serra *et al* [191].

Briefly, on a 96-well black plate, a mixture was added to each well consisting of 5.18 M AAPH, 4 x 10^{-3} mM of disodium fluorescein (DF) (both prepared in PBS 75 mM, pH: 7.4) and the sample to a final volume of 0.2 mL. A calibration line was performed with the compound, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), in a range of concentrations from 0 µM to 40 µM.

The final dilution value of the EOs and Hds samples used to make the readings were obtained after several attempts until a concentration was on the range of values of the Trolox curve. The samples fluorescence was read in a microplate reader (FLx800 from Biotek®), the plate was incubated for 10 minutes at 37ºC, and fluorescence was measured every minute, 40 minutes at 37ºC (at 485 nm excitation and 527 nm emissions). The fluorimeter control software used was Gen5. The net area under the curve (AUC) of the standard (Trolox) and samples were calculated. The standard curve was obtained by plotting Trolox concentrations against the average net AUC of each concentration measurements.

The final ORAC values were calculated using the regression equation between Trolox concentration and the net AUC. The results were expressed in micromoles of Trolox equivalent antioxidant capacity

per gram of EO/Hd (µmol TEAC/g EO/Hd). All data were presented as mean ± standard deviation (SD) of two replicates.

2.4.3 In vitro antioxidant activity

The ability of EOs and Hds samples to reduce the ROS production was determined using a well-characterized probe, 20,70-Dichlorofluorescein diacetate (H_2-DCFDA; Life Technologies, UK) nonfluorescent cell-permeable compound used as a marker of oxidative stress. Once inside the cell, it is cleaved by endogenous esterases to H_2-DCF, thus preventing the back-diffusion of the dye into the extracellular space. The de-esterified product becomes the highly fluorescent compound 20,70-dichlorofluorescein (DCF) on ROS oxidation [122,192,193].

The procedure was performed as described by Silva *et al* [194], with some adaptations. Briefly, the HaCaT cell line (Cell line Service GmbH, Germany) was seeded at 2 x 104 cells per well in 96- well plates with 100 µL of the cell culture medium per well incubated for 24 h at 37 ºC. Cells were pre-incubated for 30 minutes with 20 µM of H_2-DCFDA, in the dark, at 37ºC. Then the probe solution was removed, and a fresh medium was added containing the different samples to be tested. Hydrogen peroxide (H_2O_2) solution (500 µM) was used to induce ROS in cells; 1mg/mL of ascorbic acid was used as positive control and a culture medium as a negative control. Cells were incubated in the presence of the treatments for 1h at 37 ºC before the addition of 500 µM H_2O_2.

The DCF levels were determined using a fluorescence microplate reader at excitation 485 nm and emission 520 nm wavelengths (FLUOstar BMGLabtech, Ortenberg, Germany).

Data from six replicates were reported as the relative mean of % ROS reduction determined by relative fluorescence units (RFU) of culture medium with H_2O_2 as 100% and the % ROS reduction as:

$$\left[\mathbf{100} - \left(\frac{\boldsymbol{fluorescence\ of\ sample\ exposed\ cells}}{\boldsymbol{fluorescence\ of\ unexposed\ control\ from\ the\ same\ experiement}}\right)\right] \times \mathbf{100}$$

The data were expressed as mean and standard deviation (mean ± SD) of experiments (n = 8). Statistical evaluation of data was performed using a one-way analysis of variance (ANOVA). Tukey–Kramer multiple comparison test (GraphPad PRISM 5 software, La Jolla, CA, USA) was used to compare the difference between the groups, and a $p < 0.05$ was accepted as significant.

2.5 Determination of Antimicrobial Activity

The minimum inhibition concentration (MIC) was determined to evaluate the antimicrobial activity of the EOs and Hds against Gram-positive and Gram-negative bacteria, yeast and mold.

2.5.1 Microbial strains

The EOs and Hds were tested against a large panel of microorganisms. The bacteria were acquired from international culture collections ATCC and obtained from the collection of ADEIM/Faculty of Pharmacy, University of Lisbon (FFUL). The microbial strains selected for the study were: *Staphylococcus aureus* ATCC 6538, *Bacillus subtilis* ATCC 6633, *Pseudomonas aeruginosa* ATCC

9027, *Escherichia coli* ATCC 8739, *Candida albicans* ATCC 10231 and *Aspergillus brasiliensis* ATCC 16404. ATCC is considered the most relevant and representative bacteria and pathogenic yeast strains (**Table 2.2**).

Table 2.2 Microorganisms strains used in the study

Gram-positive bacteria	Gram-negative bacteria	Yeast	Fungi
Staphylococcus aureus ATCC 6538	*Pseudomonas aeruginosa* ATCC 9027	*Candida albicans* ATCC 10231	*Aspergillus brasiliensis* ATCC 16404
Bacillus subtilis ATCC 6633	*Escherichia coli* ATCC 8739		

2.5.2 Evaluation of antimicrobial activity

2.5.2.1 Determination of minimum inhibitory concentration by the microdilution method

The minimum inhibitory concentration (MIC) assay determines the lowest concentration of an antimicrobial agent that prevents a microorganism visible growth. The MIC determinations for each EO and Hd were performed by the broth microdilution method.

The minimum inhibitory concentration (MIC) was assessed by the microdilution method for six reference strains, namely: Gram-positive (*Staphylococcus aureus* ATCC 6538 and *Bacillus subtilis* ATCC 6633) and Gram-negative (*Pseudomonas aeruginosa* ATCC 9027 and *Escherichia coli* ATCC 8739). The selected yeast was the *Candida albicans* ATCC 10231 and the fungi was *Aspergillus brasiliensis* ATCC 16404. Initially, the microbial suspensions of each strain were prepared in PBS to a final concentration of 1.5 x 10^8 in colony-forming units (CFU/mL) (turbidity value corresponding to 0.5 in the McFarland standard scale). Then, 100 µL Mueller Hinton (MH) for bacteria and Sabourad dextrose broth (SBD) for the yeast and mold were added to each well of a 96-well microplate. Afterwards, 100 µL of the EO and Hds solutions (1 mg/mL) prepared in the appropriate culture media were added to the first well followed by a twofold serial dilution to obtain final concentrations ranging from 500 mg/mL to 0.48 µg/mL. Finally, 10 µL of a bacterial/fungi suspension diluted in the appropriate culture medium were added to each well to obtain a final concentration of 10^5 CFU/mL. The microplates were incubated at 35-37ºC for 24h for bacteria or 48h for yeast, and at 22ºC for 5 days for *Aspergillus brasiliensis*. Bacterial growth and culture medium were used as controls. The MIC was defined as the lowest concentration where no visible growth was observed, and the growth was monitored by measuring OD_{600nm} in a microplate reader (Varioskan™ multimode microplate reader). All experiments were performed in triplicate

2.6 Sensory evaluation

2.6.1 Participants

Sensory evaluation was conducted from March until November 2020. The aim of this study was to evaluate the sensorially of *Eucalyptus globulus*, *Pinus pinaster*, *Pinus pinea* and *Cryptomeria japonica* EOs in skin care emulsions.

Sensory double-blind evaluation was performed in 100 volunteers. The protocol was approved by the local Ethical Committee (nº 1_2022) and respected the Helsinki Declaration and Good Clinical

Practice studies on cosmetic products. All participants gave their informed consent. A structured questionnaire -annex 1 and 2 was used to collect data about the perception and applicability of different emulsions, and they were encoded with different colours (pink, blue, green, orange, and purple). Emulsions were prepared with 0.5% EO of selected samples. Portuguese participants were invited to identify and classify the odours of five selected emulsions into the following categories: without odour, slightly perceptible, perceptible, very perceptible, intense odour, pleasant and unpleasant smells. In addition, the applicability of each odour was evaluated.

2.6.2 Emulsions' Preparation

The emulsions were developed in pharmaceutical technology laboratory of Faculdade de Farmácia da Universidade de Lisboa. Five EOs were selected from each species under study except for *P. pinaster* EO, in which we chose two samples from different producers. To prepare the emulsions, the oily phase (decyl oleate, cetyl alcohol, ceteareth-11, ceteareth-20 and paraffinum liquidum) and aqueous phase (purified water and glycerin) were heated separately to 75 ºC. Then the oily phase was added to the water phase and the system was mixed (130r.p.m) with constant agitation until 30 ºC in a VMI bench mixer. Finally, the EOs were added and manually mixed. The emulsions were formulated with 0.5% of EO from the selected samples. The excipients used in formulations and the percentage composition of the emulsions prepared with the EOs are described in **Table 2.3**.

Table 2.3 Qualitative and quantitative (%, w/w) composition of the emulsions prepared with *Eucalyptus globulus*, *Pinus pinaster*, *Pinus pinea* and *Cryptomeria japonica essential oils*

INCI*	Trade name	Function	(%, w/w)	Company/Origin
Phase A				
Ceteareth-11	Eumulgin B1®	Non-ionic O/W emulsifier	1.5	SABO – DS Produtos Químicos **L.da** (S. Domingos de Rana – Portugal)
Ceteareth-20	Eumulgin **B2®**	Non-ionic O/W emulsifier	1.5	SABO – DS Produtos Químicos **L.da** (S. Domingos de Rana – Portugal)
Cetyl alcohol	Cetyl alcohol	Thickenner	2.0	Laboratório de Farmácia Galénica - Portugal
Paraffinum liquidum	Mineral oil	Emolient	2.5	Mosselman - Belgium
Decyl oleate	Tegosoft DO®	Lipophilic emollient	4.5	Evonik - Germany
Phase B				
Glycerin	-	Humectant	5.0	Lacrilar - Portugal
Purified Water	-	Solvent	82.0	Millipore®, Elix 3
Phase C				
Essential Oil	-	Fragrance	0.5	Portugal

*Ingredients' names according to the International Nomenclature of Cosmetics Ingredients (INCI).

2.6.3 Experimental protocol Quiz

The sensory characteristics of the emulsions were evaluated by a group of inexperienced volunteers. The volunteers (n=100) were females and males aged in the range of]18 - 60[. The academic object of the research was reported to the participants, maintaining the privacy of the people and the confidentiality of the information collected.

The sensory questionnaire was divided into two sections: **Section 1.** corresponding to the **Emulsions' Odour**, which aimed to classify emulsions according to the olfactory preferences of the selected volunteers and **Section 2.** that was relative to the **Emulsions' Applicability** and had as main objective to evaluate the acceptability of the emulsions considering the probability of purchasing different personal care products (perfume; air freshener; massage cream; toothpaste; shampoo and candy) with the odour and colour of the respective emulsion. Emulsions were prepared with 0.5% EOs from selected samples according to the protocol published by Neves *et al*. [190]. The formulations of the different samples are coded with different colours (pink, blue, green, orange, and purple). The identification of EOs emulsions with the respective EOs codes and formulation colour is illustrated in the **Table 2.4**. The emulsions were delivered in person after being properly disinfected. The procedure consisted of four main steps namely:

1. Explain the objective, the experimental protocol and read/sign the "Informed consent" document to the volunteers (present in the annexes, **Annex 1**);
2. Provide the five emulsions samples with the different colours to each volunteer;
3. Request the completion of the online questionnaire (**Annex 2 and 3**, print-version in English and Portuguese);
4. Proceed with the collection for later disposal of all samples.

Table 2.4 Identification of EOs emulsions used in the sensory questionnaire

Species EOs	Selected EOs Code	Formulation Colour
E. globulus	Eg_OE_1_G	Orange
P. pinaster	Pp_OE_1_G	Blue
	Pp_OE_2_P	Purple
P. pinea	Ppi_OE_1_B	Green
C. japonica	Cj_OE_1_M	Pink

2.6.4 Specific inclusion and exclusion criteria of subjects

The selection of participants in the study was made considering the following inclusion and exclusion criteria. The inclusion criteria for selecting participants include people of both genders, female and male and aged between]18,60[. The exclusion criteria include people who had or have an infection with SARS-CoV-2 in which the olfactory part was affected. Also include people with associated respiratory problems or olfactory diseases that can compromise the sensory questionnaire results.

2.6.5 Statistics Analysis

The data collected through the sensory questionnaire during the experimental study were subjected to statistical treatment, using the statistical program IBM® SPSS® software (Statistical Package for the Social Sciences version 27 for Windows 10).

Chapter 3. Results and Discussion

3.1 Essential Oils and Hydrolates Volatiles Composition

3.1.1 Eucalyptus globulus EO

Chromatographic analyses of *E. globulus* EOs resulted in the identification of 44-49 constituents. The components identified in the samples of *E. globulus* EO are grouped into five classes: monoterpene hydrocarbons; oxygen-containing monoterpenes; sesquiterpene hydrocarbons; oxygen-containing sesquiterpenes and others (**Table 3.1).**

Table 3.1 Percentage composition of *Eucalyptus globulus* essential oils

Components	RI	Eg_OE_1_G	Eg_OE_2_B	Eg_OE_3_O	Eg_OE_4_E	Eg_OE_5_P	Eg_OE_6_S
Isovaleraldehyde	637	0.1	0.4	t	t	t	0.1
Isoamyl alcohol	722	t	t	t	t	t	t
Isovaleric acid	847	t	t	t	t	t	
Isoamyl acetate	882	t	t	t	t	t	t
α-Thujene	924	t	t	t	t	t	t
α-Pinene	930	13.2	13.3	11.0	21.8	14.7	13.8
α-Fenchene	938	t	0.1	t	0.1	0.1	0.1
Camphene	938	t	0.1	t	0.1	0.1	0.1
Thuja-2,4(10)-diene*	940	t	t	t	t	t	t
β-Pinene	963	0.4	0.3	0.4	0.7	0.4	0.3
Dehydro-1,8-cineole	973	t	t	t	t	t	t
β-Myrcene	975	0.2	t	1.0	0.7	0.1	0.0
α-Phellandrene	995	0.3	0.1	0.2	0.2	0.3	0.3
α-Terpinene	1002	t		0.1	0.1	t	t
p-Cymene	1003	1.2	0.8	0.2	0.6	1.6	1.7
1,8-Cineole	1005	65.2	63.2	59.5	53.9	58.2	49.4
Limonene	1009	8.2	17.2	13.7	16.6	12.5	18.0
cis-β-Ocimene	1017	0.3	t	0.2	0.5	0.1	t
trans-β-Ocimene	1027	t	0.1	t	0.1	t	
γ-Terpinene	1035	0.5	t	0.7	0.9	0.2	0.2
2,5-Dimethyl styrene	1059	t	t	t	t	0.2	0.2
Terpinolene	1064	t	t	0.2	0.1	0.1	0.1
Nonanal	1073	t	t	t	t	t	t
Linalool	1074	t		t	t	t	t
Isopentyl isovalerate	1080	t	t	t	t	0.1	0.1
endo-Fenchol	1085	t	t	t	t	0.1	0.1
α-Campholenal	1092	0.2	0.1	t	t	0.1	0.1
trans-Pinocarveol	1106	1.6	1.9	t	0.3	3.0	3.1
Pinocarvone	1121	1.3	1.2	t	0.1	1.3	1.3
δ-Terpineol	1134	0.1	t	0.1	0.1	0.1	0.2
Borneol	1138	0.1	t	0.1	0.1	0.1	0.2
Terpinen-4-ol	1148	0.3	t	0.5	0.3	0.2	0.2
α-Terpineol	1159	0.6	0.2	2.0	1.3	0.3	0.7
Myrtenol	1168	t	t	t	t	0.1	0.2
cis-Piperitol*	1182	t	t	t	t	t	t
cis-carveol	1202	0.1	t	t	t	0.1	0.1
Carvone	1210	t	t		t	t	0.1
Geraniol	1236	0.2		0.5	t	t	t
α-Terpenyl acetate	1334	2.2	t	5.4	0.2	0.8	0.9
Geranyl acetate	1370	t		0.2	t	t	t

Components	RI	Eg_OE_1_G	Eg_OE_2_B	Eg_OE_3_O	Eg_OE_4_E	Eg_OE_5_P	Eg_OE_6_S
β-Caryophyllene	1414	0.6	t	t	t	0.2	0.2
Aromadendrene	1428	1.2	0.4	0.4	0.4	3.1	4.3
α-Humulene	1447	0.3	t	t	t	t	0.1
allo-Aromadendrene	1456	0.3	t	0.2	0.1	0.6	0.8
Phenethyl isovalerate	1468	t	t	0.1	t	t	t
Germacrene-D	1474	t		t		t	0.1
Viridiflorene	1487	t	t	0.2	t	0.2	0.3
Globulol	1566	0.3	0.2	0.7	0.2	0.5	1.0
% of identification		99.0	99.6	97.6	99.5	99.5	99.4
Group components							
Monoterpene hydrocarbons		24.3	32.0	27.7	42.5	30.2	34.6
Oxygen-containing monoterpenes		71.9	66.6	68.3	56.3	64.4	56.6
Sesquiterpene hydrocarbons		2.4	0.4	0.8	0.5	4.1	5.8
Oxygen-containing sesquiterpenes		0.3	0.2	0.7	0.2	0.5	1.0
Others		0.1	0.4	0.1	t	0.3	0.4

Retention Index (RI) calculated in-lab, relative to $C_9 - C_{17}$ n-alkanes on the DB-1 column, t: trace ($< 0.05\%$).

