INSTITUTO POLITÉCNICO DE BRAGANÇA Escola Superior Agrária

Extraction and Analysis of Plant Volatiles

Hanine Hached

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Supervised by

Dr. Márcio Carocho

Dr. Josiana Vaz

Dr. Feriel Rezouga

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Abbreviations and Acronyms

8505C: Thyroid carcinoma cell line

95-D: High metastasis human lung cancer cell line

A549: Human lung adenocarcinoma cell line

AGS: Human gastric adenocarcinoma cell line

ATCC: American type culture collection

ATP: Adenosine triphosphate

ATPase: Adenosine triphosphatases

CAA: Cellular antioxidant activity

CaCo2: Human colorectal adenocarcinoma cell line

DPPH: 2,2-diphenyl-1-picrylhydrazyl

DMAPP: Dimethylallyl pyrophosphate

DNA: Desoxyribonucleic acid

EC_{50}: Half maximal effective concentration

E.O: Essential oil

FDA: Food and drug administration

GC-MS: Gas chromatography–mass spectrometry

GI_{50}: Concentration for 50% of maximal inhibition of cell proliferation

GRAS: Generally recognized as safe

HCT 116: Human colorectal carcinoma cell line

HD: Hydro distillation

HL-60: Human acute myeloid leukaemia cell line

IC_{50}: Half maximal inhibitory concentration

ICAM-1: Intracellular cell adhesion molecule 1

IL-12 : Interleukine 12

IL-1β : Interleukine 1 beta

IL-3 : Interleukine 3

IL-6 : Interleukine 6

iNOS: Inducible nitric oxide synthase

IP-10: Interferon γ-induced protein 10

IPP: Isopentenyl pyrophosphate

IκBα: Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha

LPS: Lipopolysaccharide

MAHD: Microwave-assisted hydro distillation

MBC: Minimal bactericidal concentration

MCF-7: Human breast cancer cell line

MDA: Malondialdehyde

MDA-MB-231: Human breast carcinoma cell-line

MEP: Methylerythritol phosphate

MFC: Minimal fungicidal concentration

MIC: Minimum inhibitory concentration

MMP-9: Matrix metallopeptidase-9

MOLT-4: Human acute T lymphoblastic leukaemia cell line

MV3: Human melanoma cell line

MVA: Mevalonic acid

NAT: N-acetyltransferase

NCI: National Cancer Institute

NCI-H292: Human lung mucoepidermoid carcinoma cells

NCI-H446: Small cell lung cancer cell line

NCI-H460: Human large-cell lung carcinoma cell line

NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells

NIST: National institute of standards and technology

NO: Nitric oxide

NSCLC: Non-small-cell lung carcinoma cell line

PLP2: Porcine liver primary cell culture

ROS: Reactive oxygen species

RP: Reducing power

RSM: Response surface methodology

SRB: Sulforhodamine B

SPCA-1: Human lung cancer cell line

SW620: Colorectal cancer cell line

TNF-α: Tumor necrosis factor alpha

TPA: Topical 12-*O*-tetradecanoylphorbol-13-acetate

WEHI-3: Murine leukaemia cell line

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Abstract

As worldwide population is growing, the demand on food increases exponentially, which results in food overproduction, food spoilage, and in foodborne diseases. This issue has arisen many concerns related to public health, economy, and the environment, which led to exhaustive research on possible solutions to minimize costs and ensure safer food products that respond to consumers' demand for clean-label foods with less collateral damages on the environment.

Essential oils (E.Os) and volatiles are plant secondary metabolites and are considered as promising food preservatives seen their remarkable biological activities including antioxidant, antimicrobial, antiproliferative, and anti-inflammatory activities.

E.Os from three aromatic plants: eucalyptus (*Eucalyptus globulus* Labill.), peppermint (*Mentha x piperita* L.), and pine (*Pinus pinaster*) were extracted by hydro distillation (HD) and microwave-assisted hydro distillation (MAHD), both optimized with response surface methodology (RSM), which generally showed higher yields in E.Os obtained with MAHD influenced primarily by solid/liquid ratio, with eucalyptus giving the highest yield from the three studied plants.

Chromatographic identification resulted in high amounts of monoterpenes, followed by sesquiterpenes in both eucalyptus and peppermint E.Os, while pine E.O was proven to be richer in diterpenes. These volatile compounds led to interesting bioactive properties in all of the studied E.Os, especially the ones extracted using hydro distillation.

The extracts showed an inhibitory capacity against the majority of the tested bacterial strains, specifically against Methicillin-resistant Staphylococcus aureus (MRSA), which is a multi-resistant bacteria. Also, a remarkable antifungal activity against *Aspergillus brasiliensis* and *Aspergillus fumigatus*.

In terms of antiproliferative activity, the E.Os extracted through HD demonstrated higher tumoral cell growth inhibition against the majority of the studied cancer cell lines, with pine E.O standing out with the lowest GI_{50} value, and none of the tested E.O showed cytotoxicity against normal cell line studied in this case (PLP2).

The E.Os also revealed an anti-inflammatory capacity that stood out mainly with those extracted using HD. This work highlights the promising use of essential oils as headspace preservatives for food packaging.

Keywords: Essential oils, volatiles, *Eucalyptus globulus* Labill., *Mentha x piperita* L., *Pinus pinaster*, hydro distillation, microwave-assisted hydro distillation antioxidant, antimicrobial, antiproliferative, anti-inflammatory.

Resumo

Com o crescimento da população mundial, a necessidade de alimentos aumenta exponencialmente, o que resulta em sobreprodução de alimentos, desperdícios e doenças transmitidas por alimentos.

Esta questão tem despertado muitas preocupações relacionadas com a saúde pública, economia e meio ambiente, o que levou a investigação sobre possíveis soluções para minimizar custos e garantir produtos alimentares mais seguros e que atendam à procura dos consumidores por alimentos sustentáveis e com menos danos ao ambiente.

Os óleos essenciais (O.Es) e moléculas voláteis são metabolitos secundários de plantas e são considerados conservantes de alimentos promissores devido às suas notáveis atividades biológicas, incluindo atividades antioxidantes, antimicrobianas, antiproliverativas, e anti-inflamatórias

O.Es de três plantas aromáticas: eucalipto (*Eucalyptus globulus* Labill.), hortelã-pimenta (*Mentha x piperita* L.) e pinheiro (*Pinus pinaster* L.) foram extraídos por hidrodestilação (HD) e hidrodestilação assistida por microondas (MAHD), ambas otimizada com a metodologia de superfície de resposta (RSM), que apresentou geralmente maiores rendimentos em O.Es. obtidos com MAHD influenciados principalmente pela relação sólido/líquido, onde o eucalipto apresentou o maior rendimento das três plantas estudadas.

A identificação cromatográfica resultou em altas quantidades de monoterpenos, seguidos de sesquiterpenos em O.Es de eucalipto e hortelã-pimenta, enquanto o O.E. de pinheiro provou ser mais rico em diterpenos. Esses compostos voláteis levaram a interessantes propriedades bioativas em todos os E.Os estudados, especialmente os extraídos por hidrodestilação.

Os extratos mostraram capacidade inibitória contra a maioria das bacterias testadas, especificamente contra *Staphylococcus aureus* resistente à meticilina (MRSA), que é uma bactéria multirresistente. Além disso, uma notável atividade antifúngica foi detetada contra *Aspergillus brasiliensis* e *Aspergillus fumigatus*.

Em termos de atividade antiproliferativa, os O.Es extraídos por HD demonstraram maior inibição do crescimento de células tumorais contra a maioria das linhagens de células

cancerígenas estudadas, destacando-se o O.E. de pinheiro com o menor valor de GI_{50}, e nenhum dos O.E. testados apresentou citotoxicidade contra a linha não tumoral estudada (PLP2).

Os O.E. também revelaram uma capacidade anti-inflamatória que se destacou principalmente com os extraídos com HD. Este estudo revela uma boa possibilidade dos óleos essenciais poderem vir a ser estudados como conservantes alimentares em embalagens alimentares.

Palavras-chave : óleos essenciais, voláteis, *Eucalyptus globulus Labill.*, *Mentha x piperita* L., *Pinus pinaster*, hidrodestilação, hidrodestilação assistada por microondas, antioxidante, antimicrobiano, antiproliferativo, anti-inflamatório.

1. Introduction

1.1 Secondary metabolites

Secondary metabolites of plants are vital bioactive compounds synthesized during primary and intermediary metabolism. Their production requires more energy than the production of primary metabolites, which explains the importance of the secondary metabolites as they stimulate the plant's defence mechanism against biotic and abiotic stresses as well as its signalling mechanisms to ensure their survival and reproduction (Cardoso et al., 2019; Jamloki et al., 2021).

Plant secondary metabolites have different industrial applications as high added value bioactive molecules in as many industries as the pharmaceutical, nutraceutical, cosmetic industries, as well as in the production of food additives, antioxidants, antimicrobial compounds, fragrances, colours, and a wide range of other products (Shih and Morgan, 2020). In addition to their potent antimicrobial and antioxidant activities, plant secondary metabolites exhibit interesting antiproliferative and anti-inflammatory activities (Gomes et al., 2019).

1.1.1 Types of secondary metabolites

Plant secondary metabolites can be divided into three main groups according to their origin of synthesis: terpenoids and terpenes, derived from non-nitrogenous compounds, and they represent the major group of plant secondary metabolism with approximately 30.000 compounds, followed by alkaloids that represent approximately 21.000 compounds and are mainly derived from precursors of various amino acids, but only coniferous alkaloids are biosynthesized by the polyketide biosynthetic pathway. Coming at the third place, phenolic compounds and phenolic acids, constituting approximately 8.000 compounds that are derived from non-nitrogenous compounds and are biosynthesized either by the shikimic acid pathway or by the malonate/acetate pathway (Jamloki et al., 2021).

1.1.1.1 Terpenes and Terpenoids

Terpenes are hydrocarbons produced from combination of several isoprene units (C_5H_8) and are synthesized in the cytoplasm of plant cells via the mevalonic acid (MVA)

pathway starting from acetyl-CoA. Terpenes have a hydrocarbon backbone which can be rearranged into cyclic structures by special enzymes called cyclases, and that leads to forming monocyclic or bicyclic structures (Hyldgaard et al., 2012). They are volatile organic compounds possessing odorous characteristics (Dashora et al., 2021). The main terpenes are monoterpenes (C10H16) and sesquiterpene (C15H24), but longer chains such as diterpenes (C20H32), triterpenes (C30H40), and more, also exist (Hyldgaard et al., 2012). Monoterpenes consist of two isoprene units, and they are the most representative molecules constituting 90% of essential oils extracted from a myriad of plants (Ribeiro et al., 2018).

Volatile terpenes like isoprenes, monoterpenes, sesquiterpenes, phenylpropenes, and some diterpenoids constitute the largest class of plant volatile terpenoid compounds, used in the plant-environment communication and as a response to several biotic and abiotic conditions (Boncan et al., 2020). These volatile compounds can be found in essential oils extracted from a myriad of plants (Abbas et al., 2017).

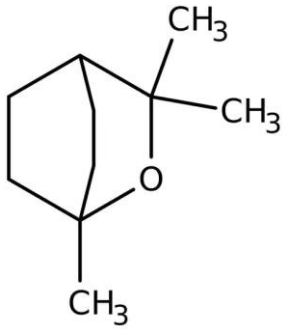

Figure 1. Structure of a monoterpene: 1,8 – cineole (Eucalyptol) (thermofisher.com)

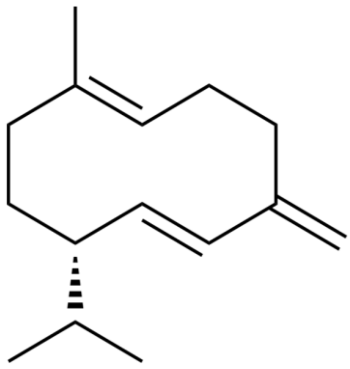

Figure 2. Structure of a sesquiterpene: Germacrene-D (medchemexpress.com)

Terpenoids, on the other hand, are terpenes with additional oxygen molecules or with enzymatically moved or removed methyl groups (Ribeiro et al., 2018), and they represent one of the largest and most abundant classes of plant secondary metabolites. They are unsaturated hydrocarbons and are synthesized from the five-carbon precursor units isopentenyl pyrophosphate (IPP) and its functional isomer dimethylallyl pyrophosphate (DMAPP). The biosynthesis of terpenoids is stimulated by plant growth regulators, and they play a huge role in the plant's communication with the environment, in its growth and development, and in its defence mechanisms against biotic and abiotic stress conditions (Tholl, 2015). These compounds have been used for a long time as insecticides, herbicides, pharmaceuticals, food additives, and cosmetical compounds for their interesting antiproliferative and antimicrobial activities (Jamwal et al., 2018; Tholl, 2015). Their biological activities rely on their aromatic feature as well as the presence of the functional hydroxyl group (Ribeiro et al., 2018).