Monoterpenes dominated in all analysed *E. globulus* EOs, ranging from 91 to 99%. From monoterpenes group, oxygen-containing monoterpenes dominated in all *E. globulus* EO, ranged from 56 to 72%, then monoterpene hydrocarbons ranged from 24 to 43% and sesquiterpenes ranged from 0.2 to 6%. A fraction designated by others comprises compounds that are not terpenes, occurring in EO in trace amounts (t) to 0.4 (**Table 3.1**). 1.8-Cineole (eucalyptol) was the main component of the *E. globulus* EO samples, obtained from the leaves, ranging from 49 to 65%. α-Pinene (11-22%), limonene (8-18%), and α-terpenyl acetate (t-5%) were other relevant compounds identified in the samples of *E. globulus* EO (**Table 3.1**). This result follows previous studies, as shown in **Table 3.2**, where some quantitative differences were observed in the amounts of 1.8-cineole (55-84%).

Table 3.2 Chemical composition of *Eucalyptus globulus* EO samples, obtained in the present study and comparison with other authors from Portugal, India, Brazil, and Ethiopia

Main Components	***Eucalyptus globulus* EO (%)**								
	Leaves	Leaves and branches	Leaves	Leaves and branches	Leaves	Aerial parts	Leaves	Leaves	Leaves
α-Pinene	11-22	16	19	16	13	20	12	4	11
β-Pinene	0-1						19		
1,8-Cineole	49-65	70	63	64	75	64	55	84	77
Limonene	8-18	3	4					8	
α-Terpenyl acetate	t-5	1	1						
β-Eudesmol			t				5		
Reference	Present study	Faria et al. [195]	Silvestre et al. [196]	Luís et al. [156]	Vieira et al. [197]	Miguel et al. [198]	Joshi et al. [199]	Maciel et al. [200]	Abdo [201]

t : traces (<0.05%), main components (≥5%).

α-Pinene has also been identified in prior EOs studies, with percentage variations (4-20%). β-Eudesmol (t-5%) has been identified in some studies and was not present in this study[196,199]. Most of the

compounds identified in the *E. globulus* EO samples were in the range indicated by the ISO standards, which means that the EO complies with the international standard[202]. Shiferaw *et al* [203] reported that the highest content of the 1.8-Cineole compound was obtained for the oldest *E. globulus* tree, which suggests that differences in the ages of the trees can cause variations in oil contents, which may be a possible justification for the differences in this compound observed in previous studies (**Table 3.2**).

3.1.2 Pinus pinaster EO

Between 63 and 68 components were identified in the analyses *Pinus pinaster* EOs. The components are grouped into six classes: monoterpene hydrocarbons; oxygen-containing monoterpenes; sesquiterpene hydrocarbons; oxygen-containing sesquiterpenes, diterpene hydrocarbons and others (**Table 3.3**).

Table 3.3 Percentage composition of the samples of *Pinus pinaster* essential oil

Components	RI	Pp_OE_1_G	Pp_OE_2_P	Pp_OE_3_S
Hexenal	866			t
cis-3-Hexen-1-ol	868		t	t
Tricyclene	921	t	t	t
α-Thujene	924	t	t	t
α-Pinene	930	28.0	44.6	36.5
α-Fenchene	938	0.2	0.4	0.4
Camphene	938	0.2	0.4	0.4
Thuja-2,4(10)-diene*	940	0.1	0.1	0.3
Sabinene	958	t	t	t
β-Pinene	963	28.5	23.0	18.8
2-Pentyl furan	973	t	t	t
β-Myrcene	975	11.0	5.0	5.9
α-Phellandrene	995	0.1	t	0.2
δ-3-Carene	1000	6.6	2.1	1.8
α-Terpinene	1002	0.1	t	0.1
p-Cymene	1003	0.1	0.2	0.2
β-Phellandrene	1005	1.1	0.8	1.2
1,8-Cineole	1005		0.8	0.6
Limonene	1009	4.5	3.9	3.3
cis-β-Ocimene	1017	t	t	t
trans-β-Ocimene	1027	0.6	0.2	0.5
γ-Terpinene	1035	0.1	t	t
2,5-Dimethyl styrene	1059	t	0.1	0.1
Terpinolene	1064	1.1	0.3	0.7
Nonanal	1073	t	0.3	0.1
Linalool	1074	t	0.1	0.1
Isopenthyl isovalerate	1080	t	t	t
endo-Fenchol	1085	0.1	0.1	0.1
trans-Pinocarveol	1106	t	0.4	0.1
cis-Verbenol	1113	t	0.1	t
trans-Pinocamphone	1121	t	0.1	0.1
trans-Pinocarvone		t	0.1	0.3
Borneol	1138	t	0.1	0.2
Terpinen-4-ol	1148	t	t	0.1
p-Cymen-8-ol	1148		t	t
Myrtenal	1153	t	0.1	0.1

Components	RI	Pp_OE_1_G	Pp_OE_2_P	Pp_OE_3_S
α-Terpineol	1159	0.3	0.5	0.6
Myrtenol	1168		0.2	0.1
Methyl thymol	1210	0.1	t	0.1
Hexyl isovalerate	1225	t	0.1	0.1
Linalyl acetate	1245	0.1	0.1	0.1
Bornyl acetate	1265	0.1	0.1	0.1
Thymol	1275	t		t
trans-Pinocarvyl acetate	1278	t	t	t
Tridecane	1300	t	t	t
α-Cubebene	1345	0.2	0.4	0.4
Geranyl acetate	1370	0.1	0.1	0.1
Thymol	1275	t		t
trans-Pinocarvyl acetate	1278	t	t	t
Tridecane	1300	t	t	t
α-Cubebene	1345	0.2	0.4	0.4
Geranyl acetate	1370	0.1	0.1	0.1
α-Ylangene	1371	0.1	0.1	0.1
α-Copaene	1375	0.3	0.4	0.6
β-Bourbonene	1379	t	0.2	t
Longifolene	1399	0.4	0.9	1.4
β-Caryophyllene	1414	4.5	5.0	8.7
β-Copaene	1426	0.1	0.2	0.3
Aromandendrene	1428		0.1	
α-Humulene	1447	0.6	0.7	1.2
Phenethyl 2-methyl butyrate	1467	0.1	0.1	0.1
Phenethyl isovalerate	1468	0.3	0.1	0.4
γ-Muurolene	1469	0.7	1.0	1.4
Germacrene-D	1474	6.3	1.7	5.6
α-Muurolene	1494	0.2	0.3	0.5
γ-Cadinene	1500	0.4	0.4	0.4
trans-Calamenene	1505	t	0.2	0.1
δ-Cadinene	1505	1.1	1.0	2.1
trans-Calamenene	1505	t	0.2	0.1
δ-Cadinene	1505	1.1	1.0	2.1
α-Cadinene	1529	t	t	0.1
trans-α-Bisabolene	1536	0.1	0.1	0.1
β-Caryophyllene oxide	1561	0.1	0.7	0.2
Abietatriene	2045	t	0.2	t
Abietadiene	2060	0.4	0.2	0.5
Abieta-8(14),13(15)-diene*	2116	0.1	t	0.1
% of identification		99.1	98.4	97.7
Group components				
Monoterpene hydrocarbons		82.3	81.0	70.3
Oxygen-containing monoterpenes		0.8	2.9	2.8
Sesquiterpene hydrocarbons		15.0	12.7	23.0
Oxygen-containing sesquiterpenes		0.1	0.7	0.2
Diterpene hydrocarbons		0.5	0.4	0.6
Others		0.4	0.7	0.8

Retention Index (RI) calculated in-lab, relative to C_9-C_{17} n-alkanes on the DB-1 column, t: trace (<0.05%).

The samples of *P. pinaster* consisted mainly of monoterpenes hydrocarbons ranging from 70 to 82%, and lower contents of oxygen-containing monoterpenes ranged from 1 to 3%. Sesquiterpene hydrocarbons ranged from 13 to 23%, oxygen-containing sesquiterpenes ranged from 0.1 to 1% and others from 0.4 to 1% (**Table 3.3**). From the three samples of *P. pinaster* EO, two were dominated by

α-pinene (37-45%), and the third showed similar amounts of α-pinene and β-pinene (28% and 29%, respectively) (**Table 3.3**). These results are according to previous reports in which α and β-pinene were the dominant compounds with significant percentage variations (21-62% and 1-52%, respectively) (**Table 3.4**).

Table 3.4 Chemical composition of *Pinus pinaster* EO samples obtained in the present study and comparison with other authors, from Portugal, Italy, Greece and Tunisia

Main Components	***Pinus pinaster* EO (%)**							
	Needles	Needles	Needles	Needles	Needles	Branches	Cones	Needles
α-Pinene	27-45	25-42	27	62	29	40	25	21
β-Pinene	19-28	36-52	20	23	22	23	30	1
β-Myrcene	5-11		6		5	12	4	T
δ-3-Carene	2-7	t-18						
Limonene	3-5		8		4	9	2	1
α-Terpineol	0-1	4-8	1		1	t	3	
Longifolene	0-1		4		1	8	21	
β-Caryophyllene	5-9	t-2	t		13	t	t	15
Germacrene-D	2-6		3		4	t		19
Reference	Present study	Rodrigues et al. [90]	Carmo and Frazão [204]	Miguel et al. [205]	Macchioni et al. [165]	Macchioni et al. [165]	Macchioni et al. [165]	Petrakis et al. [166]

t : traces (≤ 0.05%), main components (≥ 5%).

Furthermore, other compounds with significant percentage values have been identified in the EO of *P. pinaster*, namely, β-myrcene (5-11%); δ-3-carene (2-7%); limonene (3-5%); α-terpineol (0.3-1%); longifolene (0.4-1%); β-caryophyllene (5-9%); germacrene-D (2-6%) (**Table 3.3**). These results are in accordance with previous reports for *P. pinaster* EO from Portugal and from other producers' countries (**Table 3.4**). Most of the compounds identified in the *P. pinaster* EO samples were in the range indicated by the ISO standards, which means that the EO complies with the international standard [206]. All studies mentioned in **Table 3.4**, were found to have α and β-pinene as the dominant compounds, except for Petrakis *et al* [166]. Petrakis *et al* [166], characterized the chemical composition of the needle oil of *P. pinaster*, growing in Greece and had as main dominant compound the α-pinene (21%), following the germacrene-D (19%) and β-caryophyllene (15%). Some compounds were identified in higher percentages, than in the present study, such as δ-3-carene (t-18%) [90]; germacrene-D (19%) [166]; α-terpineol (3-8%) [90,165]; β-caryophyllene (13-15%) [165,166]; limonene (8-9%) and longifolene (4-21%) [165,204]. Faria and Rodrigues [207] proved the existence of a high chemical variability in the composition of the *P. pinaster* and *P. pinea* EOs and that the main compounds responsible for this chemical variability were the α and β-pinene, trans-β-caryophyllene, germacrene D and β-myrcene.

The genetic characteristics as well as the high influence of different climatic conditions are factors known to influence the phenotypic characteristics of the pine trees and may be a possible justification for the chemical variability presented by this species [208]. There are several factors that the chemical composition of EOs depends on, such as the physiological parameters of the plant, climatic conditions (e.g. climate, pests, pollution, diseases, soil type, among others) and geographic location [209]. Faria and Rodrigues [207] also suggested that the pines are exposed to various environmental conditions associated with the species geographical variation, demonstrating that EOs chemotypes are a source of variation among species. Furthermore, they reported the chemical variability of EOs from *P. pinaster* and *P. pinea* of various provinces and concluded that *P. pinaster* EO composition variability depended more on geographic localization than in the *P. pinea* EO [207].

3.1.3 Pinus pinea EO

In the *Pinus pinea* EO sample, 50 constituents were identified, as described in **Table 3.5.** The components are grouped into five classes: monoterpene hydrocarbons; oxygen-containing monoterpenes; sesquiterpene hydrocarbons; oxygen-containing sesquiterpenes and others.

Table 3.5 Percentage composition of the samples of *Pinus pinea* essential oil

Components	RI	Ppi_OE_1_B
2-*trans*-Hexenal	866	t
cis-3-Hexen-1-ol	868	0.2
Hexanol	881	t
Tricyclene	921	t
α-Thujene	924	t
α-Pinene	930	7.6
Camphene	938	0.1
Thuja-2,4(10)-diene*	940	t
Sabinene	958	t
β-Pinene	963	1.2
β-Myrcene	975	2.1
α-Phellandrene	995	0.1
p-Cymene	1003	0.3
1,8-Cineole	1005	3.8
β-Phellandrene	1005	3.8
Limonene	1009	72.8
cis-β-Ocimene	1017	0.1
trans-β-Ocimene	1027	t
γ-Terpinene	1035	t
2,5-Dimethyl styrene	1059	t
Terpinolene	1064	0.1
Nonanal	1073	t
Linalool	1074	t
endo-Fenchol	1085	t
cis-Limonene oxide	1095	0.2
trans-Limonene oxide	1112	0.1
Cryptone*	1143	0.3
Terpinen-4-ol	1148	0.1
α-Terpineol	1159	0.3
trans-Carveol	1189	0.2
cis-Carveol	1202	0.1
Carvone	1210	0.2
Methyl thymol	1210	0.6
Bornyl acetate	1265	0.1
α-Terpenyl acetate	1334	0.6
α-Longipinene	1338	0.5
Longifolene	1399	1.0
β-Caryophyllene	1414	1.0
β-Copaene	1426	0.0
Aromadendrene	1428	0.6
α-Humulene	1447	0.2
trans-β-Farnesene	1455	0.1
allo-Aromadendrene	1456	0.1
Phenethyl 2-methyl butyrate	1467	0.1

Components	RI	Ppi_OE_1_B
γ-Muurolene	1469	t
Germacrene-D	1474	t
Viridiflorene	1487	0.1
β-Caryophyllene oxide	1561	0.3
Globulol	1566	0.2
Viridiflorol	1569	t
% of Identification		99.2
Group Components		
Monoterpene hydrocarbons		88.2
Oxygen-containing monoterpenes		6.6
Sesquiterpene hydrocarbons		3.6
Oxygen-containing sesquiterpenes		0.5
Others		0.3

Retention Index (RI) calculated in-lab, relative to C_9-C_{17} n-alkanes on the DB-1 column, t: trace (<0.05%).

In the sample of *P. pinea*, EO monoterpenes dominated with a percentage of 95%. in which the monoterpene hydrocarbons had a higher percentage value (88%), following the oxygen-containing monoterpenes (7%) and with lower contents of sesquiterpenes (4%) and others (0.3%) (**Table 3.5**). Limonene (73%) was the main component in *P. pinea* EO (**Table 3.5**) what agrees with previous studies, despite percentual variations (36-63%) (**Table 3.6**). α-Pinene (8%) was another relevant compound, and some authors also identified the compound with a variety of values from 4 to 19% (**Table 3.6**). Myrcene [165], β-caryophyllene and β-phellandrene [160,165] were reported, for *P. pinea* EO, in some studies in higher percentages (**Table 3.6**) than those obtained in the present study (**Table 3.5**). Faria and Rodrigues [207], in addition to having proven the high chemical variability existing in the composition of *P. pinea* EO, as mentioned above, found that it was mainly due to the compound limonene.

Table 3.6 Chemical composition of *Pinus pinea* EO samples obtained in the present study and comparison with other authors from Portugal, Italy, Tunisia and Turkey

Main Components	***Pinus pinea* EO (%)**						
	Needles	Needles	Needles	Branches	Cones	Needles	Needles
α-Pinene	8	10-13	6	6	19	6	4
β-Pinene	1	10-25	2	2	2	1	2
Myrcene	2		3	2	1	1	2
β-Phellandrene	4		7	8		t	7
Limonene	73	52-61	59	63	62	36	55
β-Caryophyllene	1	1	4	6	3	1	4
Reference	Present study	Rodrigues et al. [90]	Macchioni et al. [165]	Macchioni et al. [165]	Macchioni et al. [165]	Nasri et al. [210]	Demirci et al. [160]

t: traces (<0.05%), main components (≥5%).

3.1.4 Cryptomeria japonica EO

Seventy-nine components were identified in *Cryptomeria japonica* EO. The components are grouped into a larger group of classes, namely, eight classes: monoterpene hydrocarbons; oxygen-containing monoterpenes; sesquiterpene hydrocarbons; oxygen-containing sesquiterpenes; diterpene hydrocarbons; oxygen-containing diterpene; phenylpropanoids and others (**Table 3.7**). The main components of the sample of *C. japonica* EO were monoterpene hydrocarbons (66%) and diterpene hydrocarbons (16%).

Sesquiterpenes obtained a percentage of 9% and phenylpropanoids and others with trace values (**Table 3.7**). In the sample of *C. japonica* EO, the α-pinene dominated with 26% (**Table 3.7**). This result follows previous studies, as shown in **Table 3.8**, with some quantitative differences concerning the percentage values (3-39%). Sabinene (18%) and phyllocladene (14%) were other relevant compounds identified in the *C. japonica* EO (**Table 3.7).** This partly agrees with other studies since the sabinene was identified by most of the previous studies (1-24%) (**Table 3.8**). β-Cedrene [211], thujopsene [211], α-elemol [212] and kaur-16-ene [211,212] were compounds identified by other authors from producing countries that were not found in this study (**Table 3.8**).

Table 3.7 Percentage composition of the samples of *Cryptomeria japonica* essential oil

Components	RI	Cj_OE_1_M
Trycyclene	921	0.3
α-Thujene	924	1.3
α-Pinene	930	26.1
α-Fenchene	938	0.6
Camphene	938	1.8
Sabinene	958	18.1
β-Pinene	963	2.0
β-Myrcene	975	4.4
α-Phellandrene	995	0.1
δ-3-Carene	1000	1.2
α-Terpinene	1002	1.6
p-Cymene	1003	0.4
β-Phellandrene	1005	0.7
Limonene	1009	3.8
cis-β-Ocymene	1017	t
trans-β-Ocymene	1027	t
γ-Terpinene	1035	2.6
trans-Sabinene hydrate	1037	0.2
2,5-Dimethyl styrene	1059	t
Terpinolene	1064	1.0
cis-Sabinene hydrate	1066	0.1
Linalool	1074	0.2
trans-Thujone	1081	t
1-Octen-3-yl-acetate	1086	t
α-Campholenal	1092	0.1
trans-*p*-2-Menthen-1-ol	1099	0.1
Camphor	1102	t
cis-*p*-2-Menthen-1-ol	1114	0.1
trans-Pinocamphone	1121	t
Borneol	1138	t
cis-Pinocamphone	1134	t
Terpinen-4-ol	1148	2.3
α-Terpineol	1159	0.1
cis-Piperitol*	1182	t
trans-Piperitol	1189	t
α-Fenchyl acetate	1200	t
Piperitone	1211	t
Geraniol	1236	t
Linalyl acetate	1245	0.2
trans-Anethole	1254	t
Bornyl acetate	1265	1.8
cis-Verbenyl acetate	1266	0.1

Components	RI	Cj_OE_1_M
α-Terpenyl acetate	1334	0.2
α-Cubebene	1345	t
Geranyl acetate	1370	t
α-Copaene	1375	t
β-Bourbonene	1379	t
β-Elemene	1388	0.1
β-Caryophyllene	1414	t
β-Copaene	1426	0.1
α-Humulene	1447	t
trans-β-Farnesene	1455	t
γ-Muurolene	1469	0.1
Germacrene-D	1474	0.3
α-Muurolene	1494	0.1
β-Bisabolene	1500	0.2
γ-Cadinene	1500	0.2
δ-Cadinene	1505	0.5
α-Cadinene	1529	t
Elemol	1530	4.4
trans-Nerolidol	1549	t
Germacrene-D-4-ol*	1557	0.2
Cedrol	1574	t
Anydrooplopanone	1576	0.1
10-epi-γ-Eudesmol	1593	0.1
γ-Eudesmol	1609	0.6
trans-Muurolol	1616	0.1
α-Muurolol	1618	0.1
β-Eudesmol	1620	0.9
α-Eudesmol	1634	1.1
Cryptomerione*	1686	t
Oplopanoyl acetate*	1808	t
Rimuene	1814	0.0
Isopimara-9(11),15-diene	1821	0.5
Isokaurene*	1977	0.5
Sandaracopimara-8(14),15-diene	1956	0.7
Phyllocladene	2006	13.8
Kaurene	2044	0.5
Nezukol*	2112	0.7
% of Identification		97.4
Group components		
Monoterpene hydrocarbons		66.0
Oxygen-containing monoterpenes		5.5
Sesquiterpene hydrocarbons		1.6
Oxygen-containing sesquiterpenes		7.6
Diterpene hydrocarbons		16.0
Oxygen-containing diterpene		0.7
Phenylpropanoids		t
Others		t

Retention Index (RI) calculated in-lab, relative to C_9-C_{17} n-alkanes on the DB-1 column, t: trace (<0.05)

Differences in the chemical composition of *C. japonica* EO may be related to the different origins of the plant species, the age of the trees, the habitats, or even the extraction methods used [213]. Furthermore, in general, the chemical composition of EOs might be affected by certain factors, such as endogenous or exogenous. The endogenous factors are related to the anatomical and physiological characteristics of the plants associated with the chemical variation among different parts of the plant and from genetically affiliated factors [214].

Alternatively, the exogenous factors or environmentally regulated factors, such as light, precipitation, growing site and soil may modify the qualitative/quantitative amount of the volatiles in the EOs [214]. Also, it is widespread occur EO chemotypes in forest trees [207].

Table 3.8 Chemical composition of the samples of *Cryptomeria japonica* EO obtained in the present study and comparison with other authors from Portugal, Japan, China, and Corsica

Main Components	***Cryptomeria japonica* EO (%)**							
	Foliage	Foliage	Foliage	Cones	Foliage	Leaves	Foliage	Cones
α-Pinene	26	9-39	10-30	23	3-13	8	19	12-25
Sabinene	18	5-24	1-20	17	nd-17		20	13-17
β-Myrcene	4	2-6						
δ-3-Carene	1	t-3						
Limonene	4	1-11	1-12	2	1-6	2		
Terpinen-4-ol	2	1-5	t-9	24	t-4	1	7	3-7
β-Cedrene					1-13			
Thujopsene					8-10			
δ–Cadinene	1		1-7	t	1-12	2	1	t
Elemol	4	1-9	t-13	1	3-7	1	11	13-20
α-Elemol						20		
γ-Eudesmol	1							
β-Eudesmol	1	t-2						
α-Eudesmol	1	t-3						
Phyllocladene	14	t-22	4-27	1				
Kaurene	1	t-6	t-21		nd-9	2	7	5-10
Kaur-16-ene					nd-4	15		
Reference	Present study	Figueiredo et al. [87]	Moiteiro et al. [182]	Faria et al. [215]	Nakagawa et al. [211]	Xie et al.[212]	Garcia et al. [216]	Garcia et al. [216]

t: traces (<0.05%), main components (≥5%), nd: not detected.

Bang, Lewis and Villas-Boas [217] suggested that environmental conditions such as seasonal differences could affect the chemical composition of the *C. japonica* EO. For that reason, the chemical composition of the EOs can depend on several factors, which may justify the differences observed in previously mentioned studies (**Table 3.8**).

3.1.5 Hydrolates volatiles

3.1.5.1 Eucalyptus globulus Hd

Chemical analyses of *E. globulus* HVs identified 46-58 constituents. The compounds identified are grouped into four classes: monoterpene hydrocarbons, oxygen-containing monoterpenes, oxygen-containing sesquiterpenes and others (**Table 3.9**).

E. globulus HVs were predominantly composed of monoterpenes ranging from 93-99%. Oxygen-containing monoterpenes dominated in all the samples of *E. globulus* Hd ranged from 84-92%, following the monoterpene hydrocarbons ranged from 2-14%, while oxygen-containing sesquiterpenes ranged from traces to 1%, and others from traces to 2% (**Table 3.9).** Like with *E. globulus* EO samples three of the HVs were dominated by 1,8-cineole (54-80%), **Table 3.9.** The fourth sample was dominated by *trans*-pinocarveol (37%). The second main component varied according to the sample, two samples showing high percentages of α-terpineol (17% and 25%), one limonene (7%) and the fourth sample

myrtenol (12%) (**Table 3.9**).

Table 3.9 Percentage composition of the hydrolates volatiles from *Eucalyptus globulus*

Components	RI	Eg_Hd_1_G	Eg_Hd_2_O	Eg_Hd_3_E	Eg_Hd_4_P
2-*trans*-Hexenal	866	t	t	t	t
Isovaleric acid	867	t	t	t	t
cis-3-Hexen-1-ol	868	t	t	t	t
2-Methyl butyric acid	871	t		t	t
cis-2-Hexen-1-ol	882	t	t	t	t
n-Hexanol	883	t	t	t	t
2-Acetyl furan*	897				t
Terbutyl isovalerate	924	t		t	t
Benzaldehyde	927		t		
α-Pinene	930	t		t	t
Hexanoic acid	968	t	t	t	t
Benzyl alcohol	1002		t	t	t
Benzene acetaldehyde	1004		t	t	t
2-Ethyl-1-hexanol	1004				2.3
1,8-Cineole	1005	80.2	55.5	53.5	4.5
Limonene	1009	7.3	14.1	6.7	1.5
Acetophenone	1017	t	t	t	
cis-Linalool oxide (furanoid)	1045	t	0.1	t	t
trans-Linalool oxide (furanoid)	1059	t	0.1	t	t
Phenyl ethyl alcohol	1067	t	0.1		t
Linalool	1074	t	0.5	t	t
endo-Fenchol	1085	t	t	t	t
α-Campholenal	1092	t	t	t	0.4
Nopinone	1093	t	t		t
trans-*p*-2-Menthen-1-ol	1099	t	t	t	t
Cosmene*	1102	t		t	t
trans-Pinocarveol	1106	4.9	0.4	8.0	36.6
cis-Verbenol	1114	t	0.1	t	
cis-*p*-2-Menthen-1-ol	1114	t	t	t	4.6
Pinocarvone	1121	1.6	0.1	t	0.3
Benzyl acetate	1123		t		
δ-Terpineol	1134	t	1.2		
Borneol	1138	t	1.2		3.7
p-Cymen-8-ol	1148	0.9	t	t	2.4
Terpinen-4-ol	1148	t	3.0	2.2	
Myrtenal	1153		t	t	5.5
α-Terpineol	1159	2.7	17.2	24.7	5.3
Verbenone	1164	t	t	t	
Myrtenol	1168		t	t	12.0
trans-Carveol	1189	t	0.1	t	2.4
cis-Carveol	1202	1.1	0.1	2.8	8.6
2-Hydroxy-3-pinanone	1206				t
Carvone	1210	t		t	t
Neral	1210	t	t	t	
Citronellol	1211	t	0.5		
cis-Piperitone epoxide	1211	t	0.4	t	0.9
2-Phenyl ethyl acetate	1228	t	t	t	
Geraniol	1236	t	2.6	t	t
m-Acetanisole	1237	t	0.5	t	
Geranial	1240	t	0.2	t	t
Linalyl acetate	1254		t	t	t
Thymol	1275	t	t	t	1.5

Components	RI	Eg_Hd_1_G	Eg_Hd_2_O	Eg_Hd_3_E	Eg_Hd_4_P
2-Methoxy-4-vinylphenol	1285	t	t	t	t
Carvacrol	1286	t	t	t	t
p-Acetanisole *	1311	t	t	t	
Exo-2-hydroxy cineole acetate *	1323	t	0.8		2.4
α-Terpenyl acetate	1334		t	t	
Geranyl acetate	1370	t	t	t	
Perilla alcohol isopentyl ether	1389		t	t	t
Spathulenol	1551	t	t	t	
Viridiflorol	1569	t	t	t	
Globulol	1566	t	t	t	t
Ledol	1580		0.2	t	
γ-Eudesmol	1609	t	0.1	t	
β-Eudesmol	1620	t	0.2		
α-Eudesmol	1634	t	0.1		
% Identification		98.8	99.2	97.8	94.7
Group components					
Monoterpene hydrocarbons		7.3	14.3	6.7	1.5
Oxygen-containing monoterpenes		91.5	83.8	91.1	91.0
Oxygen-containing sesquiterpenes		t	0.5	t	t
Others.		t	0.6	t	2.3

Retention Index (RI) calculated in-lab, relative to C_9-C_{17} n-alkanes on the DB-1 column, t: trace (<0.05).

In a previous study, Ndiaye *et al* [218] evaluated the chemical composition of EOs and Hds from three distinct *Eucalyptus* species (*Eucalyptus camaldulensis*, *Eucalyptus alba* and *Eucalyptus tereticornis*). The HVs demonstrated a chemical composition rich in oxygenated molecules in which 1,8-cineole (31-53%) was its dominant compound as well as in its corresponding EOs, which agrees with the one found in the present study. In addition, they also identified other relevant compounds present in smaller amounts, and which were also described in the present study, such as *trans*-pinocarveol (3-19%), α-terpineol (3-9%) and *cis*-carveol (3-5%). Paolini *et al* [219] characterized the commercial HVs from typically Mediterranean species, including *E. globulus*. 1,8-cineole was, again, the dominant compound of the *E. globulus* HVs, having obtained higher percentages compared to the EO, which is according to the present study.

In this study, most of the percentage values of the compounds: 1,8-cineole, *trans*-pinocarveol, α-terpineol and *cis*-carveol, were higher in the *E. globulus* HVs samples than in their corresponding EO, which was also evident in the study mentioned previously [219]. Other compounds have been identified in *E. globulus* HVs but not in their EO. This suggests that during extracting volatiles from Hds, hydrophilic oxygenated molecules may have passed [218]. Furthermore, the dominance of monoterpene hydrocarbons in EO samples but not in HVs can be justified due to their low water solubility compared to oxygen-containing monoterpenes [220].