Terpenoids are highly diversified, they can be subdivided into alcohols, esters, aldehydes, ketones, ethers, phenols, and epoxides (Hyldgaard et al., 2012). There are two independent pathways used by the plants: the primarily cytosolic mevalonic acid pathway from which originate the volatile sesquiterpenes (C15), and the plastidial methylerythritol phosphate (MEP) pathway to produce the two building blocks of terpenoids IPP and DMAPP, and which provides the precursors for the biosynthesis of the volatile hemiterpenes, monoterpenes, and diterpenes. Another pathway called the shikimic acid pathway, leads to the production of phenylpropenes, which are the second largest group of plant volatiles and are considered one of the major compounds of essential oil. They are a subfamily of compounds under phenylpropanoids that are synthesised in plants using phenylalanine, containing a six-carbon aromatic phenol group and a three-carbon propene tail from cinnamic acid (Marei and Abdelgaleil, 2018; Hyldgaard et al., 2012; Tholl, 2015; Verdeguer et al., 2020).

The research conducted by Marei and Abdelgaleil, (2018) on the antifungal activity of monoterpenes and phenylpropenes, proved that trans-cinnamaldehyde, eugenol, and (–)-menthone revealed promising antifungal properties against eight plant pathogenic fungi: *Aspergillus niger*, *Alternaria solani*, *Botrytis cinerea*, *Fusarium oxysporum*, *Fusarium solani*, *Penicillium digitatum*, *Phytophthora infestans*, and *Rhizoctonia solani*. Another study conducted by Aoudou et al. (2010) has also proven that (–)-citronellol and (–)-terpinen-4-ol cause the mycelial growth inhibition of food spoilage fungi: *Aspergillus* spp., *Penicillium* spp.,

and *Fusarium* spp.). Wang et al. (2018) reported antimicrobial, antifungal, and repellent activities of volatile phenylpropenes (methyleugenol, safrole, and 3,5-dimethoxytoluene) against phytopathogens such as *Lasioderma serricorne* and *Liposcelis bostrychophila*.

Diterpenes such as Taxol (paclitaxel) and its derivatives are proven to have remarkable pharmaceutical activities, mainly antitumoral activity. They are commercialized and largely used in the treatment of numerous types of cancer such as mammary, ovarian, and prostate cancer as well as non-small-cell lung carcinoma (Jamwal et al., 2018).

1.1.1.2 Alkaloids

Alkaloids are among the most abundant plant secondary metabolites and are found in numerous plants. Their basic nature contains nitrogen, and are derived from amino acids, purines, pyrimidines, or terpenes (Jerzykiewicz, 2007). These compounds are known for being highly diverse structure wise, but they have one feature in common which is the presence of nitrogen. They can either be monomers or can be homo or hetero oligomers, and, on a biosynthetic level, they're divided into three main categories: true, proto-, and pseudo-alkaloids (Casciaro et al., 2020). True- and proto-alkaloids have an amino acid as a precursor, but they differ by the presence of nitrogen in the heterocycle in the case of true alkaloids, and its presence in a side chain in proto-alkaloids (Ribeiro et al., 2018). While pseudoalkaloids feature a basic carbon skeleton not deriving from an amino acid, and they include terpene-like, steroid-like, and purine-like alkaloids (Casciaro et al., 2020).

Plants use alkaloids as a way of protection against predators and pathogens. In fact, there are many specialized groups of alkaloids that confer antimicrobial and antiproliferative characteristics to plants (Maital and Jaitak, 2019). These compounds are usually found in essential oils (Preedy, 2016), being their exceptional biological activity provided by the ability to form hydrogen bonds with enzymes, receptors, and proteins, due to the presence of a proton accepting nitrogen atom and one or more protons donating amine hydrogen atoms (Casciaro et al., 2020). For instance, Berberine, a natural isoquinoline alkaloid with low toxicity, capable of penetrating all cell lines, presents interesting properties of being anti-inflammatory, antibacterial, and especially antiviral, all while protecting the liver (Warowicka et al., 2020). It has the ability to inhibit the replication and halt the viral infection caused by various viruses

such as influenza virus, herpes simplex virus, human cytomegalovirus, human papillomavirus, and chikungunya virus (Warowicka et al., 2020).

In addition, Berberine exhibits remarkable anti-tumoral acitivity by halting the cell cycle during its early phases and by tumor cell apoptosis, as well as by the decrease of cancer cells migration and the inhibition of tumor cell metastasis and invasion caused by endoplasmic reticulum stress and autophagy (Thawabteh et al., 2019). Other alkaloids like camptothecin, vincristine and vinblastine are generally employed in cancer treatments due to their antiproliferative activity (Thawabteh et al., 2019).

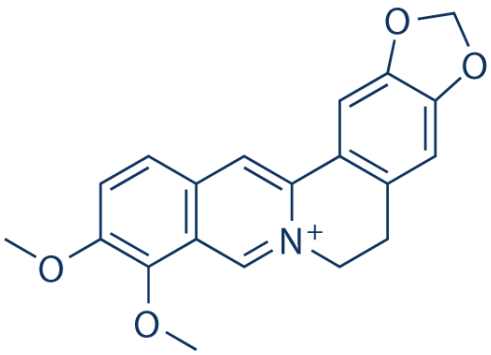

Figure 3. Berberine chemical structure (Selleckchem.com)

β-carboline alkaloids, derived of indole, have also been proven to show remarkable antioxidant, antimicrobial, antiproliferative, antiviral and anti-inflammatory activities, with low toxicity in certain well-studied doses (Xie et al., 2021).

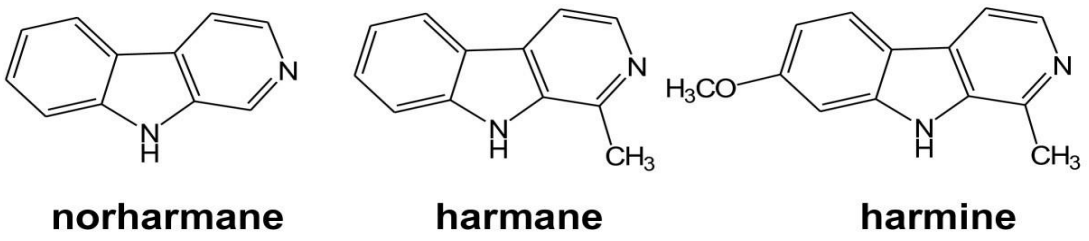

Figure 4. Examples of β-carboline alkaloids (Alomar et al., 2013)

The antiproliferative activity of *β*-carboline alkaloids such as harmine is highlighted by the selective action on cancer cells through the inhibition of essential enzymes such as topoisomerase, as well as through intercalting cancer cell's DNA which causes DNA malfunctions, leading to apoptosis and autophagy, and could go far into preventing and reversing DNA synthesis of cancer cells (Xie et al., 2021). Moreover, other *β*-carboline alkaloids like harmaline, harmane, and 1-carbomethoxy-β-carboline present potent anti-inflammatory activity, and compounds such as norharman, harmane, and tetrahydro-β-carbolines are good scavengers of hydroxyl radicals and antioxidants thanks to their hydroxyl group, and they are also known for being stable even under heat (Xie et al., 2021). These properties make of *β*-carboline alkaloids interesting bioactive compounds to be incorporated as natural food preservatives or even to be used for human health treatement (Xie et al., 2021). As a matter of fact, a study conducted by Darabpour et al. (2011) showed the antimicrobial effect of *Peganum harmala* against numerous strains of multi-drug resistant gram-positive bacteria such as *Bacillus anthracis*, *B. cereus*, *B. pumilus*, *Staphylococcus aureus*, *S. epidermidis*, *Listeria monocytogenes* and *S. pyogenes*, and gram-negative bacteria like *Pseudomonas aeruginosa*, *Brucella melitensis*, *Proteus mirabilis*, *Salmonella typhi*, *Escherichia coli*, and *Klebsiella pneumoniae*, since the extract from the seeds and the roots of *Peganum harmala* is a source of *β*-carboline alkaloids such as harmaline, haman, harmalol, and harmine.

Furthermore, bioactive amaryllidaceae alkaloids have an interesting antiproliferative properties, mainly antiproliferative, antibacterial, as well as antioxidant and anti-inflammatory activities (Chen et al., 2018). The study conducted by Chen et al. (2018), reported the antiproliferative and cytotoxic activity of amaryllidaceae alkaloids (4,8-dimethoxy-cripowellin C, 4,8-dimethoxycripowellin D, 9-methoxy-cripowellin B, and 4-methoxy-8-hydroxy-cripowellin B, and cripowellin) extracted from *Crinum latifolium*, against seven lung cancer cell lines (A549, NCI-H446, NCI-H460, NCI-H292, 95-D, and SPCA-1). In addition, the same alkaloids were praised for their antimicrobial activity against three gram-positive bacteria: *Streptococcus pneumoniae, Staphylococcus aureus* and *Staphylococcus epidermidis*, and five gram-negative bacteria: *Klebsiella pneumoniae, Pseudomonas aeruginosa*, *Haemophilus influenzae*, *Enterobacter cloacae*, and *Shigella dysenteriae*. Moreover, these alkaloids possess a remarkable anti-inflammatory activity through the inhibition of cyclo-oxygenase 1 (COX 1) and cyclo-oxygenase 2 (COX 2), as well as an antioxidant activity by reducing free radicals.

1.1.1.3 Phenolic compounds

Phenolic compounds are a heterogenous group of plant secondary metabolites found in a myriad of plants. There are approximately 8000 currently known phenolic and polyphenolic metabolites. Their diversity and heterogeneity confer plants the ability to defend against various pathogens and pests (Sarkar and Shetty, 2014). In terms of structure, phenolic compounds have an aromatic ring with one or more hydroxyl substitutes, including their functional derivatives (Carocho and Ferreira, 2012). They contribute to the development of plants as well as to their sensory and nutritional quality, tolerance to various abiotic and biotic stresses (Sarkar and Shetty, 2014). In fact, phenolic acids, flavonoids, and tannins represent the most common and abundant groups of phenolic metabolites. They are volatile molecules known for their remarkable antioxidant potential since they function as chelators and eliminate free radicals (Carocho and Ferreira, 2012), and they're also characterized with antimicrobial, cytotoxic, and anti-inflammatory activities, as well as with their great potential of protection against Ultraviolet radiations (Sarkar and Shetty, 2014). These properties make phenolics such relevant compounds in food preservatives, in human health management, in natural pesticides and in cosmetics (Sarkar and Shetty, 2014; Wu et al., 2021).

The wide use of phenolic compounds is due to their chemical stability, easy identification, widespread distribution, and chemical variability. In addition, they enhance the bioactive nutraceutical properties of food to provide relevant chronic disease management strategies (Sarkar and Shetty, 2014).

A study done by Melguizo-Rodríguez et al. (2021) highlighted the antimicrobial properties of olive oil phenolic compounds such as apigenin and luteolin, as well as its phenolic acids such as p-coumaric acid, ferulic acid and caffeic acid that all managed to suppress the growth of *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, *Proteus* spp., and *Candida Albicans*.

Another study conducted by Mizgier et al. (2016) on the antioxidant and anti-inflammatory activities of the phenolic compounds and phenolic acids extracted from red cabbage and purple carrot, showed that these extracts were rich in acylated anthocyanins, caffeoylquinic acid, and hydroxycinnamic acid derivatives, which resulted in their ability to

inhibit COX-1 and COX-2 as an anti-inflammatory response, as well as to scavenger free radicals as an antioxidant property.

According to numerous studies, it is known that oxidative stress leads to the formation of cancer cells (Muller et al., 2019). A plethora of research was conducted on the ability of various phenolic compounds and phenolic acids to inhibit the growth of tumoral cells, and it has been proven that flavonoids like genistein cause the antiproliferation and cell-cycle arrest of human lung cancer cells SPCA-1 as well as the induction of apoptosis. Luteolin, on the other hand, has an apoptotic effect and causes the reduction of cell motility and cell migration in A549 cancer cells. Phenolic acids such as caffeic acid and ferulic acid applied on A549 cancer cells showed a decrease in tumoral cells proliferation and cell adhesion and migration as well as a decrease in the production of superoxide anion (Muller et al., 2019).

1.2 Importance of volatiles from aromatic plants

1.2.1 Essential Oils and Volatiles

Plants have the ability to synthesize liquid lipophilic and infrequently hydrophilic aromatic secondary metabolites like essential oils that are usually extracted by steam or hydro-distillation, or can also be extracted with organic solvents, with supercritical fluids, or even by different mechanical ways, from all of the plant organs such as flowers, fruits, seeds, roots, stems, leaves, or even the whole plant. Generally, they're accumulated in secretory cells, oil and resin ducts, and glandular trichomes (Chávez-González et al., 2016; Ramsey et al., 2020; Sakkas and Papadopoulou, 2017).