3.1.5.2 Pinus pinaster Hd

In *P. pinaster* HVs, 38 compounds were identified in **Pp_Hd_1_G** and 42 in **Pp_Hd_2_P**. The constituents are grouped into five classes: monoterpene hydrocarbons, oxygen-containing monoterpenes, oxygen-containing sesquiterpenes, phenylpropanoids and others (**Table 3.10**).

The samples of *P. pinaster* HVs were predominantly composed of oxygen-containing monoterpenes (95%), and lower contents of monoterpenes hydrocarbons ranged from traces to 4%. The oxygen-containing sesquiterpenes, phenylpropanoids and others had trace values. *P. pinaster* HVs samples had

α-terpineol as the dominant compound (38-44%), followed by verbenone (18-29%). The **Pp_Hd_1_G** sample had other relevant compounds present in smaller amounts such as, 1,8-cineole (5%), terpinen-4-ol (8%), *p*-cymen-8-ol (8%), perilla alcohol (7%) and thymol (7%) (**Table 3.10**). These results are under previous reports in which α-terpineol was the main HVs compound of a species from genus *Pinus* (ex: *Pinus cembra*) with percentage variations between 28-38% [221].

Table 3.10 Percentage composition of the hydrolate volatiles from *Pinus pinaster*

Components	RI	Pp_Hd_1_G	Pp_Hd_2_P
2-*trans*-Hexenal	866	t	t
cis-3-Hexen-1-ol	868	t	t
cis-2-Hexen-1-ol	882	t	t
n-Hexanol	883	t	t
Benzaldehyde	927	t	t
α-Pinene	930	t	t
β-Pinene	963	t	t
Benzyl alcohol	1002	t	t
Benzene acetaldehyde	1004	t	t
2-Ethyl-1-hexanol	1004		t
1,8-Cineole	1005	5.0	t
Limonene	1009	4.3	
cis-Linalool oxide (furanoid)	1045		t
trans-Linalool oxide (furanoid)	1059		t
Phenyl ethyl alcohol	1067		t
Linalool	1074	t	t
endo-Fenchol	1085	t	t
trans-*p*-2-Menthen-1-ol	1099	t	t
Camphor	1102	t	t
trans-Pinocarveol	1106	t	t
cis-Verbenol	1114	t	t
cis-*p*-2-Menthen-1-ol	1114	t	14.0
neo-Isopulegol	1116		14.0
trans-Pinocamphone	1121	t	t
Pinocarvone	1121	t	t
Isoborneol	1132	t	t
cis-Linalool oxide (pyranoid)	1132		t
Borneol	1138	t	t
Terpinen-4-ol	1148	7.5	
p-Cymen-8-ol	1148	7.5	t
Myrtenal	1153	t	
α-Terpineol	1159	43.8	38.1
Verbenone	1164	17.9	28.7
trans-Carveol	1189	t	t
Geraniol	1236	t	t
Perilla alcohol	1274	6.6	t
Thymol	1275	6.6	t
Carvacrol	1286	t	t
Geranyl acetate	1370	t	t
Methyl eugenol	1377	t	t
Phenethyl 2-methybutyrate	1467	t	t
Phenethyl isovalerate	1468		t
Globulol	1566	t	t
epi-α-Muurolol	1616	t	t
α-Cadinol	1616	t	t
% Identification		99.1	94.7

Components	RI	Pp_Hd_1_G	Pp_Hd_2_P
Group components			
Monoterpene hydrocarbons		4.3	t
Oxygen-containing monoterpenes		94.8	94.7
Oxygen-containing sesquiterpenes		t	t
Phenylpropanoids		t	t
Others.		t	t

Retention Index (RI) calculated in-lab, relative to C_9-C_{17} n-alkanes on the DB-1 column, t: trace (<0.05).

P. cembra HVs was mainly composed of oxygen-containing monoterpenes while its EO presented, predominantly, monoterpene hydrocarbons, which is according to the one found in the present study. Once again, it was proved that compounds belonging to the oxygen-containing monoterpenes dominated in *P. pinaster* Hd samples due to the fact that monoterpenes hydrocarbons have a lower solubility in water [220]. Thus, the high percentage value of the compound α-terpineol can be justified due to its high solubility in water (710 mg/L) when compared to the α and β-pinene (2.49 mg/L and 4.89 mg/L, respectively) [222].

3.1.5.3 Cryptomeria japonica Hd

In *C. japonica* HVs, 44 constituents were identified. They are grouped into five classes: monoterpene hydrocarbons, oxygen-containing monoterpenes, oxygen-containing sesquiterpenes, diterpene hydrocarbons and others (**Table 3.11**).

Table 3.11 Percentage composition of the hydrolate volatiles from *Cryptomeria japonica*

Components	RI	Cj_Hd_1_M
cis-3-Hexen-1-ol	868	t
n-Hexanol	883	t
n-Nonane	900	t
α-Pinene	930	t
n-Heptanol	952	t
1-Octen-3-ol	961	t
β-Pinene	963	t
β-Myrcene	975	t
α-Phellandrene	995	t
Benzene acetaldehyde	1002	t
α-Terpinene	1002	t
p-Cymene	1003	t
1,8-Cineole	1005	6.3
Limonene	1009	2.3
Acetophenone	1017	t
γ-Terpinene	1035	t
cis-Linalool oxide (furanoid)	1045	t
Fenchone	1050	t
trans-Linalool oxide (furanoid)	1059	t
Linalool	1074	2.6
trans-*p*-2-Menthen-1-ol	1099	2.8
Camphor	1102	0.4
cis-*p*-2-Menthen-1-ol	1114	2.5
Pinocarvone	1121	0.7
cis-Pinocamphone	1134	0.3

Components	RI	Cj_Hd_1_M
Borneol ethyl ether	1138	0.7
Terpinen-4-ol	1148	56.2
α-Terpineol	1159	4.6
Citronellol	1211	0.2
Linalyl acetate	1245	0.5
Bornyl acetate	1265	0.8
trans-Pinocarvyl acetate	1278	t
Carvacrol	1286	0.1
trans-Carvyl acetate	1305	t
α-Terpenyl acetate	1334	t
Vanillin	1358	t
Elemol	1530	4.2
Anydrooplopanone	1576	t
γ-Eudesmol	1609	1.6
β-Eudesmol	1620	1.6
Valerianol	1623	0.2
α-Eudesmol	1634	2.1
Oplopanoyl acetate*	1808	t
Phyllocladene	2006	4.8
% Identification		95.4
Group components		
Monoterpene hydrocarbons		2.3
Oxygen-containing monoterpenes		78.7
Oxygen-containing sesquiterpenes		9.6
Diterpene hydrocarbons		4.8
Others.		0.0

Retention Index (RI) calculated in-lab, relative to C_9-C_{17} n-alkanes on the DB-1 column, t: trace (<0.05).

The main components of the *C. japonica* HVs were oxygen-containing monoterpenes (79%), then oxygen-containing sesquiterpenes (10%), diterpene hydrocarbons (5%), monoterpene hydrocarbons (2%) and others (0%). Terpinen-4-ol was the dominant compound in the *C. japonica* HVs sample (56%) (**Table 3.11**). This result follows previous studies such as Nakagawa *et al* [211], which evaluated the chemical composition of EO and HVs from *C. japonica* and realized that the main compound of *C. japonica* HVs, obtained from branches with leaves, were terpinen-4-ol ranged from of 32-37%.

The presence of α-terpineol compound in percentage values is more significant in the *C. japonica* Hd (5%) than the trace values obtained in its corresponding EO which may be related to being a compound with a high solubility in water, belonging to oxygen-containing monoterpenes, as mentioned above. Furthermore, 1,8-cineole was identified only in the *C. japonica* Hd and not in its corresponding EO, which may be related to the fact that it is also an oxygen-containing monoterpene being a water-soluble compound (3.5 x 103 mg/L) [220]. This can be explained by the EOs that are characterized by being composed of complex molecules of volatile organic compounds that are insoluble in water [223] and on the other hand, Hds are co-products with greater solubility in water, since they are richer in oxygenated compounds which have a higher solubility in water [224].

3.2 Antioxidant Activity

The antioxidant capacity for the samples of EOs and Hds assessed through the different assays such as DPPH, ORAC, and intracellular ROS measurement summarized in **Tables 3.12**; **3.13** and **Figures 3.1**; **3.2**; **3.3** and **3.4**.

3.2.1 DPPH and ORAC assays

In the DPPH method, the EOs studied generally showed a weak antioxidant activity, except the sample **CJ_OE_1_M** (23.1 mg/mL), which demonstrated a considerable antioxidant activity followed by the sample **Pp_OE_1_G** (55.2 mg/mL). The lowest antioxidant activity was observed for the **EG_OE_2_B** (647.3 mg/mL) sample. In contrast, EOs showed a greater antioxidant capacity with the ORAC method, emphasizing the samples **Pp_OE_3_S** (565450.6 µmol TE/g), **Pp_OE_2_P** (355575.7 µmol TE/g) and **Cj_OE_1_M** (224877.9 µmol TE/g). The lower values of antioxidant capacity were obtained for the samples **Eg_OE_2_B** (53669.2 µmol TE/g) and then by the **Eg_OE_5_P** (86174.9 µmol TE/g) (**Table 3.12** and **Figure 3.1**). Regarding Hds, they did not present antioxidant activity for the DPPH method and exhibited lower values of antioxidant activity than the respective EOs, in the ORAC method. The sample **Eg_Hd_4_O** (1129.7 µmol TE/g) had the highest antioxidant capacity of the Hd samples (**Table 3.13** and **Figure 3.2**).

Considering the method used to evaluate the antioxidant activity, both EOs and Hds showed different results. The EOs and Hds ORAC assay showed higher antioxidant activities when compared to the results obtained from DPPH assay. These methods in several studies have shown different results, which can be related to the assays evaluating the antioxidant capacity from different mechanisms [225]. Therefore, they are classified into two distinct groups considering the reaction mechanisms: the hydrogen atom transfer (HAT) and the electron transfer (ET). The ORAC method is included in the HAT group since it encompasses a competitive reaction scheme between antioxidant substances and a fluorescence probe (fluorescein) for a radical, commonly the peroxyl radical that results from AAPH [128]. The DPPH method, in turn, belongs to the ET group, which is based on a single electron transfer reaction in which the DPPH radical reacts by itself as a radical and as a probe [225].

Furthermore, some of the chemical compounds identified in the studied EOs and Hds may have antioxidant properties and therefore be related to the antioxidant capacity of the extracts.

The *C. japonica* EO had the highest antioxidant capacity in the DPPH assay (**Table 3.12** and **Figure 3.1**). and the third best in the ORAC assay (**Table 3.12** and **Figure 3.2**). The antioxidant activity of *C. japonica* EO and Hd evaluated by various testing methods is poorly described in the literature. However, Ho et al [213] evaluated the antioxidant activity, using the DPPH method, of EO obtained from different parts of the *C. japonica* plant, leaf, heartwood, sapwood, bark and twigs. The IC50 values obtained were higher than those described in this study, being that the sapwood oil showed the best radical scavenging capability, and the leaf oil demonstrated the poorest antioxidant capacity. So, our sample of *C. japonica* EO obtained from foliage seems to have a better antioxidant capacity in the DPPH method, as it had a lower IC50 value. The main compound of the *C. japonica* EO (α-pinene) did not show an antioxidant activity against DPPH and AAPH radicals in previous studies. Therefore, the DPPH antioxidant activity would be due to the presence of appreciable amounts of compounds that are described in the literature as having an antioxidant activity against free radicals such as DPPH, namely, sabinene [226,227], β-myrcene [228] and γ-terpinene [229,230].

Table 3.12 The antioxidant capacity for the EOs samples of *Eucalyptus globulus*, *Pinus pinaster*, *Pinus pinea* and *Cryptomeria japonica*

Sample Code of EOs*	DPPH[a]	ORAC[b]
Eg_OE_1_G	197.6 ± 20.4	113245.9 ± 15003.8
Eg_OE_2_B	647.3 ± 5.7	53669.2 ± 8659.3
Eg_OE_3_O	151.8 ± 0.0	171891.9 ± 25388.4
Eg_OE_4_E	-	113884.2 ± 14067.0
Eg_OE_5_P	-	86174.9 ± 9813.9
Eg_OE_6_S	246.7 ± 24.5	160532.2 ± 16659.3
Pp_OE_1_G	55.2 ± 0.9	161208.7 ± 24896.4
Pp_OE_2_P	-	355575.7 ± 30254.3
Pp_OE_3_S	-	565450.6 ± 70377.8
Ppi_OE_1_B	195.7 ± 22.9	165063.9 ± 20907.1
Cj_OE_1_M	23.1 ± 0.2	224877.9 ± 25680.9
Ascorbic Acid	36.0 ± 1.1	-

*For the sample code of EOs, see **Table 2.1**. [a] radical scavenging activity (IC^{50}, mg/mL); [b] Trolox equivalents/extract (µmol TE/g). DPPH positive control: Ascorbic Acid (µg/mL). Values are means ± SD

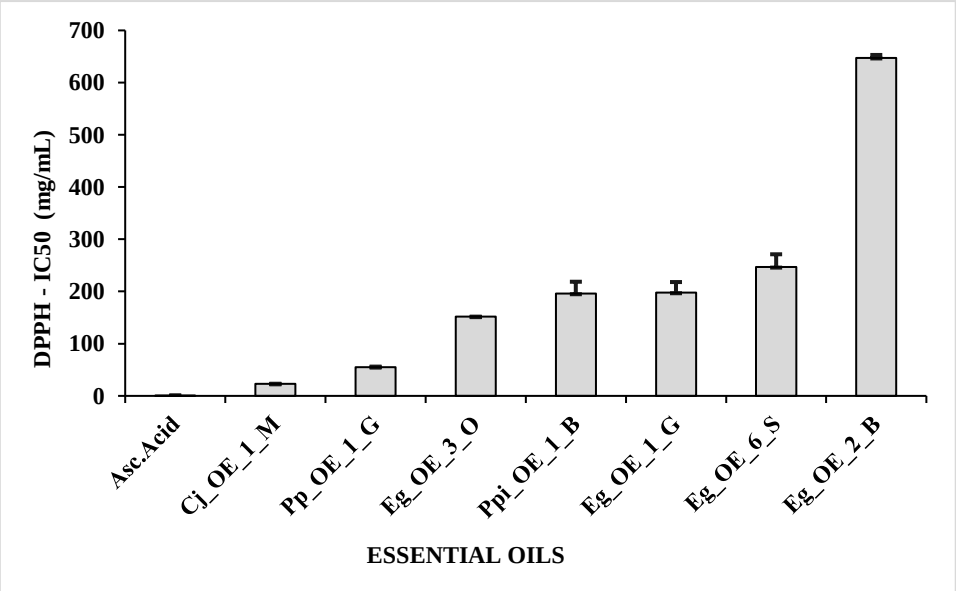

Figure 3.1 Antioxidant activity of essential oils extracts from *E. globulus*, *P. pinaster*, *P. pinea* and *C. japonica* by DPPH, IC^{50}, mg/mL. Positive control: Ascorbic Acid (µg/mL). Values are means ± SD

The γ-terpinene has also been shown to have antioxidant capacity against the AAPH radical. In addition, there are other compounds in the *C. japonica* EO, in smaller amounts, that are described in the literature as having antioxidant activity against the AAPH radical and may explain the antioxidant capacity that the oil demonstrated for the ORAC method, namely, terpinen-4-ol, β-pinene and limonene [230]. It is important to note that right after *C. japonica* EO, in the DPPH assay, one of the *P. pinaster* EO samples had the lowest IC50 value, namely, **Pp_OE_1_G**. The **Pp_OE_1_G** was the sample that,

among the three, showed a higher percentage of the compound β-myrcene, which was previously described as having antioxidant activity against the DPPH radical [228].

One of the *P. pinaster* EO samples obtained the highest antioxidant capacity in the ORAC assay, namely, **Pp_OE_3_S** (**Table 3.12** and **Figure 3.2**). The EO of *P. pinaster* is poorly described in its antioxidant activity using the ORAC method. However, Mediavilla *et al* [145] evaluated forest species antioxidant activity, including *P. pinaster* and *P. sylvestris* EOs using the ORAC method. The *P. pinaster* EO demonstrated a higher antioxidant activity than the *P. sylvestris* EO, which obtained the lowest ORAC value concerning all species studied. In contrast, our ORAC results revealed higher antioxidant activity values than those described above for the EO from *P. pinaster*.

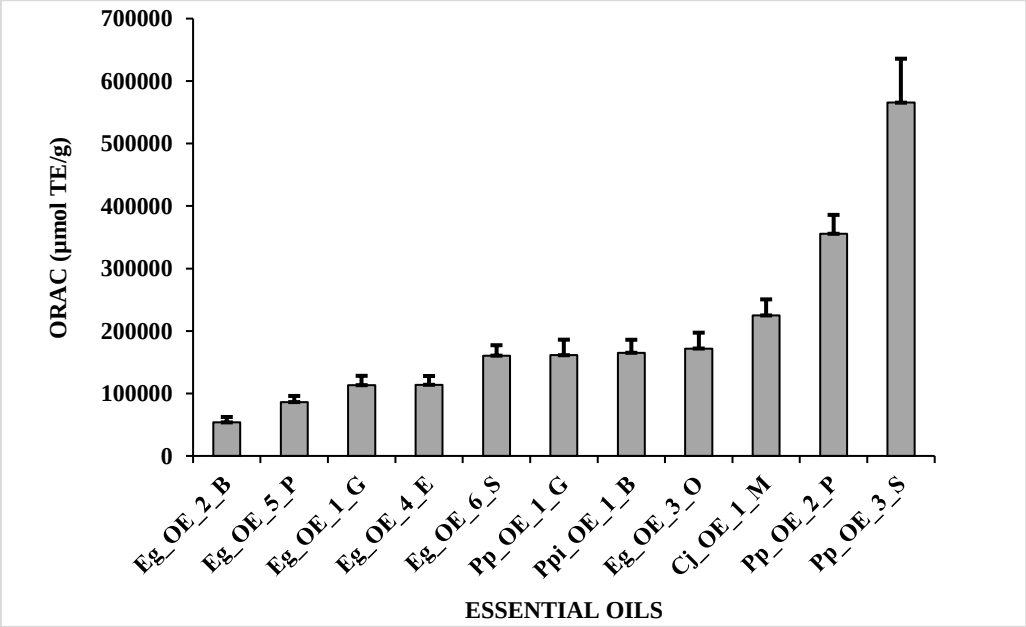

Figure 3.2 Antioxidant activity of essential oils extracts from *E. globulus*, *P. pinaster*, *P. pinea* and *C. japonica* by ORAC, Trolox equivalents/extract (µmol TE/g). Values are means ± SD

The strong activity of the *P. pinaster* EO sample cannot be explained again by the dominant compound since α-pinene also does not have antioxidant activity against the AAPH radical. Thus, it can be explained by the presence in smaller amounts of antioxidant compounds described in the literature. For example, the β-caryophyllene compound that was analysed individually for their antioxidant capacity in the ORAC assay, and was the one that obtained the highest ORAC value compared to the others in the Cutillas *et al* [230] study. Furthermore, our sample that had the highest antioxidant capacity (**Pp_OE_3_S**) for ORAC assay was the one with the highest percentage of β-caryophyllene compound (9%) among the three. This may be a justification for having the highest ORAC value. However, EOs are very complex mixtures, and their antioxidant activities may be related to more than one compound. So the different compounds of EOs can act synergistically or antagonistically, which originates an action resulting from the interaction among the various constituents [198].

The **Eg_Hd_2_O** sample of *E. globulus* Hd had the highest antioxidant capacity in the ORAC assay (**Table 3.13** and **Figure 3.3**). The antioxidant capacity demonstrated by the mentioned *E. globulus* Hd sample may be related to the presence of a higher percentage of limonene compound (14%), in relation to the other samples of the species, which was mentioned above for its antioxidant capacity against AAPH radicals [230]. Furthermore, the presence of compounds in smaller amounts such as the case of terpinen-4-ol (3%), that was previously reported to have shown antioxidant efficacy against AAPH

radicals [230], may also justify the antioxidant capacity demonstrated by the **Eg_Hd_2_O** sample. Two samples of *E. globulus* EO had the lowest antioxidant capacity in both assays. The major components found in this *E. globulus* EO samples were 1,8-cineole and α-pinene that are described in the literature as compounds with a weak antioxidant activity against DPPH and AAPH radicals. Although the DPPH and ORAC methods have different mechanisms to assess the antioxidant activity, a trend in the studied extracts is notorious. In general, the EO species of *C. japonica* and *P. pinaster* demonstrated a greater antioxidant capacity compared to *E. globulus* EO samples.

Table 3.13 The antioxidant capacity for the Hds samples of *Eucalyptus globulus*, *Pinus pinaster* and *Cryptomeria japonica*

Sample Code of Hds*	DPPH[a]	ORAC[b]
Eg_Hd_1_G	-	84.1 ± 10.0
Eg_Hd_2_O	-	1129.7 ± 100.6
Eg_Hd_3_E	-	454.6 ± 39.7
Eg_Hd_4_P	-	238.5 ± 24.5
Pp_Hd_1_G	-	212.2 ± 16.9
Pp_Hd_2_P	-	295.1 ± 44.4
Cj_Hd_1_M	-	131.1 ± 10.8
Ascorbic Acid	36.0 ± 1.1	-

*For the sample code of Hds, see **Table 2.1**. [a] radical scavenging activity (IC^{50}, mg/ml); [b] Trolox equivalents/extract (µmol TE/g). DPPH positive control: Ascorbic Acid (µg/mL). Values are means ± SD

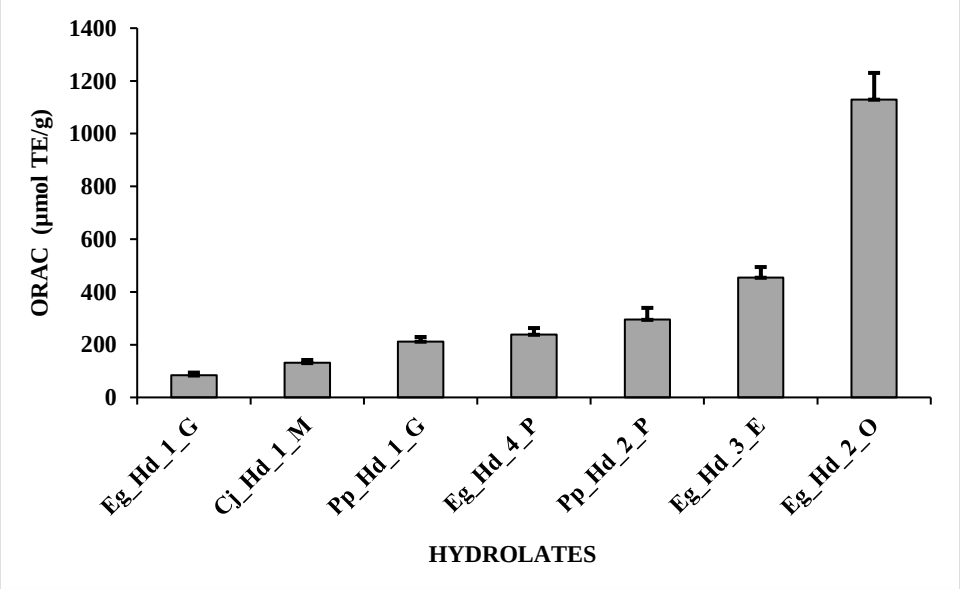

Figure 3.3 Antioxidant activity of hydrolates extracts from *E. globulus*, *P. pinaster*, *P. pinea* and *C. japonica* by ORAC, Trolox equivalents/extract (µmol TE/g). Values are means ± SD

3.2.2 Intracellular ROS measurement

In the intracellular ROS measurement, for a concentration of 10% (v/v), the EOs samples present a low capacity of reduction of % ROS, compared to the samples of Hds. Some of them potentiate its formation, such as **Ppi_OE_1_B** and **Eg_OE_3_O** (**Figure 3.4**). However, *C. japonica* EO showed the highest antioxidant activity against peroxyl radicals, probably due to the presence of antioxidant compounds such as sabinene [226,227], and others present in smaller amounts such as terpinen-4-ol[231], β-myrcene [228] and γ-terpinene [229,230]. The decline of the percentage of ROS by Hds extracts, for the same concentration, was not significantly different from 1 mg/mL ascorbic acid ($p \geq 0.05$), except for **Eg_Hd_4_O** with a lower value of reduction of ROS (47±7%) that is significantly different from ascorbic acid ($p \leq 0.05$). The EOs and Hds samples were both tested for a low concentration of 1% (v/v), and at this concentration, the samples were not able to reduce H_2O_2 induced ROS formation. *C. japonica* Hd, showed the best antioxidant capacity against peroxyl radicals (**Figure 3.5).** This antioxidant capacity demonstrated by *C. japonica* Hd may be related to its main chemical compound, terpinen-4-ol, which revealed antioxidant capacities in the study by Souza *et al* [231].

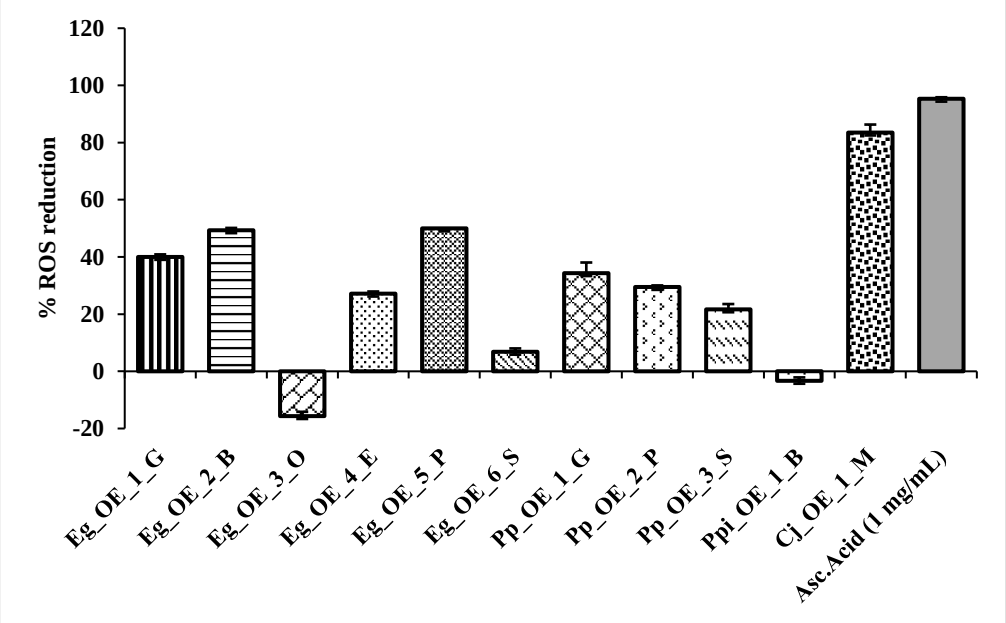

Figure 3.4 Percentage of intracellular ROS reduction by essential oils extracts at 10% (v/v) in HaCat cell line exposed to 500 µM of H_2O_2 (mean ± SD; n=6) (positive control: Ascorbic Acid 1mg/mL and negative control: culture medium)

However, it may also be related to compounds present in smaller amounts that have antioxidant properties, such as β-eudesmol, which in Kim [232] was suggested as a possible cosmeceutical compound with antioxidant and ROS-scavenging capabilities. One of the samples of *P. pinaster* Hd (**Pp_Hd_1_G**) showed a significant antioxidant capacity just after the *C. japonica* Hd (**Figure 3.5**). This could be related to the presence of compounds in smaller amounts with antioxidant properties, namely, terpinen-4-ol, which previously showed its antioxidant capacity, and the compounds thymol and perilla alcohol. Thymol is a phenolic compound that has already been shown in studies of EOs to have antioxidant capabilities higher than other volatiles and to have the ability for minimize oxidative stress [233].

Furthermore, Ambrosio *et al* [234] demonstrated that the perilla alcohol is a compound with a high antioxidant capacity.

Perhaps, the presence of these compounds, in **Pp_Hd_1_G,** with antioxidant properties mentioned above, may justify this having a relatively higher antioxidant capacity than the other sample of *P. pinaster* (**Pp_Hd_2_P**) since this one presented these compounds but in trace amounts. Two other Hd samples showed the same antioxidant capacity as the **Pp_Hd_2_P** sample, namely **Eg_Hd_4_P** and **Eg_Hd_1_G** (**Figure 3.5**). The antioxidant activity demonstrated by the **Eg_Hd_4_P** sample may perhaps be explained by that it was the only *E. globulus* Hd sample that did not present 1,8-cineole as the dominant compound. 1,8-Cineole is described, in the literature, as a compound that has a poor antioxidant capacity [235]. Furthermore, it is the only sample, concerning the others, that presents appropriate amounts of certain compounds with antioxidant properties described in the literature, such as myrtenol [236], *cis*-carveol [237] e thymol [233]. Thymol has previously been mentioned as a compound with antioxidant capacity, and myrtenol has been described in *in vitro* studies as having an antioxidant activity by inhibiting lipid peroxidation and, consequently, scavenging hydroxyl radicals [236].

Cis-carveol compound was studied by Hritcu *et al* [237] and realized that this compound revealed antioxidant activity and an ability to minimize oxidative stress. These possible explanations of the antioxidant capacities revealed by the Hds samples may not only be due to the action of a single chemical compound with antioxidant properties but rather due to a synergistic effect combined between more than one compound [238].

Hds only demonstrated antioxidant activity against the intracellular ROS assay which may be related to, usually, showed lower antioxidant capacities, compared to EOs. Furthermore, they are more dilute extracts since they have water in their constitution [31]. For this reason, they can have different volatile compounds due to the different solubility in water that their compounds have and therefore, they may have different biological properties than EOs [239]. The presence of water in its constitution may be one of the reasons for having antioxidant activity only in the intracellular ROS assay. As it is an aqueous solution, it can facilitate the dissolution process in the cells.