About 60 plant families like *Lamiaceae*, *Myrtaceae*, *Poaceae*, *Alliaceae*, *Apiaceae*, *Asteraceae* and *Rutaceae* produce around 3000 known essential oils of which only 300 are frequently used in different applications (da Silva et al., 2021). Plants use volatiles as a mean of communication with their environment for different purposes such as fertilization and reproduction, repelling insects, attracting predators to kill herbivores, and overall providing protection against pathogens. In addition, they have long been used for their antimicrobial, antiseptic and antioxidant properties in human health maintenance and in food preservation (Cagliero et al., 2021, Chávez-González et al., 2016; Ramsey et al., 2020; Sakkas and Papadopoulou, 2017).

Essential oils contain approximately 500 compounds of low molecular wight such as volatile terpenoids and terpenes, aliphatic and aromatic substances like phenols and other oxygen-substituted derivates. Their production depends on the origin and species of the plant and the time it was harvested, as well as on biotic and abiotic factors, and the way they are produced, extracted, and conserved. The nature of the compounds determines the characteristics and the biological activities of essential oils (Ramsey et al., 2020; Sakkas and Papadopoulou, 2017; Verdeguer et al., 2020). Early in history, essential oils were used as herbal medicines, fragnances, aphrodisiacs, and in aromatherapy.

Nowadays, these volatile secondary metabolites have a wide spectrum of uses ; they're found in everyday products like fragnances, soapes, detergents, cosmetic products, food additives, pharmaceuticals, insecticides, air-freshners and many other products, and they are not only used for their distinct smell but also for their antimicrobial, antioxidant, anti-inflammatory, antiproliferative, and insect-repelling properties (Hyldgaard et al,, 2012; Ramsey et al., 2020). The wide range of essential oil applications originate from the high demand on natural products, and it is estimated by Grand View Research Inc. that in 2015 the worldwide essential oil market was worth around 3.4 billion US dollars (Lainez-Cerón et al., 2021).

1.2.2 Mechanisms of action of essential oils

1.2.2.1 Antioxidant mechanism of action of essential oils

Phenolic compounds like flavonoids, and phenolic acids like gallic acid are the main bioactive molecules that confer to the plants the needed protection against oxidative stress and damage. They exhibit their antioxidant potential through various action mechanisms such as increasing antioxidant enzymes activity, chelating ions, eliminating ROS, and inhibiting lipid peroxidation (Ridaoui et al., 2022). For instance, preventive oxidants inhibit the the formation of free lipid radicals, thus ensure the halt of free radical oxidation reactions anions. On the other hand, chain-breaking antioxidants don't allow the propagation of the autoxidation chain reaction in cells.

Also, metal chelators play a role in converting metal pro-oxidants like iron and copper derivates into stable products that don't increase cellular oxidative damage anions (Carocho and Ferreira, 2012). Natural antioxidants also act as inhibitors of pro-oxidative enzymes such

as lipooxigenases, inhibit oxidases, increase levels of uric acid, and reduce nitrosative stress inside the cells as well as the production of superoxide anion (Carocho and Ferreira, 2012).

1.2.2.2 Antimicrobial mechanism of action of essential oils

The antimicrobial properties of essential oils are mainly due to the volatile compounds' hydrophobicity, which, among several action mechanisms, allows interaction with the lipidic components of the microorganisms' cell membrane. This interaction mainly affects the integrity of the membrane on both structural and functional levels (da Silva et al., 2021).

Polyphenols are hydrohobic essential oil compounds that interact the most with bacterial membranes because they can incorporate themselves deep into the lipid bilayer causing the disruption of the plasma membrane on a structural level. This is due to the fact that polyphenols mainly cause pH fluctuations, formation of pores, and delocalization of membrane proteins (Alvarez-Martínez et al., 2021).

The antibacterial mechanism of flavonoids can be carried out in different ways, which encompasses the impairment of bacterial cytoplasmic membrane caused by formation of pores and increase in membrane rigidity, inhibition of the energetic metabolism, and disfunction of nucleic acids synthesis. More mechanisms such as the inhibition of cell wall and cell membrane synthesis have also been reported (Ribeiro et al., 2018). As for phenolic acids, their antibacterial mechanism of action includes enzyme inhibition and repression of nucleic acids' synthesis in both gram-positive and gram-negative bacteria. This is due to the toxicity provided by the number of hydroxyl groups on the phenol group. The toxicity is proportional to the number of hydroxyl groups (Ribeiro et al., 2018).

In the case of tannins, which are water soluble polyphenols, their antimicrobial mechanism of action may be attributed to the inhibition of extracellular microbial enzymes, deprivation of the substrates required for microbial growth, inhibition of oxidative phosphorylation which has a direct action on the microbial metabolism, or even the deprivation of essential metal ions through their chelating action over many metal ions (Ribeiro et al., 2018).

Like phenolic compounds, terpenes also target the cell membrane. Some terpenes that contain a phenolic hydroxyl group, have the ability to pass through the bacterial plasma

membrane and inhibit vital enzymes like adenosine triphosphatases (ATPase). They can also bind more important molecules like adenosine triphosphate (ATP) and cations like K+, and carry them outside the cell. These mechanisms cause a disarrangement of the membrane potential and, eventually, cell lysis (Alvarez-Martínez F.J. et al., 2021).
Other terpenes, such as thymol and cinnamaldehyde, affect the fluidity of the membrane by disrupting its sturcture through integrating themselves into the polar-head region of the phospholipid bilayer which increases the rigidity of the bacterial plasma membrane. And in the case of gram-negative bacteria, some terpenoid compounds, such as monoterpenoids, can induce the release of lipopolysaccharides molecules situated on the outer membrane, causing membrane desintegration (Di Pasqua et al., 2007; Nazzaro et al., 2013).

Aromatic planar quaternary alkaloids such as berberine and harmane are able to intercalate with nucleic acids which causes abnormal cell division and, eventually, cell death (Ribeiro et al., 2018). Gram-positive bacteria such as *Staphylococcus aureus*, for instance, are the most sensitive to essential oils seen that they're more hydrophobic than gram-negative bacteria such as *Escherichia coli*, which is due to the thick layer of peptodoglycane covering their outer membrane by approximately 90-95%, making it two to three times thicker than the peptidoglycane layer of gram-negative bacteria. In addition, the outer membrane is a unique feature of gram-negative bacteria, it lies outside of the thin peptidoglycan layer and mainly restricts the diffusion of hydrophobic compounds from essential oils and only allows the transfer of hydrophyllic solutes through transmembranne proteins that work as porine channels. This makes gram-negative bacteria less targeted by essential oil compounds and drugs such as antibiotics (da Silva et al., 2021; Nazzaro et al., 2013; Sakkas and Papadopoulou, 2017).

Similar to the antibacterial mechanism of action of essential oils, antifungal mechanism of action targets cell membrane on a structural and functional level through the action of terpenes and terpenoids (Nazzaro et al., 2017). For instance, cinnamaldehyde inhibits the enzymes responsible for cell wall synthesis in *Saccharomyces cerevisiae* by functioning as a non-competitive inhibitor of β-(1,3)-glucan synthase and a mixed inhibitor of chitin synthase isozymes (Hyldgaard et al., 2012). In addition, antifungal properties go far into the cell organelles to inhibit the efflux pumps as well as the mitochondrial membrane potential and mitochondrial respiration activity through the accumulation of reactive oxygen species (ROS), leading to the inhibition of sporulation and germination processes in fungi as well as the

destabilisation of the synthesis of mycotoxins, which eventually results in cell death (Nazzaro et al., 2017).

1.2.2.3 Antiproliferative mechanism of action of essential oils

The bioactive compounds in essential oils, mainly terpenes, also demonstrate a promising antiproliferative activity with lower costs and, especially, less collateral effects than chemical compounds (Beeby et al., 2020), making over 60% of the currently available and significantly effectve anticancer medicines derived from or contain natural products (Magalhães et al., 2021).

In fact, previous studies have reported the apoptosis and promotion of antiproliferative effect provided by monoterpenes and sesquiterpenes in cancer cells (Beeby et al., 2020), and they also impair tumor initiation and prevent carcinogenesis (Magalhães et al., 2021). Others reported mechanism of action of certain phenolic compounds, acting by inhibition of proliferation (cell cycle arrest, induction of apoptosis) through depolarization of mitochondrial membrane, increase of oxidative stress inside cancer cells, and inhibition of angiogenesis, or even by immunomodulation (Magalhães et al., 2021).

More studies have highlighted the antimutagenic activity of phenolic compounds by impairing tumor initiation and growth through the inhibition of carcinogens-induced oxidative stress, reduction of phase I enzymes such as cytochrome P450 and increase of phase II enzymes such as glutathione S-transferase from drug metabolism to block the activation of procarcinogens and to foster the elimination of carcinogens, respectively, as well as the stimulation of DNA repair (Magalhães et al., 2021). Similar mechanisms of action have been reported in alkaloids such as berberine, which inhibits numerous enzymes, including COX-2, N-acetyltransferase (NAT), and telomerase. It also inhibits NF-κB and activates the synthesis of intracellular ROS, causes apoptosis by mitochondrial-dependent pathway, and activates p53 gene which results in apoptosis and cell cycle arrest (Thawabteh et al., 2019).

1.2.2.4 Anti-inflammatory mechanism of action of essential oils

In addition to their remarkable antioxidant, antimicrobial, and antiproliferative properties, natural bioactive compounds extracted from essential oil have always shown a remarkable anti-inflammatory property along with less toxicity and less adverse effects on the

long-term run, which is the reason why they have been incorporated for centuries in traditional medicines and in phytomedicines to treat a myriad of inflammatory diseases, symptoms, and sores, as well as to heal wounds (Zhang et al., 2020).

Frankincense essential oil, for example, has been reported to show significant potential in reducing levels of interferon γ-induced protein10 (IP-104) and intracellular cell adhesion molecule 1 (ICAM-1) which are important inflammatory biomarkers (Zhang et al., 2020). In general, essential oils like frankincense or those extracted from Myrrh, *Radix aucklandiae*, *Matricaria chamomilla*, *Jasminum sambac*, and *Syzygium aromaticum* display the ability to suppress inflammatory responses even better than conventional drugs, and that's through the inhibition of signs of inflammation such as the formation of Prostaglandin E2 (PGE-2) by repressing the COX-2 expression, as well as the transactivation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) via stabilization of nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (IκBα) as a form of protection against topical 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced inflammation (Zhang et al., 2020).

Some of the potent molecules found in the essential oils make them a better, safer, and a more effective substitute to chemical anti-inflammatories when delivered adequately (Zhang et al., 2020).

Other bioactive compounds found in essential oils like thymol and carvacrol, control the phosphorylation of multiple signalling molecules which results in supressing key mediators of inflammation (García-Salinas et al., 2020).

1.2.3 Aromatic Plants

1.2.3.1 Eucalyptus (*Eucalyptus globulus* Labill.)

Eucalyptus globulus Labill., or Bluegum Eucalyptus, also known as Tasmanian Eucalyptus, belongs to the *Myrtaceae* family, and is one of the most known eucalyptus tree species, reaching up to 60 m. It is native to Tasmania in Australia and cultivated in many other countries (Skolmen and Ledig, 1990).

Figure 5. Eucalyptus globulus Labill. tree (http://www.biorede.pt/page.asp?id=1850)

Figure 6. Leaves, flowers, and fruits of *Eucalyptus globulus* Labill. (http://www.biorede.pt/page.asp?id=1850)

Eucalyptus leaves are perennial and odorous due to the essential oil that is produced and accumulated in secretory cells (Barbosa et al., 2016). Usually, the eucalyptus oil is extracted using steam or hydro distillation and it is either a colourless or pale-yellow liquid (Bello et al., 2021). Moreover, studies conducted by Pan et al. (2020) and Manuel et al. (2021) on *Eucalyptus globulus* leaves showed that eucalyptus essential oil is rich in monoterpenes such as eucalyptol (1,8-cineole) as a major component (67.29%), and could be higher than 80% in some cases (Barbosa et al., 2016), followed by pinocarvone (11.33%), pinocarveol (11.11%) (Manuel et al., 2021), α-pinene (9.80%), dihydrocarvone (5.15%), *D*-limonene (2.59%), camphene (2.43%), fenchyl alcohol (1.83%), citral (0.20%) which has potent anticancer, antimicrobial, and antioxidant activities.

The oil also contains some sesquiterpenes such as *β*-caryophyllene (2.42%), δ-cadinene (1.52%) (Pan et al., 2020), Virdiflorol (5.22%) (Manuel et al., 2021), *β*-eudesmol, globulol, and spathulenol (Jinbiao et al., 2010), as well as a few phenolic acids (2.93%) such as quinic acid (2.4%) and protocatechuic acid (0.15%) which is known for its antibacterial and antioxidant effects (Pan et al., 2020).

The eucalyptus essential oil has bacteriostatic, insecticidal, herbicidal, as well as antifungal effects (Jinbiao et al., 2010; Pan et al., 2020). For instance, the study by Pan et al. (2020) showed results of an antifeedant activity on *Henosepilachna vigintioctopunctata* (Fabricius) due to the repellent potential of protocatechuic acid and citral, while another study conducted by Mareggiani et al. (2008) proved the insecticidal activity of *Eucalyptus globulus* essential oil against *Aphis gossypii* (Hemiptera, Aphididae) adults. In general, the gram-positive pathogenic bacteria *Staphylococcus aureus, Staphylococcus intermedius, B. subtilis*, and other *Staphylococcus* species, the gram-negative *Escherichia coli, Shigella,* as well as a few *Salmonella* species, and the yeast species *Candida albicans*, were reported to be sensitive to *Eucalyptus globulus* essential oil and other essential oils extracted from different eucalyptus species (Barbosa et al., 2016).

α-terpineol, a monoterpene found in eucalyptus essential oil, is one of the known bioactive compounds that have an antiproliferative property, which was studied by Lahmadi et al. (2021) along with various other bioactive compounds like trans-myrtanol, myrtenol, and decanoic acid, all of which were extracted from *Eucalyptus torquata* Luehm and *Eucalyptus salmonophloia* F. Muell., to evaluate their cytotoxic activity against human colorectal cancer cell-line SW620 and MDA-MB-231 cancer cell lines. The study resulted in a significant dose-

dependent inhibition against the viability of the cancer cell lines, with the essential oils being more cytotoxic to SW620 colon carcinoma cells.

Besides the antiproliferative activity, eucalyptus essential oil possesses such a remarkable anti-inflammatory activity, which is why it has always been highly used to treat inflammatory symptoms and diseases as an alternative remedy (Nakamura et al., 2020). In fact, eucalyptol (1,8-cineole), the main component of eucalyptus oil, was proved to block arachidonic acid metabolism in blood monocytes from patients with asthma and inhibits lipopolysaccharide (LPS)-induced interleukin 1 beta (IL-1β) production by human monocytes, as well as suppresses the production of histamine, interleukin 4 (IL-4), and interleukin 13 (IL-13) (Nakamura et al., 2020). Moreover, it was praised for soothing and improving skin irritation symptoms of patients with atopic dermatitis (Nakamura et al., 2020).

The biological activities of eucalyptus essential oil are the reason behind its incorporation in numerous pharmaceutical products. Along with its characteristic smell, cosmetic and perfumery industries have also incorporated the essential oil in everyday products such as air fresheners, toothpastes, mouthwashes, detergents, disinfectants, soaps, skin cleansers and moisturizers (Barbosa et al., 2016).

1.2.3.2 Peppermint (*Mentha x piperita* L.)

Peppermint (*Mentha x piperita* L.) is a 30-90 cm tall herbaceous, rhizomatous, perennial, and aromatic plant that belongs to the *Lamiaceae* family. It is a hybrid of water mint (*Mentha aquatica*) and spearmint (*Mentha spicata*), and native to the Mediterranean region (Engels et al., 2006), naturalized in northern America, and cultivated in many countries around the world (McKay and Blumberg, 2006).

Figure 7. Peppermint plant (left) and peppermint leaf (right) (Mühlbauer and Müller, 2020)

Amongst the different mint species, peppermint is the most known and utilised in different applications. It has a long history of being used as a traditional phyto-medicine by civilisations as ancient as Egyptian, Greek, and Romans. Whether as brewed dried leaves or as an essential oil, its therapeutic values include treating ulcers, gastrointestinal complications, coughs, and colds. Its values extend to having analgesic effects as well as antiviral, antioxidant, and anticancer properties (Badea et al., 2019; Muntean et al., 2019). Peppermint (*Mentha x piperita L.*) leaves contain 0.5 - 4% of essential oil (Abdel-Wareth et al., 2019) that can be extracted by different methods (distillation, solvent extraction, extraction with supercritical gases, mechanical methods). They also contain flavonoids, tannins, polyphenol carboxylic acids, triterpene, tocopherols, carotenoids, and minerals, which contribute to the antimicrobial and antioxidant properties of peppermint (Muntean et al., 2019).

Terpenoids are the most abundant compounds found in the peppermint oil, and they're present as monoterpenes, hemiterpenes, or as sesquiterpenes and as their derivatives. These compounds are known to be active against bacteria and fungi (Ben Hsouna et al., 2019). Peppermint essential oil is mainly produced in the United States, India, Russia, and China (de Groot and Schmidt, 2016). It has cooling and vasoconstrictive properties giving it a broad spectrum of pharmaceutical applications, mainly for relieving muscle aches and spasms, treating headaches and toothaches, healing respiratory tract infections, soothing neuralgia, and stimulating immunity (de Groot and Schmidt, 2016; McKay and Blumberg, 2006). In addition, peppermint essential oil is applied in the food industry as a flavour in candies, ice cream, chewing-gum, chocolate, and many other food products. Also, thanks to its carvone content, *Mentha x piperita L.* essential oil is used in the cosmetic and perfumery industries for its refreshing smell and sensation in fragrances, toothpastes, mouthwashes, soaps, lotions, skin

products, shampoos, among others (de Groot and Schmidt, 2016). The main constituents of peppermint essential oil, extracted by distillation, are monoterpenes such as menthol (25%-78%) and mentone (14%-36%), followed by cineol (3.5-14%), menthylacetate (2.8%-10%), and isomenthone (1.5%-10%) (Abdel-Wareth et al., 2019). More monoterpene compounds can also be found such as menthofuran, limonene, pulegone, eucalyptol, and carvone (Muntean et al., 2019). Peppermint oil also contains phenolic compounds such as rosmarinic acid and various flavonoids mainly eriocitrin, luteolin and hesperidin (McKay and Blumberg, 2006).

According to Badea et al. (2019), nnumerous studies have reported the strong antibacterial activity of peppermint oil against *Enterococcus faecium*, *Salmonella choleraesuis*, *S. aureus*, and *B. subtilis*. As well as antifungal activity against *Aspergillus albus* and *dermatophytic* fungi. The study conducted by Badea et al. (2019) proved the antimicrobial effect of peppermint essential oil against four gram-positive bacterial strains, four gram-negative bacterial strains, and one fungal strain (*Candida parapsilosis*).

The high content of non-volatile phenolic compounds such as flavonoids, the monoterpenes, and the oxygenated sesquiterpenes content contribute significantly to the antioxidant and anti-inflammatory activities of *Mentha x piperita L*. (Alsaraf et al., 2021). A study conducted by Li et al. (2017) on the ethanol extracts of *Mentha x piperita* L. proved that the leaves extracts demonstrated an anti-inflammatory activity which was translated by the suppression of the production of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), nitric oxide (NO), and PGE-2, as well as an antioxidant activity that was demonstrated through a high scavenging activity. Moreover, the volatile compounds in peppermint essential oil, such as D-carvone and DL-limonene, can be considered as cytotoxic agents against various non-tumor and cancer cell lines (Alsaraf et al., 2021). The *in-silico* results of the study carried out by Alsaraf et al. (2021) showed that D-Carvone was observed to be most active against thyroid carcinoma cell line (8505C) and it might produce beneficial effects against numerous other types of cancer. On the other hand, DL-limonene was predicted to be most active against skin melanoma in addition to thyroid carcinoma, brain glioma, leukaemia, breast cancer, and pancreatic carcinoma.

1.2.3.3 Pine (*Pinus pinaster*)

The genus Pinus encompasses about 800 species worldwide, majorly found in the northern hemisphere (Kadri et al., 2015). *Pinus pinaster* is the most common pine genus spread in the Mediterranean region. Over 4 million hectares of *Pinus pinaster* forests are located in France, Italy, the Iberian Peninsula, and in western north African countries such as Tunisia, Algeria, and Morocco. This species is grown on mineral soil and is known to remarkably adjust to uncommon soils such as sandy, acidic, and indigent soils (Dob et al., 2005; Kadri et al., 2015). In addition, *P. pinaster* is adapted to moderate and habitual Mediterranean climate as it is a heliophile species and grows in slightly humid conditions, which consequently makes it very resistant to mild summer droughts but sensitive to frosty winters (Kadri et al., 2015). For this reason, this pine species has been cultivated as an exotic species in countries such as Portugal and France for more than 6 centuries (Tümen et al., 2018).

The best *Pinus pinaster* stands are found up to an altitude of 400 metres. They can reach over 800 meters, but the occasionally rough weather conditions of wind and snow lead to broken crowns as well as to deformed and poorly-developed pine trees. The trees themselves can reach a height of 30 meters and live to as long as 80 – 100 years seen that they regenerate easily through seeds (utad.pt).

Figure 8. *Pinus pinaster* trees (https://jb.utad.pt/especie/Pinus_pinaster)

Pinus pinaster Ait. is the most abundant conifer tree, dominating the Portuguese pine forests along with *Pinus pinea*, and is ranked third in forest species with largest occupied area

in mainland Portugal (Rodrigues et al., 2016). *P. pinaster* Ait. represents a column in the Portuguese national economy as it is used for construction, furniture, panels, and energy generation, while the resin is primarily destinated for manufacturing oils, varnishes, and adhesives (Ramos et al., 2022).

In terms of essential oils, pine oils have long been used in different industries such as cosmetics, food and beverages, and in the pharmaceutical field (Tümen et al., 2018). As a matter of fact, pine needles essential oil has been used for centuries in traditional medicine to treat several diseases including metabolic diseases, neurological pathologies, respiratory tract infections and cardiovascular diseases by lowering cholesterol levels and enhancing microcirculation (Gabaston et al., 2017; Ferreira-Santos et al., 2020; Fkiri et al., 2019). In addition, pine needles essential oil has potent antioxidant and anti-inflammatory activities seen that it is rich in phenolic acids, flavanols and flavonoids such as cinnamic acid, catechin, quercetin, and in mono-, sesqui- and diterpene hydrocarbons, especially α -pinene and β -caryophyllene (Gabaston et al., 2017; Ferreia-Santos et al., 2020; Ottavioli et al., 2008).

Figure 9. *Pinus pinaster* needles (https://jb.utad.pt/especie/Pinus_pinaster)

Pinenes such as α-pinene, β-pinene are highly found in pine extracts and essential oil, and are known for their interesting biological activities which makes them widely used in various applications. These phytochemicals are generally recognised as safe (GRAS), therefore, they are recognised by the FDA as compounds that can be used in food products (Dziedziński et al., 2021).

In 2007, Ahn et al. conducted a study on the addition of pine bark extract a to meat, and it resulted in the improvement of oxidative stability of cooked beef thanks to a highly-antioxidant compound called pycnogenol. In the same year, Choi et al. studied the addition of fermented pine needle extract syrup to bread, which resulted in the extension of its shelf-life by inhibiting the growth of aerobic bacteria and moulds on the bread surface.

Fkiri et al., (2019) reported that the essential oil of two varieties of *P. pinaster* inhibits the growth of Gram-positive bacteria such as *S. aureus* and *B. cereus*, while the Gram-negative strains of bacteria were more resistant as it was reported by Utakod et al. (2017). The study conducted by Fkiri et al., (2019) also reported the absence of growth inhibition of *Candida albicans*, *Aspergillus flavous*, and *A. niger* essential oils of *P. pinaster* did not inhibit the growth (Fkiri et al., 2019).

1.3 Use of plant volatiles and essential oils against foodborne pathogens

1.3.1 Foodborne pathogens

Since the old days till these modern days, foodborne infections and intoxications have been considered an alarming threat to the public health, food industries, and economy (Ji et al., 2021).

Foodborne pathogens are pathogenic microorganisms (bacteria, viruses, fungi, and some parasites) that cause contamination in food products by releasing their toxins and are, consequently, responsible of human infections and intoxication due to the consumption of contaminated food products (Campini et al., 2021).

Bacteria represent the main causing agent of food contamination and human infection diseases related to the consumption of contaminated food, by 66% (Abebe et al., 2020). Symptoms of food poisoning are usually gastrointestinal and are characterized by vomiting, diarrhea, nausea, abdominal spasms, loss of appetite, and sometimes severe complications. The most common bacteria strains responsible of foodborne contamination and diseases are *S. aureus*, *E. coli*, *Salmonella* spp., *Campylobacter* spp., and *L. monocytogenes* (Abebe et al., 2020).

Fungi like *Aspergillus* spp., *Fusarium* spp., and *Penicillium* spp. are frequent and cause spoilage in numerous food products as well as release their toxins, which distort the

organoleptic properties and can potentially lead to food poisoning (Ji et al., 2021). Moreover, the presence of the toxins released from these fungi, such as aflatoxins, are considered a health concern not only for their infectiousness, but also for their carcinogenic effect (Juárez et al., 2016).

Other microorganisms such as viruses (4%) and parasites (4%) are also considered foodborne pathogens, but not as frequent as bacteria (Abebe et al., 2020).

1.3.2 Use of plant volatiles and essential oils as food preservatives

Following the demand for safe and high-quality food, the food industry has conducted considerable amounts of studies on methods to prevent spoilage and foodborne diseases, seen that microbial spoilage is amongst the major concerns in food industry, and it can occur during production, processing, distribution, and preparation of food products (Basak et al., 2021). The drawbacks of this problem reach public health and economy, calling for more innovative, effective, and eco-friendly preservation methods. One of the main and predominant methods for food preservation is the application of chemical preservatives because of their low cost and availability. However, on the long-term, chemical food preservatives display a serious impact on human health (carcinogenic, teratogenic, and toxic effects) and on the environment (Basak et al., 2021).