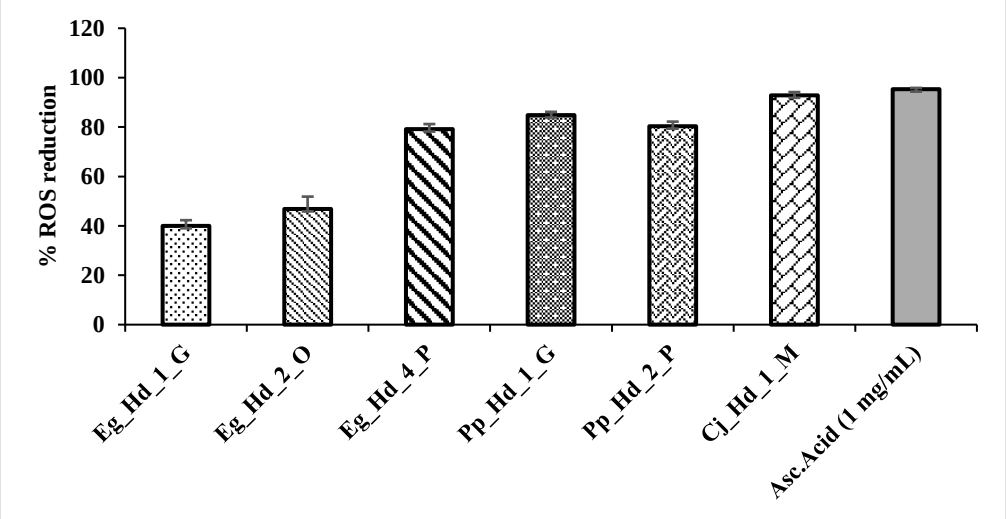

Figure 3.5 Percentage of intracellular ROS reduction by hydrolates extracts at 10% (v/v) in HaCat cell line exposed to 500 µM of H_2O_2 (mean ± SD; n=6) (positive control: Ascorbic Acid 1mg/mL and negative control: culture medium)

3.3 Antimicrobial Activity

3.3.1 Evaluation of antimicrobial activity

The method of microdilution plate was used to determine the MIC of all EOs and Hds studied. The MIC values were determined for *E. globulus, P. pinaster*, *P. pinea,* and *C. japonica* EOs and Hds, and the results are described in **Table 3.14.** In the case of the *P. pinea* species, only the MIC values for the EO were determined since it was the only extract provided by the producer.

Initially, a concentration of 100 µg/mL was used for the EOs and Hds samples and, consequently, successive dilutions were carried out. The culture medium and the culture medium with the bacteria/fungi suspension were used as a control, without dilution. All *E. globulus* EO samples exhibited significant antimicrobial activity against *B. subtilis*, with **Eg_OE_5_P** presenting the lowest MIC (1.95 µg/mL), while for S. aureus the MIC observed when this OE was used was 62.5 µg/mL. On the other hand, the results revealed that for Gram-negative bacteria, the *E. globulus* EO samples had a better efficacy against the *E. coli* ATCC 8739 strain, which means that this was the most susceptible strain among the Gram-negative ones, while that the strain of *P. aeruginosa* ATCC 9027 was the most resistant. Most *E. globulus* EO in the tested concentrations range were not effective against *P. aeruginosa* except **Eg_OE_3_O**, with a MIC of 31.25 µg/mL. **Eg_OE_2_B** and **Eg_OE_5_P** showed the highest activities against *E. coli* and were also the most active against the pathogenic yeast *Candida albicans*. For the strain of *A. brasiliensis* ATCC 16404, none of the *E. globulus* samples had antifungal efficacy (**Table 3.14**). These results agree with studies carried out in the literature. Cimanga *et al* [240] studied the chemical composition and antimicrobial activity of several EOs, including *E. globulus*, concluding that this oil did not show any inhibitory efficacy against the *P. aeruginosa* strain, being considered one of the most resistant clinical bacteria. Furthermore, the *B. subtilis* strain was regarded as one of the most susceptible strains against the studied EOs and, on the other hand, *S. aureus* was one of the most resistant to them.

Bachir and Benali [157] analysed the antibacterial activity against *E. coli* and *S. aureus* strains of *E. globulus* EO, and it was noticed that it revealed antibacterial efficacy against the strains. The antibacterial activity demonstrated by the extract was justified by the presence of certain chemical compounds such as 1,8-cineole and α-pinene. 1,8-Cineole is a compound mentioned in the literature due to its strong antimicrobial properties against important pathogens [157]. In this study, the main dominant compound of all *E. globulus* EO samples was 1,8-cineole, followed by α-pinene and limonene. For this reason, part of the antimicrobial effectiveness attributed to these samples may be due to these compounds that have proven antimicrobial properties. However, they are complex mixtures, so the antimicrobial activity of a given extract may not be attributed to one compound *per se*. Furthermore, the dominant constituent of an extract may not be directly related to a high antimicrobial efficacy, but it may be due to the presence of compounds in smaller amounts that have antimicrobial properties. In addition, having a particular compound in high concentrations does not mean having an antimicrobial efficacy for all strains considered. It is also important to consider the possibility of the occurrence of synergistic and antagonistic effects by the constituents [241].

In general, the *P. pinaster* EO samples revealed, and antimicrobial activity lower than that of *E. globulus* EO. The highest activity was observed on *B. subtilis* with **Pp_OE_2_P** and **Pp_OE_3_S**, with MIC values of 15.62 µg/mL. Therefore, the *B. subtilis* ATCC 6622 strain was the most susceptible to these samples, while *S. aureus* ATCC 6538 was the most resistant. According to Gram-negative bacteria, and similarly to the *E. globulus* EO samples, it was noticeable that the *E. coli* ATCC 8739 strain was more susceptible to *P. pinaster* EO samples, which means that *E. coli* ATCC 8739 turned out to be a

more susceptible bacterium compared to *P. aeruginosa* ATCC 9027 that proved to be more resistant. No activity against *P. aeruginosa* was observed in the tested concentration range, except for **Pp_OE_2_P** for which a high MIC value (500 µg/mL) was obtained. The remaining sample of *P. pinaster* EO (**Pp_OE_1_G**) had no antimicrobial efficacy against the strains studied. Furthermore, none of the *P. pinaster* EO samples was active against *A. brasiliensis* ATCC 16404. (**Table 3.14)**. The results concerning the *P. pinaster* EO samples revealed that only one of the samples, namely, **Pp_OE_2_P**, had antimicrobial efficacy against all the strains considered. This may be related to the fact that it is the sample that shows a higher percentage value of the α-pinene compound that was mentioned above as having antimicrobial properties. However, Hmamouchi *et al* [173] evaluated the antimicrobial activity of *P. pinaster* and *P. pinea* EOs and concluded that compounds present in smaller amounts might play an essential role in their microbial efficacy. The authors also found that the EO of *P. pinaster* presented a higher antimicrobial activity against the *E. coli* strain compared to the EO of *P. pinea*, which agrees with the present study if we compare it with the sample of *P. pinaster* that showed antimicrobial efficacies for all strains studied, including against *E. coli* (**Pp_OE_2_P**). Once again, it was proven that the compounds present in lower percentage values should be considered to justify a particular antimicrobial efficacy.

The *P. pinea* EO sample showed better activity, relative to Gram-positive and negative, against *B. subtilis* ATCC 6633 and *E. coli* ATCC 8739 strains, respectively. Therefore, the *S. aureus* ATCC 6538 strain was the most resistant to Gram-positive bacteria and, in contrast, *P. aeruginosa* ATCC 9027 was the most resistant Gram-negative bacteria. Also, the *P. pinea* EO sample had no antifungal activity against *A. brasiliensis* ATCC 16404, but was effective against *C. albicans*, with a MIC of 15.62 µg/mL (**Table 3.14**). According to previous research reports about evaluating the antimicrobial activity of *P. pinea* EO, it was demonstrated that this oil was effective against several strains, including *S. aureus* ATCC 6538, as was verified in the present study.

Table 3.14 Minimum inhibitory concentrations (MICs) of *Eucalyptus globulus*, *Pinus pinaster*, *Pinus pinea* and *Cryptomeria japonica* essential oils (EOs) against Gram-positive and Gram-negative bacteria, yeast, and mold.

EOs samples	Minimum inhibitory concentration (MICs) (µg/mL)					
	S.a[1]	**B.s[2]**	**P.a[3]**	**E.c[4]**	**C.a[5]**	**A.b[6]**
Eg_OE_1_G	125	31.25	500	15.62	7.81	>500
Eg_OE_2_B	125	31.25	500	3.90	3.90	>500
Eg_OE_3_O	62.5	15.62	31.25	15.62	31.25	>500
Eg_OE_4_E	125	15.62	500	62.5	31.25	>500
Eg_OE_5_P	62.5	1.95	500	3.90	3.90	>500
Eg_OE_6_S	62.5	15.62	500	15.62	7.81	>500
Pp_OE_1_G	>500	>500	>500	>500	>500	>500
Pp_OE_2_P	31.25	15.62	500	15.62	62.5	>500
Pp_OE_3_S	>500	15.62	>500	125	125	>500
Ppi_OE_1_B	62.5	7.81	>500	125	15.62	>500
Cj_OE_1_M	>500	>500	>500	>500	>500	>500

S.a[1] - *Staphylococcus aureus* ATCC 6538; B.s[2] - *Bacillus subtilis* ATCC 6633; P.a[3] - *Pseudomonas aeruginosa* ATCC 9027; E.c[4] - *Escherichia coli* ATCC 8739; C.a[5] - *Candida albicans* ATCC 10231 and A.b[6] - *Aspergillus brasiliensis* ATCC 16404.

Hmamouchi *et al* [173] evaluated the antimicrobial activity of EOs from *P. pinaster* and *P. pinea* against several strains such as *E. coli*, *S. aureus* and *P. aeruginosa*. It proved that the *P. pinea* EO had antimicrobial activity, even though, in the majority, it was less than that of the *P. pinaster* EO. This may be related to the present study since comparing the *P. pinaster* EO sample that showed antimicrobial efficacies for all strains (**Pp_OE_2_P**) with the *P. pinea* EO sample, the *P. pinaster* EO sample had a

better antimicrobial capacity than *P. pinea* EO. Nevertheless, the variations in the antimicrobial efficacy found may be related to other factors that may influence or justify these changes, namely, the culture medium used, the method used, the origin of the botanical species, the plant age, the type of material used (dry or fresh), the amount of EO used in the test as well as the isolation technique [242].

Finally, the *C. japonica* EO sample did not demonstrate any antimicrobial, anti-yeast, or antifungal activity against the strains studied (**Table 3.14**). Nakagawa *et al* [211] reported the antimicrobial activity of EO, Hd and other residues from *C. japonica* against *E. coli* and *S. aureus* strains. They concluded that most of these extracts did not show antimicrobial efficacy, except the EO obtained from the branches and leaves of *C. japonica* EO, despite being weak, which agrees with the present study. The extracts obtained from different parts of *C. japonica* plants could have different antimicrobial efficacy and, in general, when their extracts presented an antimicrobial activity, it was weak. This study is in part in agreement with the present study as our sample did not demonstrate any antimicrobial activity against the strains selected. The existing variability of antimicrobial efficacy may also be due to differences in the chemical composition of each extract, obtained from different parts of the *C. japonica* plant [211]. The results obtained by Hds samples showed that none of the samples had antimicrobial, anti-yeast, and antifungal activities against the strains selected in the study.

3.4 Sensorial evaluation

3.4.1 Questionnaire results

3.4.1.1 Sample Characterization

Table 3.15 Sociodemographic characteristics of the 100 volunteers

Sociodemographic characteristics	All samples (n=100) n (%)
Age range (years)	
<18	6 (6%)
18-30	22 (22%)
31-40	13 (13%)
41-50	30 (30%)
51-60	20 (20%)
>60	9 (9%)
Gender	
Female	67 (67%)
Male	33 (33%)
Education	
Primary education	13 (13%)
Secondary education	26 (26%)
Higher education	61 (61%)
Region	
Countryside	70 (70%)
City	30 (30%)

The sensorial evaluation was performed in five (5) EOs, selected according to each plant species, except for *P. pinaster*, which presented two samples with very different odours and therefore, it made sense to include them both. The *E. globulus* EO sample choice, among the six available, was based on the one with the highest percentage value of 1.8-cineole since eucalyptol is the compound that confers most of the odour from *Eucalyptus* species. A total of 100 inexperienced participants were questioned, in which most of them were among 41-50 years old (30%), followed by the class that included the 18-

30 years old (22%) (**Table 3.15**).

The under 18 class had the lowest number of respondents (6%). In addition, 67% were female and 43% were male. The participants were divided into three categories based on their education level. The most common education level was higher education (66%), while the least common one was primary education (13%). The countryside was the region with the highest predominance of individuals, namely, 70 individuals. The remaining 30 individuals belong to the city (**Table 3.15**).

3.4.1.2 Section 1. Emulsions' Odour

In this section, questions were asked regarding the emulsions' odour provided to the participants. The first question in this study asked participants, "How do you evaluate the emulsions' odour?" The possible answers were rated on a scale of 1-5, namely, **1- Without odour**; **2- Slightly perceptible**; **3- Perceptible**; **4- Very perceptible** and **5- Intense odour**. Regarding the *C. japonica* (**Cj_OE_1_M**), *P. pinea* (**Ppi_OE_1_B**) and one of the samples of *P. pinaster* (**Pp_OE_2_P**) EOs emulsions', most participants (46, 38 and 42 individuals, respectively) rated them as perceptible odours. The *E. globulus* EO emulsion was considered the most intense odour among the four available, with 48 responses. Finally, for another emulsion of *P. pinaster* (**Pp_OE_1_G**) EO, about 40 individuals considered it to have a slightly perceptible odour. The second question was, "In case you identified any odour, how would you classify it?" The participants had to classify it as having a: **Unpleasant**; **Pleasant and hot odour** or **Pleasant and fresh odour**. Most respondents rated their odours as pleasant and fresh for the **Cj_OE_1_M** and **Ppi_OE_1_B** EOs emulsions', with 60 and 53 answers. The *E. globulus* (**Eg_OE_1_G**) EO emulsion was considered a very unpleasant odour compared to the other emulsions, with 36 answers. Most respondents considered the **Pp_OE_1_G** and **Pp_OE_2_P** emulsions odours unpleasant, with 41 and 44% percentage values, respectively (**Table 3.16**).

Table 3.16 Representation of participants responses regarding the characterization of emulsions odour

Section 1. Emulsions' Odour Odoriferous characterization of emulsions	**N (%)**				
Evaluation of odours	**Cj_OE_1_M**	**Ppi_OE_1_B**	**Eg_OE_1_G**	**Pp_OE_1_G**	**Pp_OE_2_P**
1: Without odour	3 (3%)	1 (1%)	0 (0%)	15 (15%)	13 (13%)
2: Slightly perceptible	37 (37%)	8 (8%)	4 (4%)	40 (40%)	28 (28%)
3: Perceptible	46 (46%)	38 (38%)	15 (15%)	28 (28%)	42 (42%)
4: Very perceptible	11 (11%)	34 (34%)	33 (33%)	14 (14%)	11 (11%)
5: Intense odour	3 (3%)	19 (19%)	48 (48%)	3 (3%)	6 (6%)
Classification of odours	**Cj_OE_1_M**	**Ppi_OE_1_B**	**Eg_OE_1_G**	**Pp_OE_1_G**	**Pp_OE_2_P**
1: Very unpleasant	0 (0%)	9 (9%)	36 (36%)	4 (4%)	5 (5%)
2: Unpleasant	22 (22%)	25 (25%)	27 (27%)	41 (41%)	44 (44%)
3: Pleasant and hot odour	15 (15%)	12 (12%)	15 (15%)	10 (10%)	13 (13%)
4: Pleasant and fresh odour	60 (60%)	53 (53%)	22 (22%)	30 (30%)	27 (27%)
Ranking in order of preference	**Cj_OE_1_M**	**Ppi_OE_1_B**	**Eg_OE_1_G**	**Pp_OE_1_G**	**Pp_OE_2_P**
1: Hateful Odour	3 (3%)	8 (8%)	32 (32%)	9 (9%)	11 (11%)
2: Unpleasant Odour	17 (17%)	18 (18%)	23 (23%)	41 (41%)	43 (43%)
3: Neither pleasant nor unpleasant	33 (33%)	31 (31%)	13 (13%)	30 (30%)	22 (22%)
4: Pleasant Odour	28 (28%)	24 (24%)	20 (20%)	18 (18%)	14 (14%)
5: Favourite Odour	19 (19%)	19 (19%)	12 (12%)	2 (2%)	10 (10%)
Do you think that emulsions belong to the same plant species?					