During the recent years, natural and organic substitutes for food preservation have been put under the spotlight for their sustainability and low toxicity on human health and on the environment (Campini et al., 2021). The volatile bioactive compounds in essential oils present a remarkable potential as antimicrobial and antioxidant in active packaging and in extending food shelf life, representing alternatives to synthetic molecules (Ben Derbassi et al., 2022).

Since they have been designated by the Food and Drug Administration (FDA) as Generally Recognized as Safe (GRAS), volatiles were incorporated in food products as food preservatives. Their ability to extend food shelf life is due to the presence of a wide range of bioactive compounds that suspend and halt the biological and biochemical process of microbial multiplication and growth, as well as eliminate free radicals and oxidative stress (Campini et al., 2021). However, the incorporation of essential oils in food products needs rigorous studies and meticulous knowledge about their properties and effects, such as the minimum inhibitory concentration (MIC), the mode of action, the range of target organisms, and the synergy between the volatile compounds and the food matrix components (Hyldgaard et al., 2012).

A study by Campini et al. (2021) was conducted on the use of encapsulated essential oils or their major isolated compounds as food preservatives. *Cinnamomum cassia* essential oil, eugenol, and linalool were encapsulated in polylactic acid to evaluate and compare their antimicrobial efficiency against some bacterial strains: *E. coli*, *S. aureus*, *L. monocytogenes*, and *Salmonella enterica subsp. enterica serovar Choleraesuis* (Salmonella). The encapsulation in polymeric walls promotes the protection of essential oil compounds against external agents and allows their controlled release. The results showed that the encapsulated active compounds of the three essential oils promoted higher stability and bacterial activity, suggesting applying the capsules in a food packaging system to assess the bacterial activity *in loco*.

Another experiment by Oral et al. (2009) investigated the use of oregano essential oil in vapor phase to preserve overwrap-packed chicken drumsticks. The volatile compounds were sprayed on absorbent pads, which resulted in a decrease of microorganism concentration while preserving sensory properties, and as a conclusion, preserving the chicken for two extra days. Also, Tunc et al. (2007) introduced a film paper imbibed with volatile compounds of carvacrol, allyl isothiocyanate, and cinnamaldehyde in a hermetically closed jar that contains an uncovered Petri dish inoculated with spores of *Penicillium notatum*. The experiment resulted in an inhibition of *P. notatum*'s growth (Lucera et al., 2016).

Still, some drawbacks of essential oils and volatiles are known, namely their effect on aroma and taste in the foods packagin they are used in. This poses a challenge for the insdustry, namely to use natural technologies for food preservation while educing their drawbacks. This work's objective is to help improve some knowledge on the use of volatiles as microbial preservatives.

Objectives

General objective

This work aims at extracting plant volatiles to use them as airborne food preservatives. These volatile compounds will be subject to *in vitro* assays in order to confirm their bioactivity and lack of toxicity.

Specific objectives

- Extraction of essential oils from three different aromatic plants
- Optimization of the two extraction methods using RSM
- Chromatographic identification of the volatile compounds
- *In vitro* analysis of the bioactivities of the volatiles

Methods

a) Implementation of extraction procedures of the most promising volatiles by hydro distillation and by microwave-assisted hydro distillation extraction.
b) *In vitro* antioxidant assays (DPPH, reducing power, and cellular antioxidant assays).
c) Microdilution method for antimicrobial assays performed on the volatiles to confirm their antibacterial and antifungal activities, following Pedrosa et al., 2021.
d) Cytotoxicity studies using AGS, CaCo2, MCF-7, and NCI-H460 cell lines, as well as PLP2 cells, following Xavier et al., 2021.
e) Anti-inflammatory activity assessment using RAW 264.7 murine macrophages, following Xavier et al., 2021.

2. Materials and Methods

2.1 Plant material collection and preparation

Eucalyptus globulus leaves were collected near Águeda, Portugal in October 2020. The samples were dried and packed in a carboard box away from light and moisture at room temperature until further analysis.

Both *Pinus pinaster* and *Mentha x piperita* L. leaves were acquired from "Cantinho das Aromáticas", an enterprise based in Porto, Portugal in October 2021. *Pinus pinaster* needles were packed in a carboard box upon arrival and left to dry naturally at room temperature and turned over every 2 days to release moisture. The samples were kept shielded from light and humidity until further analysis.

Mentha x piperita L. leaves were received dry and stored in a cardboard box. The box was kept protected from light and humidity in room temperature until further analysis.

After drying, the leaves were grinded using a shredder (Moulinex, La Moulinette 1, 2, 3 AD560120 800 W, New Borg El Arab City, Alexandria, Egypt) and then sieved to 3 particles sizes using 3 different sieves (Impact Test Equipment Ltd., Stevenston, Ayrshire, Scotland) with diameters of 3, 2, and 1 mm, by placing the sieves on top of each other starting with the largest diameter down to the smallest. The grinded samples were packed in separate clear plastic bags and stored in a cardboard box away from light and humidity at room temperature until the extraction process.

2.2. Materials and reagents

All materials were acquired from scientific retailers and all reagents beyond also being bought from scientific retailers were at least of P.A. purity unless stated for use in HPLC or GC, in which they were of higher purity.

2.3 Optimization of extraction methods

Two extraction techniques were compared, namely hydro distillation and microwave assisted extraction with prior optimization of these techniques by varying three parameters of each.

2.3.1 Hydro distillation

Hydro distillation (HD) is one of the oldest and most known traditional methods mainly used for the extraction of E.Os (essential oils) from plants, dating back 5000 years (Park & Tak 2016). In this technique, three major physiochemical processes are involved: hydro-diffusion, hydrolysis, and heat decomposition (Pan et al., 2019). Hot boiling water and steam are the primary media for releasing and transporting E.Os and volatiles from the ruptured cells into the steam. The vapor mixture is circulated along the Clevenger tubes and the indirect cooling in the condensing tube allows the condensation of the mixture, which eventually permits the separation of the E.O and water in the receiver tube. To maintain a constant level, water is always refluxed into the flask via the return tube.

All 3 E.Os from the 3 plants were extracted using a Clevenger apparatus (Vilabo, Marinha Grande, Portugal) joined to a 1000 mL distillation flask. The extraction conditions used were the ones obtained from the Design-of-Experiments using the Response-Surface-Methodology.

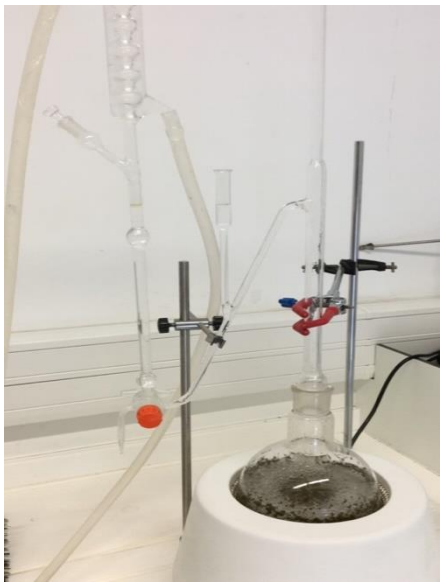

Figure 10. Clevenger apparatus used for hydro distillation

The Clevenger system was connected to a water-cooling equipment (Huber Minichiller 300 OLÉ, Huber kältemaschinenbau, Offenburg, Germany) that recycles and cools down the water outflow to 10°C and recirculates it back into the Clevenger to increase heat transfer and improve the condensation process to obtain a higher yield in E.O.

2.3.1.1 Optimization of the hydro-distillation with Response Surface Methodology

Design of experiments (DOE) was done to optimize the extraction parameters of the hydro-distilled E.Os from the 3 plants following the Box-Behnken design using Design-Expert v.11 software (Stat-Ease, Minneapolis, MN, USA). By maintaining a constant water volume of 1000 mL, a three-factor three-level design was performed. The three independent extraction parameters selected for this optimization motive were: Time (A), Particle size (B), and Solid/Liquid (S/L) ratio (C). These factors were used for optimization, with three levels each: Time (A) (180, 225, and 270 min), Particle size (B) (1, 2, and 3 mm), and S/L ratio (C) (10g/L, 30g/L, and 50g/L). The response used for optimization was the amount (mg) of essential oils obtained (R1) (Table 1).

2.3.2 Microwave-assisted hydro-distillation

Microwave-assisted hydro distillation is one of the most widely used modern techniques for extracting essential oils while saving time and energetic costs. At atmospheric pressure, the generated microwaves produce heat and elevate pressure inside the plant particles, leading to the softening of the plant tissue and the increase of heat transfer as well as the mass transfer, which allows the penetration of water in the sample. The engorgement of cells induces the bursting or rupturing of the cell wall and, eventually, the release of the E.O and volatiles into water vapour (Moradi et al., 2018).

For an accelerated extraction of essential oils, microwave-assisted hydro distillation (MAHD) was performed using (Microwave Synthesis System, NuWay-uno, Nutech Analytical Technologies Pvt. Ltd, Kolkata, India). The MAHD system was coupled to a 1000 mL flask located inside the microwave oven that provides heat for the E.O extraction and is joined to a Clevenger apparatus outside the microwave machine for condensation and oil-water separation purposes.

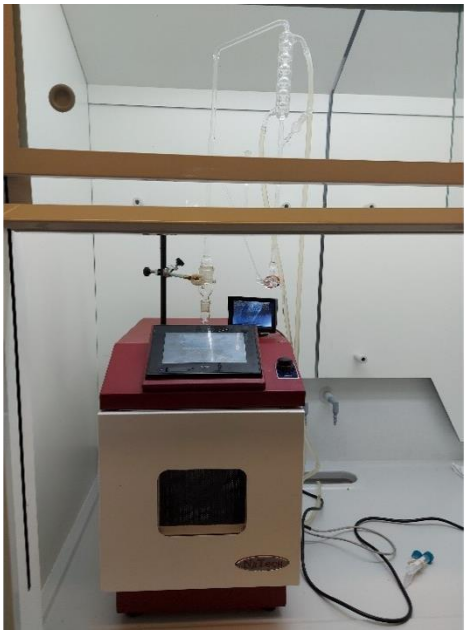

Figure 11. Microwave oven used for the MAHD

2.3.2.1 Optimization of microwave-assisted hydro distillation extraction with Response Surface Methodology

After performing a response surface analysis on the E.O yields extracted through hydro distillation from the 3 plants, an observation on the possibility of using a higher S/L ratio was made, thus, 3 different solid/liquid concentrations were implemented (40g/L, 70g/L, and 100g/L) in a constant volume of 1000 mL of distilled water. The extractions were set up for periods of time varying between 20, 40, and 60 minutes for all of the 3 plants, counted after the first drop of essential oil was obtained, at a constant temperature of 110°C and at an interval of 1500 – 1700 RPM of agitation. Three different power levels were also selected for this extraction (200, 400, and 600 W). The particle size chosen was 1 mm as it has proven to result in the highest E.O during the hydro distillation process. These parameters can be seen in Table 2. The DOE with the three independent extraction parameters selected for this optimization motive were: Time (A), Power (B), and S/L (C) using these following factors with three levels: Time (A) (20, 40, and 60 min), Power (B) (200, 400, and 600 W), and S/L ratio (C) (40g/L, 70g/L, and 100g/L).

2.3.3 Determination of yield in essential oil

For both extraction methods (HD and MAHD), the extracted yield in essential oils from the 3 plants was considered the response (R) and calculated as the difference between the vial containing the essential oil and the empty vial. The results were expressed in mg.

2.4 Gas chromatography–mass spectroscopy (GC–MS) analysis of volatile molecules

To analyse the E.Os, a GC-2010 Plus gas chromatography system (Shimadzu) with an AOC - 20iPlus automatic injector (Shimadzu) and a mass spectrometry detector with a SH - RXi - 5ms column (30 m 0.25 mm 0.25 m; Shimadzu, USA) was used (Figure 3). The following conditions were met during the process: After 4 minutes at 40 ℃, the temperature was increased 3 ℃/min to 175 ℃/min, then 15 ℃/min to 300 ℃/min and maintained for 10 minutes. For this operation, the carrier gas was helium due to being an inert gas.

Helium was used with a linear velocity of 30 cm/s set. The sample volume injected was 1 µL of the optimal point extraction for each E.O, with a split ratio of 1:10. The temperature of the injector was set to 260 ℃, for the transfer line to 280 ℃, and the ion source at 220 °C. The scanning range was 35-500 u, and the scan time was 0.3 s. The ionization energy was 70 eV. The compounds were identified by comparing the obtained spectra to those from the NIST17 mass spectral library and calculating the linear retention index (LRI) based on retention times obtained for a mixture of n-alkanes (C8 - C40, ref. 40147- U, Supelco) analysed under identical conditions. The relative peak area values based on the total ion current (TIC) values were used to quantify the various compounds. The analyses were carried out in triplicate, with the results reported as mean ± standard deviation (SD).

Figure 12. GC-MS equipment used for the analysis of volatile molecules

2.5 Bioactive properties' evaluation

The analysis of the E.Os' biological activities were performed for the purpose of evaluating their antioxidant, antimicrobial, antiproliferative, and anti-inflammatory potential to determine their aptitude to be used in food preservatives.

2.5.1 Antioxidant activity

2.5.1.1 DPPH (2,2-diphenyl-1-dicrylhydrazyl)

The antioxidant activity of the 3 E.Os (*E. globulus*, *M. piperita*, and *P. pinaster*) extracted using HD and MAHD was determined based on the ability of E.Os to donate hydrogen atoms, which leads to the decolorization of a methanol solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) when in contact with antioxidants from violet/purple color to shades of yellow (Rahman et al., 2015).

100 mg of the different essential oils (HD and MAHD) were diluted in 1 mL of methanol to prepare the mother solution with a concentration of 100 mg/mL.

As the assay was done in triplicate, 60 µL of the mother solution were poured into each one of the first 3 wells on the same line of a 96-well plate, representing the initial solution, followed by the addition of 30 µL of methanol. A successive dilution was performed. Afterwards, 270 µL of a methanolic solution containing a concentration of 6×10^{-5} mol/mL of DPPH radicals was added to all wells. The microplate was kept in the dark for 60 min to trigger the reaction, and then the absorbance was read at 515 nm using a microplate reader (SpectroStar nano, Labtech, Ortenberg, Germany) to determine EC_{50} value.

The assessment of the antioxidant power was expressed as a percentage of inhibition of the DPPH radical using the following formula:

$$\%\ inhibition = \frac{Ab - As}{Ab}\ x\ 100$$

where Ab is the absorbance of the blank and As is the absorbance of the sample.

2.5.1.2 Reducing Power

The antioxidant activity of the E.Os of the three plants extracted using HD and MAHD was determined based on the ferric iron Fe^{3+} reducing capacity as described by Martins et al., (2015). This method is based on the capacity to convert Fe^{3+} into Fe^{2+}.

In an Eppendorf tube, 20 mg of E.O were weighed to perform successive dilutions using methanol, starting with 20 mg/mL down to 0.0195 mg/mL. After adding 0.5 mL of sodium phosphate buffer solution (200 mmol/L; pH=6.6), 0.5 mL of potassium ferricyanide (1% w/v) was also added. The mixtures were incubated at 50° C for 20 min in order to promote the reaction. Then, 0.5 mL of 10% w/v trichloroacetic acid was added to terminate the reaction. 0.6 mL of the mixture was poured into a 48-well plate, and the procedure was done in duplicate with 0.6 mL of distilled water and 120 µL of iron chloride ($FeCl_3$) (0.1% w/v), with the last line of wells representing the control.

The absorbances were then measured at 690 nm using a microplate reader (SpectroStar nano, Labtech, Ortenberg, Germany).

The antioxidant power of the essential oils was estimated in comparison with Trolox as standard. The results were obtained by determination of the $EC_{0.5}$ which displays the concentration that quenches 50 percent of the oxidants.

2.5.1.3 Cellular antioxidant activity (CAA)

The particularity of the CAA assay lies in the determination of the intracellular antioxidant activity of the extracts *in vitro*. on living cells, which allows a better understanding of oxidation mechanisms.

As proposed by Wolfe & Liu (2007), the cell line used for the assessment of the cellular antioxidant activity (CAA) was the RAW 246.7 mouse macrophages (DMSMZ - Leibniz - Institut DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) maintained in an incubator at 37°C, 5% CO_2, with a humidified atmosphere, and supplemented with Dulbecco's Modified Eagle Medium (DMEM) enriched with L-glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml), fetal serum bovine (10%) and non-essential amino acids (2 mM).

The extracts (8 mg) were dissolved in distilled water (1 mL) to obtain a mother solution with a concentration of 8 mg/mL. Successive dilutions were carried out using 2',7'-dichlorodihydrofluorescein (DCFH), which is a fluorogenic synthetic molecule used for the detection of oxidative stress by being oxidized under the action of ROS, prepared with ethanol and diluted with Hanks' Balanced Salt Solution (HBSS) (50 μM), obtaining the concentrations to be tested (32.5 - 2000 μM).

After reaching confluence in the flask, the mouse macrophages were detached from the bottom using a cell scraper, and the content was transferred to a falcon tube to be centrifuged at 1200 RPM for 5 minutes. The supernatant was disposed of, and an amount of new medium was added in proportion to the size of the pellet. A solution was prepared with a cell density of 70.000 cells/mL. 300 μL of the prepared solution were poured into the blank microplates with clear bottom (SPL Lifesciences) and incubated for 48h.

The medium was eliminated after the incubation period, and the cells were washed with HBSS (2x, 100 μL), treated with different extract concentrations (200 μL; 32.5 - 2000 μM) and then incubated again for 1 hour. After that, the cells were washed twice with 100 μL HBSS, and treated with 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH) solution (100 μL; 600 μM) to induce an intracellular oxidative stress causing the formation of free radicals.

The fluorescence emitted by 2′,7′-dichlorofluorescein (DCF), product of the oxidation of DCFH by ROS, was measured at an exciting wavelength of 485 nm and an emitting wavelength of 538 nm every 5 minutes for 1 hour using a Biotek FLx800 microplate reader. Quercetin was used as a positive control, and dichlorohydroflurescein and DMEM culture medium were used as negative controls. The percentage (%) of reduction that expresses the CAA was calculated using the following formula:

$$\%\, reduction = 1 - \frac{Antiox\ (sample)}{Antiox\ (control)}\ x\ 100$$

2.5.2 Antimicrobial activity

2.5.2.1 Antibacterial activity

The clinical isolates used in this study were isolated and obtained from hospitalized patients at the Unidade Local de Saúde do Nordeste (Bragança, Portugal) and Centro Hospitalar de Trás-os-Montes and Alto Douro (Vila Real, Portugal).

The bacterial strains used were a total of five Gram-negative bacteria: *Escherichia coli* (isolated from urine), *Proteus mirabilis* (isolated from skin wound exudate), *Klebsiella pneumoniae* (isolated from urine), *Pseudomonas aeruginosa* (isolated from expectoration), and *Morganella morganii* (isolated from urine), and three Gram-positive bacteria: *Enterococcus faecalis* (isolated from urine), *L. monocytogenes* (isolated from cerebrospinal fluid) and methicillin-resistant *Staphylococcus aureus* (MRSA) (isolated from expectoration). Before analysis, all microorganisms were incubated for 24 hours at 37 ℃ in a fresh medium that ensures an exponential growth phase for the bacterial cells.

The extracts were also tested against a total of eight food contaminants (bacteria) that were purchased at Frilabo based in Porto, Portugal. Five Gram-negative bacteria, namely *Enterobacter cloacae* (ATCC 49741), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 9027), *Salmonella enterica* subsp (ATCC 13076), *Yersinia enterocolitica* (ATCC 8610) and three Gram-positive bacteria, namely *B. cereus* (ATCC 11778), *L. monocytogenes* (ATCC 19111) and *S. aureus* (ATCC 25923). The bacterial strains were incubated at 37 ºC an appropriate fresh medium for 24 h before analysis to maintain the exponential growth phase.

Following the procedure described by Pires et al., (2018), in a sterile Schott bottle, 50 mL of Muller Hinton Broth (MHB) medium and 250 µL of Tween 80 (MHB at 0.5% Tween 80) were combined to make the culture medium. In a sterile Schott bottle, 30 mL of MHB medium was added, followed by 200 µL of inoculum (standardized at 1.5 x 10^6 Colony Forming Unit (CFU)/mL). Concentrations of 5%, 2.5%, 1.25%, 0.625%, 0.313%, 0.156%, 0.078%, 0.03%, and 0.01% were used to prepare sample dilutions. In the first well of a 96-well microplate, 180 µL of MHB medium with Tween 80 and 20 µL of sample were added in duplicate. 90 µL of MHB medium containing Tween 80 was added to the remaining wells.

The samples were then serially diluted to obtain concentration ranges going from 5% to 0.01%. 100 µL of inoculum (standardized at 1.5 x 10^5 Colony Forming Units (CFU)/mL) were poured into the wells. Two negative controls, one with MHB and Tween 80, and another one with the extract, and two positive controls, one with MHB and Tween 80 and each inoculum, and one with culture medium, antibiotics, and bacteria, were prepared. For all bacteria tested, ampicillin and streptomycin were used, and methicillin was used for *Staphylococcus aureus*.

The microdilution method and *p*-iodonitrotetrazolium chloride (INT) dye were used to detect the minimum inhibitory concentration (MIC) on all bacteria, allowing a colorimetric measurement as described by Pires et al., (2018), with a few adjustments. For this, 40 µL of 0.2 mg/mL p-iodonitrotetrazolium chloride (INT), which is an indicator dye used as microbial growth indicator, were added into the wells, and then the microplates were incubated at 37 °C for 30 minutes. The minimum inhibitory concentration (MIC) of the samples was defined as the lowest concentration required to inhibit bacterial growth, as indicated by a change in colour from yellow to pink upon capturing two electrons if the microorganisms are viable (Stiefel et al., 2016).

For the determination of Minimum Bactericidal Concentration (MBC), 10 µL of liquid from each well that showed no change in colour was plated on solid medium, Blood agar (7% sheep blood) and incubated at 37 °C for 24 hours. The lowest concentration that yielded no growth determined the MBC, defined as the lowest concentration required to kill bacteria.

2.5.2.2 Antifungal activity

Antifungal activity was performed as described by Heleno et al., (2013) with some rearrangements. The fungal strains used were obtained from Frilabo, Porto, Portugal, and they were: *Aspergillus fumigatus* (ATCC 204305) and *Aspergillus brasiliensis* (ATCC 16404). The fungi were maintained on malt agar and the cultures were stored at 4 °C before being transferred toa new medium and incubated at 25 °C for 72 hours. Tostudy the antifungal activity, fungal spores were washed from the surface of agar plates with 0.85% sterile serum containing 0.1% Tween 80 (v/v). The spore suspension was adjusted with sterile saline to a concentration of approximately 1.0 x 10^5 to a final volume of 100 µl per well. Samples were first dissolved in Muller-Hinton Agar (MHA) medium containing Tween 80. Afterwards, 10 µL of each sample

was added in duplicate to the first well (96-well microplate) followed by 190 µL of malt extract medium (MEB).

90 µL of MEB was added to the remaining wells. The samples were then serially diluted to obtain a concentration range of 2.5% to 0.01%. Minimum inhibitory concentrations (MICs) were determined by serial dilution using 96-well microplates. Minimal Fungicidal concentration (MFC) was determined by serially subculturing 2 µL of the extracts dissolved in the medium and inoculated into microplates containing 100 µL of MEB per well for 72 hours followed by an incubation at 26 °C for 72 hours. The minimum fungicidal concentration was defined as MFC and indicates 99.5% killing of the original inoculum. The commercial fungicide ketoconazole (Frilabo, Porto, Portugal) was used as a positive control.

2.5.3 Antiproliferative Activity

The cell lines used to evaluate the antiproliferative activity of the tested extracts, were human cancer cell lines, namely AGS (gastric adenocarcinoma), CaCo2 (colon adenocarcinoma), MCF-7 (breast adenocarcinoma), and NCI-H460 (large-cell lung carcinoma). Non-tumor cell line PLP2 (primary pig liver culture) was also used to rule out cytotoxicity towards normal cells. AGS and CaCo2 cells were purchased from the European Collection of Authenticated Cell Cultures (ECACC), while MCF-7 and NCI-H460 cells were provided by Leibniz - Institute DSMZ. The PLP2 culture was established in the laboratory using porcine liver tissue in order to obtain tissue explants for the proliferation of non-tumor liver cells.

All cell lines, were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, glutamine (2 mM), penicillin (100 U/mL) and streptomycin (100 mg/mL). As for the Vero cells, they were maintained in DMEM medium supplemented with fetal bovine serum (10%), glutamine and antibiotics. The culture flasks were incubated at 37 °C and with 5% CO_2, under a humid atmosphere to mimic physiological conditions. When the confluence reached 70% – 80%, the cells became ready to be used for further analysis.

To obtain the stock solutions with a concentration of 8 mg/mL, 8 mg of the extracts were dissolved in 1 mL of distilled H_2O. From that, successive dilutions were performed to get the concentrations that will be tested (0.125 - 8 mg/mL). 10 µL of the previously made extractions were added to the 190 µL of the cell suspension of the cell lines tested in 96-well

microplates, after making sure the cells were well adhered to the wells. The microplates were incubated at 37 ºC and with 5% CO_2, in a humid atmosphere for 72 hours. All cell lines were tested at a concentration of 10.000 cells/well.