Section 1. Emulsions' Odour Odoriferous characterization of emulsions	**N (%)**
Positive answers	41 (41%)
Uncertainly answers	34 (34%)
Negative answers	25 (25%)

The third question was, "In your opinion, the odours of the different emulsions belong to the same plant species?". As shown in **Table 3.16**, the highest proportion of participants (41%) answered yes, which means that they consider that all emulsions belong to the same plant species. The fourth question in *Section 1. Emulsions' Odour* was "Order the emulsions, according to your preference, on a scale of 1-5, namely, **1-Without odour**; **2- Slightly perceptible**; **3- Perceptible**; **4- Very perceptible** and **5-Intense odour**." Overall, the **Cj_OE_1_M** and **Ppi_OE_1_B** EOs emulsions were the participants' favourite as they had a greater number of individuals who responded as having a pleasant odour (28 and 24%, respectively) and as favourite odour (19%) (**Table 3.16**).

The fifth question was asked to understand if one or more emulsions caused any feeling of well-being. So, for this reason a single question was asked: "Select which emulsion(s) cause you a feeling of physical or mental well-being." Most of the participants answered the **Cj_OE_1_M** EO emulsion (20%). However, a very similar percentage answered the **Eg_OE_1_G** EO emulsion or none of the emulsions (17%) (**Table 3.17).**

Table 3.17 Representation of participants responses regarding the feelings of well-being from emulsions odour

Section 1. Emulsions' Odour Feelings of well-being from emulsions odour	**N (%)**
Emulsions that caused feelings of well-being	
Ppi_OE_1_B and Eg_OE_1_G	2 (2%)
Cj_OE_1_M, Pp_OE_1_G and Pp_OE_2_P	3 (3%)
Cj_OE_1_M	20 (20%)
Cj_OE_1_M and Pp_OE_1_G	1 (1%)
Eg_OE_1_G and Pp_OE_2_P	2 (2%)
Cj_OE_1_M and Pp_OE_2_P	2 (2%)
Cj_OE_1_M and Ppi_OE_1_B	5 (5%)
Pp_OE_2_P	6 (6%)
Ppi_OE_1_B	11 (11%)
Cj_OE_1_M, Ppi_OE_1_B and Eg_OE_1_G	5 (5%)
Eg_OE_1_G	17 (17%)
Cj_OE_1_M, Eg_OE_1_G, Pp_OE_2_P and Pp_OE_1_G	1 (1%)
Cj_OE_1_M and Eg_OE_1_G	1 (1%)
Pp_OE_1_G and Pp_OE_2_P	2 (2%)
Ppi_OE_1_B and Pp_OE_2_P	1 (1%)
Cj_OE_1_M, Ppi_OE_1_B and Pp_OE_1_G	2 (2%)
Cj_OE_1_M, Ppi_OE_1_B, Pp_OE_1_G and Pp_OE_2_P	1 (1%)
Pp_OE_1_G	1 (1%)
None	17 (17%)
Feelings of well-being	
Refreshing	23 (23%)
Decongestant	15 (15%)
Decongestant, Stimulating and Refreshing	2 (2%)
Decongestant and Refreshing	4 (4%)
Relaxing, Decongestant and Refreshing	7 (7%)
Relaxing	18 (18%)

Section 1. Emulsions' Odour Feelings of well-being from emulsions odour	**N (%)**
Relaxing and Stimulating	1 (1%)
Stimulating and Refreshing	1 (1%)
Relaxing and Refreshing	4 (4%)
Stimulating	3 (3%)
Relaxing, Stimulating and Refreshing	1 (1%)
Relaxing and Decongestant	2 (2%)
Decongestant and Stimulating	2 (2%)
None	17 (17%)

The sixth and final question in *Section 1. Emulsions' Odour* was "Refers what feeling of well-being the emulsions caused you" and having been made to be answered considering the previous answer. As shown in **Table 3.17**, most participants responded refreshing and relaxing as the sensations of well-being most caused by the emulsions mentioned above (23 and 18%, respectively).

3.4.1.3 Section 2. Emulsions' Applicability

The results from *Section 2. Emulsions Applicability's* are summarized in **Table 3.18**.

Table 3.18 Representation of participants responses to purchasing a product with emulsions odour

Section 2. Applicability's of Emulsions' Odour Purchasing a product with emulsions' odours	**N (%)**					
Probability of buying a product with Cj_OE_1_M odour	**Perfume**	**Air freshener**	**Massage cream**	**Toothpaste**	**Shampoo**	**Candy**
1: Would never buy	43 43%)	12 (12%)	13 (13%)	32 (32%)	20 (20%)	49 (49%)
2: Unlikely	28 (28%)	31 (31%)	25 (25%)	36 (36%)	31 (31%)	37 (37%)
3: Likely	19 (19%)	34 (34%)	33 (33%)	17 (17%)	27 (27%)	8 (8%)
4: Quite likely	4 (4%)	5 (5%)	11 (11%)	4 (4%)	7 (7%)	2 (2%)
5: Would buy	6 (6%)	18 (18%)	18 (18%)	11 (11%)	15 (15%)	4 (4%)
Probability of buying a product with Ppi_OE_1_B odour	**Perfume**	**Air freshener**	**Massage cream**	**Toothpaste**	**Shampoo**	**Candy**
1: Would never buy	52 (52%)	22 (22%)	16 (16%)	26 (26%)	31 (31%)	43 (43%)
2: Unlikely	29 (29%)	26 (26%)	29 (29%)	27 (27%)	25 (25%)	27 (27%)
3: Likely	11 (11%)	29 (29%)	34 (34%)	27 (27%)	23 (23%)	19 (19%)
4: Quite likely	3 (3%)	11 (11%)	6 (6%)	9 (9%)	7 (7%)	4 (4%)
5: Would buy	5 (5%)	12 (12%)	15 (15%)	11 (11%)	14 (14%)	7 (7%)
Probability of buying a product with Eg_OE_1_G odour	**Perfume**	**Air freshener**	**Massage cream**	**Toothpaste**	**Shampoo**	**Candy**
1: Would never buy	63 (63%)	36 (36%)	38 (38%)	44 (44%)	37 (37%)	53 (53%)
2: Unlikely	20 (20%)	22 (22%)	25 (25%)	30 (30%)	28 (28%)	20 (20%)
3: Likely	9 (9%)	20 (20%)	17 (17%)	13 (13%)	17 (17%)	8 (8%)
4: Quite likely	4 (4%)	5 (5%)	7 (7%)	7 (7%)	7 (7%)	5 (5%)
5: Would buy	4 (4%)	17 (17%)	13 (13%)	6 (6%)	11 (11%)	14 (14%)
Probability of buying a product with Pp_OE_2_P odour	**Perfume**	**Air freshener**	**Massage cream**	**Toothpaste**	**Shampoo**	**Candy**
1: Would never buy	50 (50%)	25 (25%)	18 (18%)	39 (39%)	34 (34%)	47 (47%)
2: Unlikely	34 (34%)	35 (35%)	38 (38%)	37 (37%)	35 (35%)	35 (35%)
3: Likely	12 (12%)	28 (28%)	29 (29%)	19 (19%)	22 (22%)	13 (13%)
4: Quite likely	3 (3%)	8 (8%)	11 (11%)	5 (5%)	6 (6%)	2 (2%)
5: Would buy	1 (1%)	4 (4%)	4 (4%)	0 (0%)	3 (3%)	3 (3%)

Section 2. Applicability's of Emulsions' Odour Purchasing a product with emulsions' odours	**N (%)**					
Probability of buying a product with Pp_OE_1_G odour	**Perfume**	**Air freshener**	**Massage cream**	**Toothpaste**	**Shampoo**	**Candy**
1: Would never buy	54 (54%)	32 (32%)	27 (27%)	42 (42%)	38 (38%)	54 (54%)
2: Unlikely	32 (32%)	40 (40%)	33 (33%)	40 (40%)	34 (34%)	39 (39%)
3: Likely	10 (10%)	19 (19%)	28 (28%)	13 (13%)	18 (18%)	6 (6%)
4: Quite likely	1 (1%)	3 (3%)	6 (6%)	2 (2%)	5 (5%)	1 (1%)
5: Would buy	3 (3%)	6 (6%)	6 (6%)	3 (3%)	5 (5%)	0 (0%)

First, participants were asked whether they were likely to purchase a particular product for personal use with the odour of the selected emulsions. The question was: "Rate each of the products below, on a scale of 1-5, (**1- Would never buy**; **2- Unlikely**; **3- Likely**; **4- Quite likely** and **5- Would buy**), considering the probability of buying one with the emulsion's odour". Regarding the **Cj_OE_1_M** emulsion, participants considered that they would be more likely to purchase an air freshener and massage cream with its odour (34 and 33%, respectively) and 18% of volunteers said they would buy. Perfume and candy (43 and 49%, respectively) were considered to the majority to be the products they would never buy, with the **Cj_OE_1_M** emulsion odour. For the **Ppi_OE_1_B** emulsion, participants considered that they would likely buy air freshener and massage cream with its odour (29 and 34%, respectively) and 12 and 15% of volunteers, respectively, said they would buy. Once again, perfume and candy (52 and 43%, respectively) were considered the products they would never buy, in this case with the emulsion green odour (**Table 3.18**).

Regarding the remaining emulsions, participants considered that they would not buy any of the products with their odours, and perfume or candy were the products they would never buy with their odours. Finally, a question was asked regarding other possible applicability's' of the emulsions odours. The question was: "Do you consider that the emulsions' odour has other applicability's? If yes, mention which ones". As shown in **Table 3.19,** most participants would use the emulsions odours for cleaning products (10%). However, it should be considered that about 58 individuals did not answer the question, which can lead to the conclusion that they do not consider that the emulsions odours could have other types of applicability.

Table 3.19 Representation of participants responses to others applicability's of emulsions odour

Section 2. Applicability of Emulsions' Odour Other applicabilitys of emulsions' odour	**N (%)**
Aromatherapy and bath bombs	1 (1%)
Soaps	2 (2%)
Cleaning products	10 (10%)
Massage oils	1 (1%)
Repellents	2 (2%)
Nasal spray	1 (1%)
Incense and cleaning products	1 (1%)
Shower gel	1 (1%)
Ointment medications (analgesics)	3 (3%)
Wood Furniture Cleaning Products	1 (1%)

Section 2. Applicability of Emulsions' Odour Other applicabilitys of emulsions' odour	**N (%)**
Hand and face cream	1 (1%)
Disinfectant	1 (1%)
Body and hand cream	1 (1%)
Deodorant	2 (2%)
Candles and Soaps	1 (1%)
Shaving cream	1 (1%)
Nasal decongestant	2 (2%)
Car air freshener and cleaning products	1 (1%)
None	9 (9%)

In general, we can conclude from the sensory analysis that the participants preferred milder odours than more intense ones. There was a preference for fresher odours since the emulsions that participants considered to have a pleasant odour and which they preferred were *C. japonica* and *P. pinea* EOs emulsions, classified as having fresh odours. On the other hand, emulsions with more intense odours such as *E. globulus* EO emulsion were identified as being less pleasant. Regarding the emulsions that caused a feeling of well-being, *C. japonica* EO emulsion was the one that had the most significant prominence. In addition to having the highest percentage alone, it was almost always mentioned with others. *C. japonica* EO emulsion was also considered as having the most appreciated/pleasant odour, being the favourite one, together with the *P. pinea* EO emulsion. In contrast, *E. globulus* EO emulsion was considered to be the least appreciated and therefore the one with a more hateful odour compared to the others. Regarding the possible emulsions uses, it was noticeable that the *C. japonica* and *P. pinea* EOs emulsions odours were the only ones that could be used for air freshener and massage cream. The emulsions mentioned above were proved to be the most appreciated odours by most participants.

The odoriferous characteristics presented by EOs may be related to their chemical composition. EOs are a mixture of aromatic and non-aromatic compounds that can contribute to their scents [243]. As previously mentioned, the *P. pinaster* EO had α and β-pinene as its main constituents, which are reported in the literature as having a fresh, woody and earthy scent [76,84,86,244]. α-Pinene was also the dominant compound in *C. japonica* EO. *P. pinea* EO presented high limonene content which has strong citrus aroma [61]. In addition, *E. globulus* EO showed 1,8-cineole as its main compound which is a colourless liquid with an intense camphor-like odour [83,84]. This means that participants showed a preference for fresh and citrus scents made up of compounds such as α and β-pinene and limonene rather than EOs emulsions with more intense and stronger odours characteristics of 1,8-cineole compound. Furthermore, *C. japonica* EO revealed, previously, to have been the extract that showed the best antioxidant capacity against DPPH radicals and also demonstrated a high antioxidant capacity against AAPH radicals.

Therefore, these natural-based EOs addressed the demand for sustainable and responsibly sourced odour accepted by consumers and, combining their pleasant scents and biological properties revealed, they could be natural promising alternatives in cosmetic and phytopharmaceutical products.

Chapter 4. Final Conclusions

There has been an increasing interest in obtaining added value from forest biomass, such as the production of essential oils (EOs) and hydrolates (Hds). These extracts are a growing market as natural ingredients in the cosmetic and perfume industries. The present study meets this reality and from a point of view of sustainability and reuse of forest biomass, four EOs, from forest species: *Eucalyptus globulus*, *Pinus pinaster*, *Pinus pinea* and *Cryptomeria japonica*, obtained by national producers from the mainland of Portugal and Azores archipelago were chemically characterized and evaluated for their antioxidant and antimicrobial activities. Sensory double-blind evaluation was performed in human volunteers and a structured questionnaire was used to collect data about the perception and applicabilities of different emulsions. Emulsions containing 0.5% of EO from five selected samples were formulated.

For the four species study, the chemical analysis of EOs and HVs were performed successfully. The main constituents of EOs were: 1,8-cineole, α and β-pinene, limonene and α-pinene, for *E. globulus*, *P. pinaster*, *P. pinea* and *C. japonica*, respectively. Regarding HVs the dominant compounds were: 1,8-cineole, α-terpineol and terpinene-4-ol for *E. globulus*, *P. pinaster* and *C. japonica*. These results agree with other studies performed with this plants species in which they used the same extraction and analysis conditions.

In DPPH assay, EOs showed weak antioxidant activity, except for *C. japonica* EO which had the lowest IC50 value. Hds showed no activity against DPPH radical. In ORAC assay, EOs had a greater antioxidant activity against AAPH radical with emphasizing for two samples of *P. pinaster* EO and *C. japonica* EO. Hds demonstrated to have antioxidant activity although it is lower. To intracellular ROS measurement, EOs had a low capacity of reduction of ROS compared to Hds samples. *C. japonica* Hd showed to have the best antioxidant capacity against H_2O_2.

All *E. globulus* EO samples showed efficacy against Gram-positive and Gram-negative bacteria and yeast. *B. subtilis* ATCC 6633 and *E. coli* ATCC 8739 were the most susceptible strains for all EOs samples. The EOs did not showed antifungal activity against the *A. brasiliensis* ATCC 16404 strain. *C. japonica* and one sample of *P. pinaster* EOs showed no antimicrobial, anti-yeast, and anti-fungi activity for all studied strains. The results obtained by Hds samples also showed that none of the samples had antimicrobial, anti-yeast, and antifungal activities against all the strains.