After the end of the incubation period, previously cooled trichloroacetic acid (TCA) (10% w/v; 100 µL) was added to stop the reaction, and the plates were incubated for at 4 ºC for 1 hour. Afterwards, they were washed with distilled water and left to dry. Then, a Sulforhodamine B (SRB) solution (0.057%, m/v; 100 µL) was added, and the plates were left to stand at room temperature for 30 minutes to ensure the adherence of SRB on the tumoral cell lines. To remove non-adhered SRB, the plates were washed three times with a solution of acetic acid (1% v/v) and placed to dry. At the end, the adhered SRB was solubilized with Tris (10 mM, 200 µL) and the absorbance was read in the Biotek ELX800 microplate reader at 540 nm. The results were expressed in terms of the concentration of extract that inhibits cell growth by 50% - GI_{50}. The positive control was ellipticine.

2.5.4 Anti-inflammatory activity

8 mg of extracts were dissolved in 1 mL of H_2O to obtain a final concentration of 8 mg/mL from which successive dilutions were made, obtaining the concentrations to be tested (0.125 - 8 mg/mL).

For this assay, the RAW 264.7 mouse macrophage cell line (DMSMZ - Leibniz - Institut DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) was grown in DMEM medium, supplemented with fetal serum (10%), glutamine, and antibiotics, and incubated at 37 ºC and with 5% CO_2 and under a humid atmosphere.

Macrophages were detached to prepare a cell suspension, from which an aliquot (300 µL) with a cell density of 5 x 10^5 cells/mL and with a proportion of dead cells below 5% according to the Trypan blue exclusion test, was placed in each well. To ensure a high confluence and an adequate multiplication of the cells, the microplate was incubated for 24 hours at 37 ºC and with 5% CO_2 and under a humid atmosphere. After 24 hours, the different concentrations of the samples (15 µL, 0.125 - 8 mg/mL) were used to treat the cells, followed by an incubation of 1 hour, with the range of concentrations tested being 6.25 - 400 µg/mL.

To stimulate the anti-inflammatory response in the cells, 30 µL of the liposaccharide solution - LPS (1 mL/mL) were added, and the microplates were placed in the incubator for

extra 24 hours. Dexamethasone (50 mM) was used as a positive control and samples in the absence of LPS were used as a negative control.

Quantification of nitric oxide (NO) was performed using a Griess reagent system kit (nitrophenamide, ethylenediamine and nitrite solutions) and through the nitrite calibration curve (100 mM sodium nitrite at 1.6 mM) prepared in a 96-well plate.

Reading absorbances at 540 nm (ELX800 Biotek microplate reader, Bio-Tek Instruments, Inc., Winooski, VT, USA) and comparing them to the standard calibration line were used to calculate the amount of NO produced. The results were calculated through the graphical representation of the percentage of inhibition of nitric oxide production versus the sample concentration and expressed in relation to the concentration of each of the extracts that causes the 50% inhibition of nitric oxide production - IC_{50}.

2.6 Statistical analysis

Throughout the whole document, all data was expressed as mean ± standard deviation (SD). An analysis of variance (ANOVA) was used to analyse the samples, relying on a Tukey's test (equal variances) or a Tahmane T2 (unequal variances) for post-hoc classification in analysis involving three factors, while samples with two factors were compared using a Student's T-test. For all statistical analysis the significance was set at 0.05.

3. Results and Discussion

3.1 Optimization of essential oil extraction using hydro distillation

RSM was applied to investigate the effect of the 3 independent factors (time, particle size, and S/L ratio) on the extraction process of the 3 E.Os from *E. globulus*, *M. piperita*, and *P. pinaster* using HD. The RSM consisted of 17 experimental runs performed using the Box-Benhnken model for the response, being the E.O yield (mg) the response used to calculate the optimal points. The runs, independent factors, and response are shown in Table 1.

The *E. globulus* E.O yield ranged from 11.8 to 670.4 mg, whereas for *M. piperita* the yield ranged from 6.5 - 327.4 mg. The lowest E.O yields resulted from *P. pinaster*, ranging from 2.8 - 54 mg.

The optimal point represents the combination of the three independent factors (time - A, particle size - B, and S/L ratio - C), that yields the highest mg of essential oils. Thus, the optimal time of 256.77 min, 264.194 min, and 233.784 min, and at a ratio of 48.5782 g/L, 47.4409 g/L, and 49.4168 g/L for *E. globulus*, *M. piperita*, and *P. pinaster* respectively, as well as in an optimal particle size of 1 mm for the 3 plants.

In the aim to evaluate the significance of the model and of all the terms that constitute it, as well as to evaluate the lack-of-fit, an Analysis of variance (ANOVA) was employed. Only the terms with a *p*-value lower than 0.05 were used in the models' construction seen that the lower the *p*-value is, the higher the statistical significance. To assess the adequacy of the polynomial equation of the response, the coefficient of determination (R^2), adjusted coefficient of determination (R^2_{adj}), and adequate precision were interpreted. Lack-of-fit should be nonsignificant (*p*-value ≤ 0.05) because it measures the quality of the models' fit to the experimental data.

Table 1. Experimental runs and responses obtained under the extraction conditions defined by the Box-Behnken design for the extracted essential oil yield from *E. globulus*, *M. piperita*, and *P. pinaster* using HD, and statistical information of the models' fitting

Runs	**Independent Factors**			**Response: E.O. yield (mg)**		
	A Time (min)	B Particle size (mm)	C S/L ratio (g/L)	R_1 *E. globulus*	R_1 *M. piperita*	R_1 *P. pinaster*
1	180	1	30	293.5	139.8	32
2	225	2	30	237.6	99.2	19.2
3	225	2	30	332.9	114.5	20.7
4	270	3	30	226.7	38	5.2
5	225	3	10	11.8	6.5	9
6	225	2	30	240.6	120.9	22.1
7	225	2	30	276.6	110.6	23.5
~~8~~	225	1	10	17.2	~~25.1~~	4.5
9	270	2	50	656.8	220	50.6
10	225	3	50	296.8	147.6	49.6
11	180	3	30	256.4	96.5	8.6
12	225	2	30	290	159.7	10.5
13	180	2	10	27	24.4	2.8
14	270	1	30	319	183.3	43.7
15	225	1	50	670.4	327.4	27.4
16	270	2	10	23.5	30.2	5.9
17	180	2	50	516.8	214.9	54
Statistical data						
Model *p*-value				< 0.0001	0.0002	0.0080
Model F-value				53.74	23.47	7.27
Lack-of-fit				0.3702	0.0893	0.0678
R^2				0.9857	0.9679	0.9034
R^2_{adj}				0.9674	0.9267	0.7791
Adequate precision				23.1798	16.9288	8.8985
Coefficient of variance (%)				8.33	6.34	32.79

Figure 4 shows the 3D response surface plots that combinate the 3 factors used in the response, in this case pertaining to *E. globulus* E.O yield using HD. This optimal point was achieved by applying a square root transformation which rendered a significant quadratic

function that showed a non-significant lack of fit: $R_{E.\ globulus} = 16.55 + 0.2841A - 1.68B + 9.25C - 0.4275AB + 0.8243AC - 1.97BC + 0.9503A^2 – 0.9976B^2 - 2.92C^2$. Transformations are used to improve the fitting equations and reduce noise and lack of fit. For each optimization plot, the factor not shown is fixed at the optimal value.

The factors were varied for the model to obtain 17 runs considered as combinations that were carried out to fit the second order polynomial equation model for each plant:

$R_{E.\ globulus} = 16.55 + 0.2841A - 1.68B + 9.25C - 0.4275AB + 0.8243AC - 1.97BC + 0.9503A^2 - 0.9976B^2 - 2.92C^2$

$R_{M.\ piperita} = 4.78 - 0.0501A - 0.5211B + 1.24C - 0.3004AB - 0.0489AC + 0.1569BC + 0.0979A^2 - 0.2891B^2 - 0.5408C^2$

$R_{P.\ pinaster} = 19.20 + 1A - 12.24B + 17.51C - 3.78AB - 1.62AC + 9.25BC - 3.4A^2 + 6.57B^2 + 12.53C^2$

The emphasis on conducting an optimization study originates from the fact that, generally, the optimal combination of extraction parameters for each sample differs, showcasing the influence of each plant's native characteristics on the technique used for the extraction of E.Os.

As shown in Figure 13, the 3D response surface plots were constructed to visually interpret the effect of the combination of the 3 factors on the response, being the extracted *E. globulus* E.O yield, using HD, by applying a square root transformation which gave a quadratic/linear behaviour that showed a non-significant lack of fit.

By fixing the S/L ratio (C) at its optimal value, the effect of time (A) and particle size (B) on the E.O yield was highlighted, being the yield is highly promoted by time (A), with an increase ranging between 250 – 270 min, precisely 257 min, followed by a noticeable influence of particle size on the obtained E.O yield, being 1 mm, as shown in the left-hand side plot of Figure 4.

A higher E.O yield was also sought at higher S/L ratio, which is revealed through fixing particle size (B) at its optimal value. The variables S/L ratio (C) and time (A) interacted remarkably well and had a notable influence on the E.O yield. In fact, a maximum S/L ratio of 50 g/L and a time of 257 min collaborated in the obtainment of a high yield in E.O. The effect of S/L ratio (C) on the response was also highlighted through the interaction of both particle

size (B) and S/L ratio (C), for a higher yield was indeed obtained at a S/L ratio of 50 g/L and a particle size of 1 mm. Overall, the S/L ratio is quite a difficult factor to work with due to it being virtually impossible to obtain a curve or a plateau to determine the optimal point. This occurs due to the addition of more raw material always translating to higher yields, although the physical extraction not being possible over a specific threshold. With all 3 factors set at their optimal values the predicted yield in E.O predicts a yield of 679.437 mg, which is higher than the highest experimental runs that achieved 670.4 mg (Figure 14).

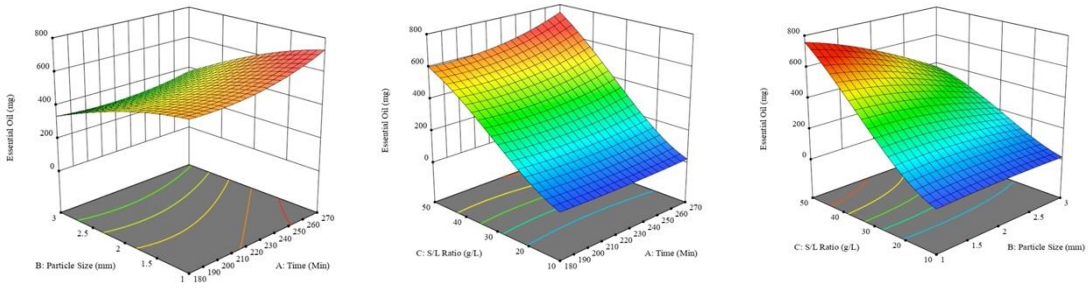

Figure 13. 3D response surface plots for the optimal point of *E. globulus* E.O extraction using HD. A-Time, B-Particle size and C-S/L ratio

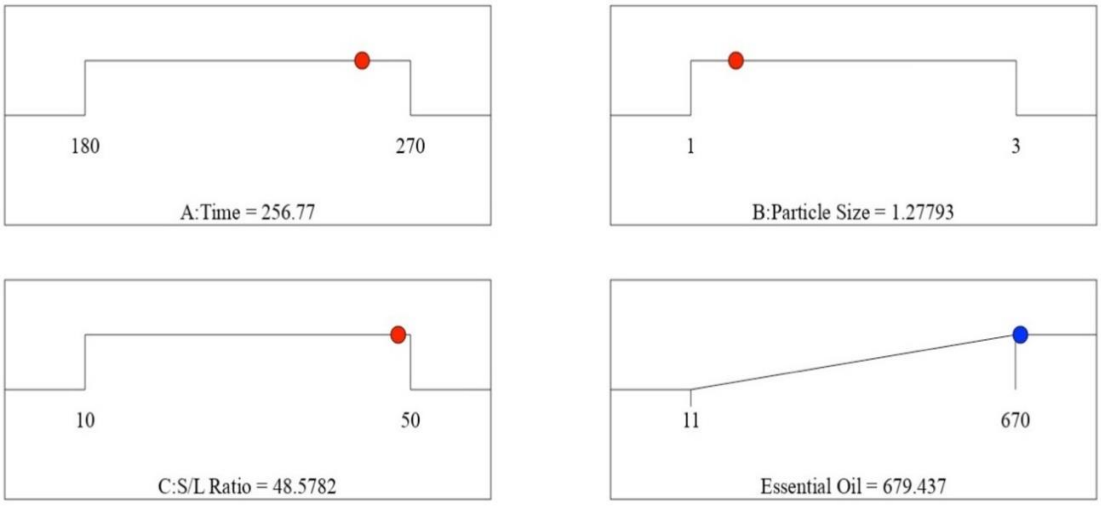

Figure 14. Graphical representation of the optimal point for the extraction of *E. globulus* E.O using HD

The 3D response surface plots shown in Figure 15 relate to *M. piperita* E.O yield, using HD, by applying a natural log transformation which rendered a quadratic equation that showed

a non-significant lack of fit: $R_{M.\ piperita}$ = 4.78 - 0.0501A - 0.5211B + 1.24C - 0.3004AB - 0.0489AC + 0.1569BC + 0.0979A^2 - 0.2891B^2 - 0.5408C^2. From the equation it is possible to see that the solid to liquid ration (C) showed the higher influence towards the optimal point.