Fresh and earthy odour from *C. japonica* EO emulsion was chosen as the most pleasant by 60% of the volunteers, followed by *P. pinea* emulsion odour with 53%, with a citrus scent. *C. japonica* emulsion was also the one chosen for improving the sense of well-being. Overall, 19% of the volunteers selected *C. japonica* and *P. pinea* emulsions as their favourites. *C. japonica* and *P. pinea* EOs proved to be the most appreciated emulsions odours by the most participant, especially to be used as air fresheners and massage creams.

In conclusion, the studied EOs and Hds showed relevant antioxidant activity and promising antimicrobial activity. Moreover, they address the demand for sustainable and responsibly sourced odour accepted by consumers.

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Chapter 6. Annexes

Annex 1. Consentimento Informado

Avaliação do odor das emulsões com diferentes óleos essenciais portugueses: *Eucalyptus globulus, Pinus pinaster, Pinus pinea* e *Cryptomeria japonica*.

Por favor, leia com atenção todo o conteúdo deste documento. Não hesite em solicitar mais informações se não estiver completamente esclarecido.

Se entender que tudo está em conformidade, então assine este documento.

Leia e rubrique cada um dos pontos de forma a demonstrar a sua compreensão e aceitação em cada uma das afirmações.

No âmbito da tese de mestrado de Ana Ruas, realizada na Faculdade de Farmácia da Universidade de Lisboa e na Faculdade de Ciências da Universidade de Lisboa sob orientação pelas Professoras Doutoras Helena Margarida Ribeiro e Ana Cristina Figueiredo venho por este meio solicitar a sua participação no estudo abaixo referido.

Afirmação	
1	As propriedades organoléticas (nomeadamente o odor e a cor) de formulações tópicas são fatores essenciais para a aceitabilidade de um produto cosmético.
2	A presença de diferentes óleos essenciais pode condicionar a aceitação do produto pois estes conferem odor e cores características do produto final.
3	O estudo pretende avaliar a classificação de cada uma das formulações de acordo com as preferências olfativas dos voluntários selecionados e avaliar a aceitabilidade das formulações tendo em conta a probabilidade de compra de diferentes produtos de higiene pessoal (perfume; ambientador; creme de massagem; pasta de dentes; champô e rebuçado) com o odor e a cor da respetiva formulação.
4	Autorizo a mestranda Ana Ruas e, ou a quem ela designe como seu representante, a realizar o questionário que permite avaliar o odor e a cor das 5 formulações tópicas.
5	Este estudo é não invasivo e não acarreta risco para a minha saúde.
6	Este estudo requer a avaliação do odor e da cor, não sendo preciso aplicar topicamente o produto.
7	Compreendo e foram-me explicadas as razões da necessidade deste estudo.
8	A mestranda respondeu às minhas questões e entendi que vou participar voluntariamente num estudo de investigação.

9	Foi-me garantido que não haverá prejuízo para os meus direitos assistenciais se eu recusar a participação no estudo.
10	Foi ainda salvaguardado, que todos os dados a serem recolhidos serão para uso exclusivo ao nível da investigação e que será mantido o anonimato.

Qualquer esclarecimento adicional ou contacto posterior, deverá ser realizado presencialmente antes da assinatura do consentimento para a orientadora Helena Ribeiro, 963080730; hribeiro@campus.ul.pt

Declaro ter lido e compreendido este documento, bem como as informações verbais queme foram fornecidas pela/s pessoa/s que abaixo assina/m. Foi-me garantida a possibilidade de, em qualquer altura, recusar participar neste estudo sem qualquer tipo de consequências. Desta forma, aceito participar neste estudo e permito a utilização dos dados que de forma voluntária forneço, confiando em que apenas serão utilizados para esta investigação e nas garantias de confidencialidade e anonimato que me são dadas pelo/a investigador/a.

Nome:_____

Assinatura:_

Data:_____/_____/_____

Representante legal (SE NÃO FOR O PRÓPRIO A ASSINAR POR IDADE OU INCAPACIDADE)

NOME:____

GRAU DE PARENTESCO OU TIPO DE REPRESENTAÇÃO:_____

ASSINATURA:____

Foi explicado à doente os procedimentos decorrentes deste estudo.

ASSINATURA DA MESTRANDA:__

Data:_____/_____/_____

ESTE DOCUMENTO É COMPOSTO DE 2 PÁGINAS E FEITO EM DUPLICADO: UMAVIA PARA O /A INVESTIGADOR /A , OUTRA PARA A PESSOA QUE CONSENTE.

Annex 2. Sensory Questionnaire (English Version)

My name is Ana Ruas. I'm a 2nd year student of the master degree in Biology of Plant Resources, at the Faculty of Sciences of the University of Lisbon (FCUL) and the Instituto Superior de Agronomia (ISA). The following questionnaire was designed following my master dissertation and is an essential addition to the work in progress, so I thank you for your availability in filling it out.

The questionnaire consists of short and objective questions that must be answered based on the samples provided. The data collected is anonymous and is used only for the mentioned purpose.

1. The aroma of many plant species has different volatile chemical compounds with the ability to stimulate our olfactory receptors. The characteristic aromas of plants are related to the quantity and diversity of these compounds in plant species.

2. Essential oils (EOs) are mixtures of volatile compounds isolated from different parts of plants and there is an increasing interest in their use for different purposes, such as in the cosmetics, perfumery, and aromatherapy industries.

Age:

- 18-30 years old
- 31-40 years old
- 41-50 years old
- 51-60 years old
- More than 60 years old

Gender:

- Female
- Male

Education level:

- Primary education
- Secondary education
- Higher education

Region:

- Country
- City

Section 1. Emulsion's odour

1. How do you evaluate the odour of the “pink” emulsion?

- Without odour
- Slightly perceptible
- Perceptible
- Very perceptible
- Intense odour

2. In case you identified any odour, how would you classify it?

- Very unpleasant
- Unpleasant
- Pleasant and fresh odour
- Pleasant and hot odour

3. How do you evaluate the odour of the “green” emulsion?

- Without odour
- Slightly perceptible
- Perceptible
- Very perceptible
- Intense odour

4. In case you have identified any odour, how would classify it?

- Very unpleasant
- Unpleasant
- Pleasant and fresh odour
- Pleasant and hot odour

5. How do you evaluate the odour of the “orange” emulsion?

- Without odour
- Slightly perceptible
- Perceptible
- Very perceptible
- Intense odour

6. In case you have identified any odour, how would you classify it?

- Very unpleasant
- Unpleasant
- Pleasant and fresh odour
- Pleasant and hot odour

7. How do you evaluate the odour of the “purple” emulsion?

- Without odour
- Slightly perceptible
- Perceptible
- Very perceptible
- Intense odour

8. In case you identified any odour, how would you classify it?

- Very unpleasant
- Unpleasant
- Pleasant and fresh odour
- Pleasant and hot odour

9. How do you evaluate the odour of the “blue” emulsion?

- Without odour
- Slightly perceptible
- Perceptible
- Very perceptible
- Intense odour

10. In case you have identified any odour, how would you classify it?

- Very unpleasant
- Unpleasant
- Pleasant and fresh odour
- Pleasant and hot odour

11. In your opinion the odours of the different emulsions belong to the same plant species?

- Yes
- No
- Maybe

12. Select which emulsion(s) cause you a feeling of physical and/or mental well-being.

- Pink
- Green
- Orange
- Purple
- Blue
- None

13. Refers what feeling of well-being the emulsions caused you.

- Relaxing
- Decongestant
- Stimulating
- Refreshing
- None

14. Order the samples, according to your preference, on a scale of 1-5 (1-Hateful odour; 2-Unpleasant odour; 3-Pleasant odour; 4-Very pleasant odour and 5-Favourite odour).

	1	**2**	**3**	**4**	**5**
Pink					
Blue					
Purple					
Green					
Orange					

Section 2. Emulsion's Applicability's

1. Rate each of the products below, on a scale of 1-5 (1- Would never buy; 2- Unlikely; 3- Likely; 4- Quite likely and 5- Would buy), considering the probability of buying one with the "pink" emulsion odour.

	1	2	3	4	5
Perfume					
Air freshener					
Massage cream					
Toothpaste					
Shampoo					
Candy					

2. Rate each of the products below, on a scale of 1-5 (1- Would never buy; 2- Unlikely; 3- Likely; 4- Quite likely and 5- Would buy), considering the probability of buying one with the "green" emulsion odour.

	1	2	3	4	5
Perfume					
Air freshener					
Massage cream					
Toothpaste					
Shampoo					
Candy					

3. Rate each of the products below, on a scale of 1-5 (1- Would never buy; 2- Unlikely; 3- Likely; 4- Quite likely and 5- Would buy), considering the probability of buying one with the "orange" emulsion odour.

	1	2	3	4	5
Perfume					
Air freshener					
Massage cream					
Toothpaste					
Shampoo					
Candy					

4. Rate each of the products below, on a scale of 1-5 (1- Would never buy; 2- Unlikely; 3- Likely; 4- Quite likely and 5- Would buy), considering the probability of buying one with the "purple" emulsion odour.

	1	2	3	4	5
Perfume					
Air freshener					
Massage cream					
Toothpaste					
Shampoo					
Candy					

5. Rate each of the products below, on a scale of 1-5 (1- Would never buy; 2- Unlikely; 3- Likely; 4- Quite likely and 5- Would buy), considering the probability of buying one with the "blue" emulsion odour.

	1	2	3	4	5
Perfume					
Air freshener					
Massage cream					
Toothpaste					
Shampoo					
Candy					

6. Do you consider that the odours in the samples have other applicability? If yes, refer which/which samples and the respective applicability.

Answer:_____

Annex 3. Questionário Sensorial (Versão Portuguesa)

O meu nome é Ana Ruas, sou estudante de 2º ano do mestrado em Biologia dos Recursos Vegetais, da Faculdade de Ciências da Universidade de Lisboa (FFUL) e do Instituto Superior de Agronomia (ISA). O seguinte questionário foi concebido na sequência da minha dissertação de mestrado e consiste num acréscimo imprescindível ao trabalho em desenvolvimento pelo que agradeço a sua disponibilidade no preenchimento do mesmo.

O questionário é composto por perguntas curtas e objetivas que devem ser respondidas com base nas amostras fornecidas. Os dados recolhidos são anónimos sendo utilizados apenas para o fim mencionado.

1. O aroma de muitas espécies vegetais apresenta compostos químicos voláteis variados com a capacidade de estimular os nossos recetores olfativos. Os aromas característicos das plantas estão relacionados com a quantidade e diversidade destes compostos nas espécies vegetais.

2. Os óleos essenciais (OEs) são misturas de compostos voláteis isolados de diferentes partes das plantas e existe um interesse acrescido na sua utilização para diversos fins como por exemplo, na indústria cosmética, perfumaria e aromaterapia.

Idade:

- 18-30 anos;
- 31-40 anos;
- 41-50 anos;
- 51-60 anos;
- Mais de 60 anos

Género:

- Feminino;
- Masculino

Nível de Escolaridade:

- Básico;
- Secundário;
- Ensino Superior

Região:

- Cidade;
- Campo

Secção 1. Aroma das Emulsões

1. Como avalia o odor da emulsão "rosa"?

- Sem odor
- Pouco percetível;
- Percetível;
- Muito percetível;
- Odor intenso

2. No caso de ter identificado algum odor, na emulsão, como o classifica?

- Desagradável;
- Pouco agradável;
- Agradável e "fresco";
- Agradável e "quente"

3. Como avalia o odor da emulsão "verde"?

- Sem odor;
- Pouco percetível;
- Percetível;
- Muito percetível;
- Odor intenso

4. No caso de ter identificado algum odor, na emulsão, como o classifica?

- Desagradável;
- Pouco agradável;
- Agradável e "fresco";
- Agradável e "quente"

5. Como avalia o odor da emulsão "laranja"?

- Sem odor;
- Pouco percetível;
- Percetível;
- Muito percetível;
- Odor intenso

6. No caso de ter identificado algum odor, na emulsão, como o classifica?

- Desagradável;
- Pouco agradável;
- Agradável e “fresco”;
- Agradável e “quente”

7. Como avalia o odor da emulsão “lilás”?

- Sem odor;
- Pouco percetível;
- Percetível;
- Muito percetível;
- Odor intenso

8. No caso de ter identificado algum odor, na emulsão, como o classifica?

- Desagradável;
- Pouco agradável;
- Agradável e “fresco”;
- Agradável e “quente”

9. Como avalia o odor da emulsão “azul”?

- Sem odor;
- Pouco percetível;
- Percetível;
- Muito percetível;
- Odor intenso

10. No caso de ter identificado algum odor, na emulsão, como o classifica?

- Agradável e fresco;
- Agradável e “quente”;
- Pouco agradável;
- Desagradável;

11. Na sua opinião os odores das diferentes emulsões pertencem à mesma espécie vegetal?

- Sim;
- Não;
- Talvez

12. Selecione qual/quais as emulsões que lhe provocaram alguma sensação de bem-estar físico e/ou mental.

- Rosa;
- Verde;
- Laranja;
- Lilás;
- Azul;
- Nenhuma

13. Refira que sensação de bem-estar a(s) emulsão(s) lhe causou.

- Relaxante;
- Descongestionante;
- Estimulante;
- Refrescante;
- Nenhuma

14. Ordene as emulsões, de acordo com a sua preferência, numa escala de 1-5 (1-Odor detestável; 2-Odor desagradável; 3-Odor agradável; 4-Odor muito agradável and 5-Odor predileto).

	1	**2**	**3**	**4**	**5**
Rosa					
Azul					
Lilás					
Verde					
Laranja					

Secção 2. Aplicabilidade das Emulsões

1. Classifique cada um dos produtos abaixo, numa escala de 1-5 (1-Nunca compraria; 2-Improvável; 3- Provável; 4- Muito provável and 5- Compraria) tendo em conta a probabilidade de comprar algum com o odor da emulsão “rosa”.

	1	2	3	4	5
Perfume					
Ambientador					
Creme de massagem					
Pasta de dentes					
Champô					
Rebuçado					

2. Classifique cada um dos produtos abaixo, numa escala de 1-5 (1-Nunca compraria; 2-Improvável; 3- Provável; 4- Muito provável and 5- Compraria) tendo em conta a probabilidade de comprar algum com o odor da emulsão “verde”.

	1	2	3	4	5
Perfume					
Ambientador					
Creme de massagem					
Pasta de dentes					
Champô					
Rebuçado					

3. Classifique cada um dos produtos abaixo, numa escala de 1-5 (1-Nunca compraria; 2-Improvável; 3- Provável; 4- Muito provável and 5- Compraria) tendo em conta a probabilidade de comprar algum com o odor da emulsão “laranja”.

	1	2	3	4	5
Perfume					
Ambientador					
Creme de massagem					
Pasta de dentes					
Champô					
Rebuçado					

4. Classifique cada um dos produtos abaixo, numa escala de 1-5 (1-Nunca compraria; 2-Improvável; 3- Provável; 4- Muito provável and 5- Compraria) tendo em conta a probabilidade de comprar algum com o odor da emulsão “lilás”.

	1	2	3	4	5
Perfume					
Ambientador					
Creme de massagem					
Pasta de dentes					
Champô					
Rebuçado					

5. Classifique cada um dos produtos abaixo, numa escala de 1-5 (1-Nunca compraria; 2- Improvável; 3- Provável; 4- Muito provável and 5- Compraria) tendo em conta a probabilidade de comprar algum com o odor da emulsão "Azul".

	1	**2**	**3**	**4**	**5**
Perfume					
Ambientador					
Creme de massagem					
Pasta de dentes					
Champô					
Rebuçado					

6. Considera que os odores das emulsões tenham outras aplicabilidades? Se sim refira qual/quais as emulsões e as aplicabilidades respetivas.

Resposta:_____