Still, the yield is remarkably impacted by time (A), for the highest yield results from time range between 255 and 270 min, precisely at 264 min, followed by a noticeable influence of particle size on the obtained E.O yield, being 1 mm the size that most yielded essential oils. This is also quite expected as smaller particle sizes allow for a higher contact surface and thus higher extractability of compounds from within the cells.

At a higher S/L ratio a higher yield in E.O is obtained proportionally. The E.O yield was highly influenced by the remarkable interaction of the two variables S/L ratio (C) and time (A). In fact, a maximum S/L ratio of 50 g/L and a time of 264 min combined to obtain a higher yield in E.O.

As shown in Figure 16, with all 3 factors set at their optimal values, the predicted yield in E.O corresponds to 329.499 mg, which is slightly higher than the highest experimental runs which only reached 327.4 mg.

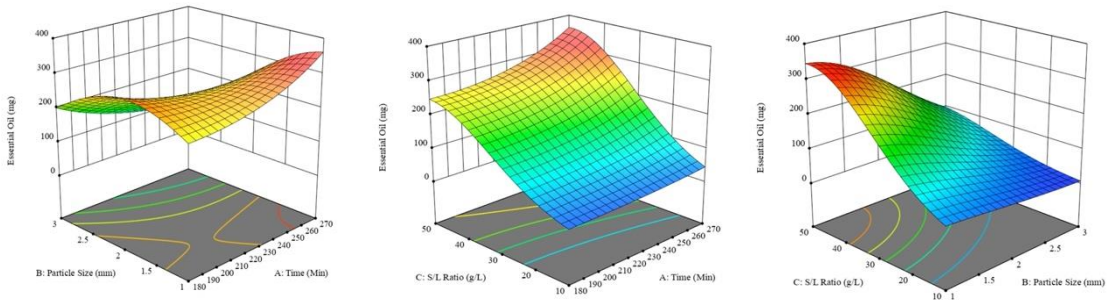

Figure 15. 3D response surface plots for the optimal point of *M. piperita* E.O extraction using HD. A-Time, B-Particle size and C-S/L ratio

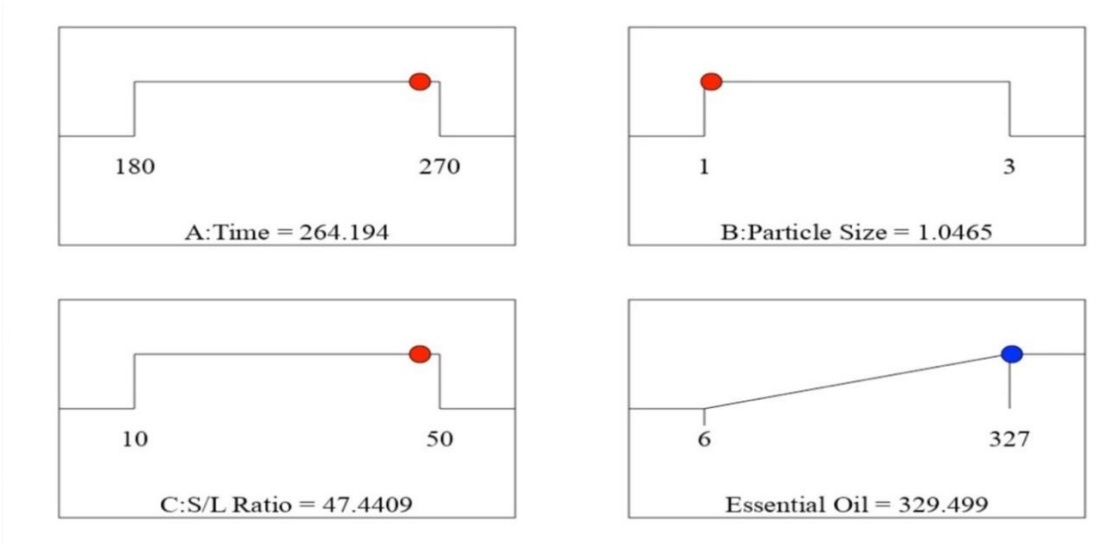

Figure 16. Graphical representation of the optimal point for the extraction of *M. piperita* E.O using HD

The 3D response surface plots shown in Figure 17 are from *P. pinaster*. The model rendered a quadratic equation: $R_{P.\ pinaster} = 19.20 + 1A - 12.24B + 17.51C - 3.78AB - 1.62AC + 9.25BC - 3.4A^2 + 6.57B^2 + 12.53C^2$. The left plot shows that time and particle size did not induce much variation in the yield, while S/L did have a strong influence in the yield (right plot).

The combination of the three optimal points results in a predicted yield of 57.618 mg, which is higher than the highest experimental yield in E.O obtained, being 54 mg (Figure 18).

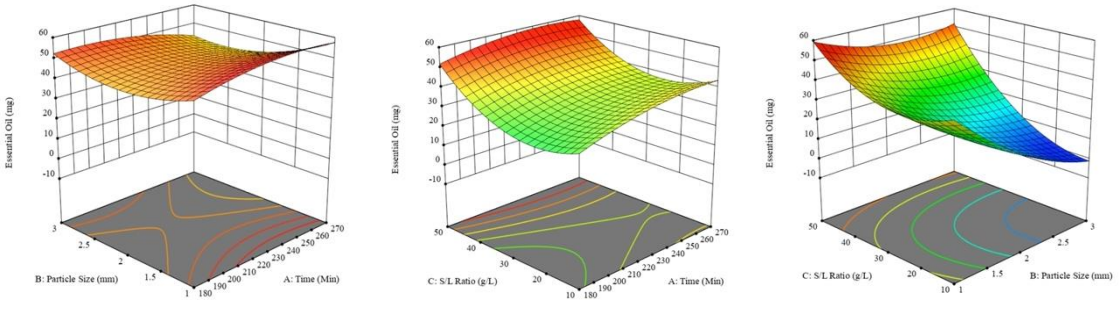

Figure 17. 3D response surface plots for the optimal point of *P. pinaster* E.O extraction using HD. A-Time, B-Particle size and C-S/L ratio

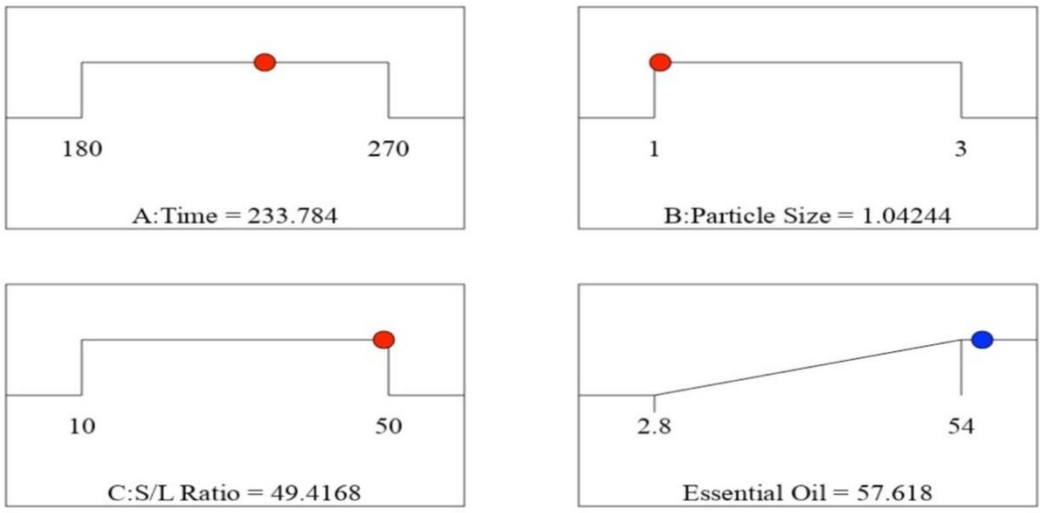

Figure 18. Graphical representation of the optimal point for the extraction of *P. pinaster* E.O using HD

The outcome of the before-discussed results implies that S/L ratio (C) is the most impactful factor in terms of E.O yield for all of the 3 plants, followed by time (A) and particle size (B).

3.2 Optimization of essential oil extraction using microwave-assisted hydro distillation

The same analysis applied for HD was carried out for the MAHD. Thus, *E. globulus* E.O yield ranged from 291.4 - 933.8 mg, considering the 17 runs for the optimization analysis, while *M. piperita* ranged from 76.7 - 212.3 mg. The lowest E.O yields resulted from *P. pinaster*, ranging from 15.7 - 62.1 mg.

The optimal point represents the highest extractability yield obtained, and is calculated using the maximization function. This resulted in an optimal time of 54.5805 min, 38.9582 min, and 52.0671 min, at a power of 393.998 W, 539.008 W, and 410.247 W, and at a ratio of 100 g/L, 100 g/L, and 99.9847 g/L for *E. globulus*, *M. piperita*, and *P. pinaster* respectively.

Table 2. Experimental runs and responses obtained under the extraction conditions defined by the Box-Behnken design for the extracted essential oil yield from *E. globulus*, *M. piperita*, and *P. pinaster* using MAHD, and statistical information of the models' fitting

Runs	**Independent Factors**			**Response: E.O yield (mg)**		
	A Time (min)	B Power (W)	C S/L ratio (g/L)	R_1 *E. globulus*	R_1 *M. piperita*	R_1 *P. pinaster*
1	40	400	70	695.9	148.1	42.3
2	60	400	40	291.4	76.7	25.1
3	40	600	100	547.5	212.3	74
4	60	200	70	430.8	114.5	35
5	40	200	100	648.5	170.11	62.1
6	20	400	40	350.1	61.7	18.3
7	40	600	70	514.5	152.8	48.4
8	20	400	40	279.9	88.09	21
9	20	200	70	280	84.3	34
10	40	600	40	416.5	88.73	27.84
11	40	400	70	781.6	142.7	41.8
12	40	400	70	666	155.8	45
13	40	200	40	280.6	54.03	15.7
14	60	600	70	570.2	126.6	53.3
15	60	400	100	933.8	192.3	70.9
16	40	400	70	578.7	146.6	46.7
17	20	400	100	853.9	178.9	59.1
Statistical data						
Model p-value				0.0471	< 0.0001	< 0.0001
Model F-value				3.77	161.42	35.84
Lack-of-fit				0.2142	0.5866	0.1481
R^2				0.8288	0.9959	0.9788
R^2_{adj}				0.6087	0.9897	0.9514
Adequate precision				7.1914	40.4116	19.2458
Coefficient of variance (%)				24.29	3.66	9.23

As resented in Figure 19, the 3D response surface plots allow a visual interpretation of the combination of the 3 factors on the response. For *E. globulus* E.O yield using MAHD rendered a quadratic function ($R_{E.\ globulus}$ = 647.34 + 57.79A + 21.78B + 205.64C + 34.88AB + 34.65C - 59.22BC - 61.55A^2 – 195.57B^2 + 21.5C^2) that displayed a non-significant lack of fit.

From this equation, it is clear that the factor with highest influence was (C) solid/liquid ratio with a value of 205.64. Thus, in the left plot, this variable was fixed at the optimal point, showing a smooth surface in which, the red zone shows the best extractive area, namely at higher temperatures and medium microwave power.

Through fixing power (B) at its optimal value, the interaction between time (A) and S/L ratio (C) was analysed, showing that higher S/L and longer extractive time promoted the yield (centre plot). Finally, the right plot revealed that intermediate power and high S/L ratios also promoted the yield.

With all 3 factors set at their optimal values, the predicted yield in E.O would correspond to 909.358 mg, which is slightly lower than the highest experimental yield in E.O obtained, being 933.8 mg (Figure 20) due to minor losses that occurred during E.O retrieval.

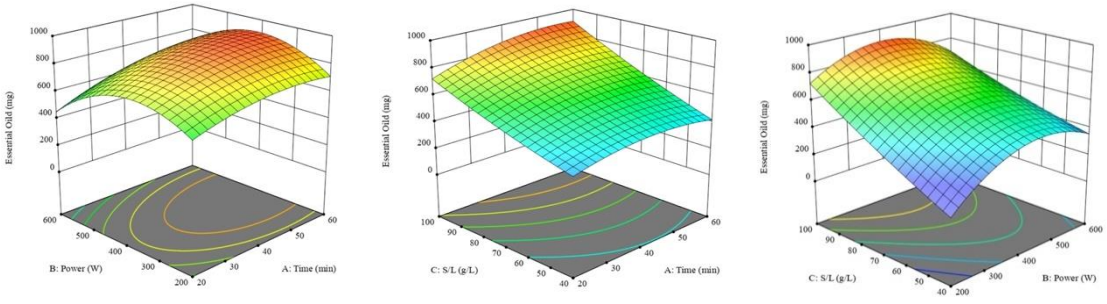

Figure 19. 3D response surface plots for the optimal point of *E. globulus* E.O extraction using MAHD. A-Time, B-Particle size and C-S/L ratio

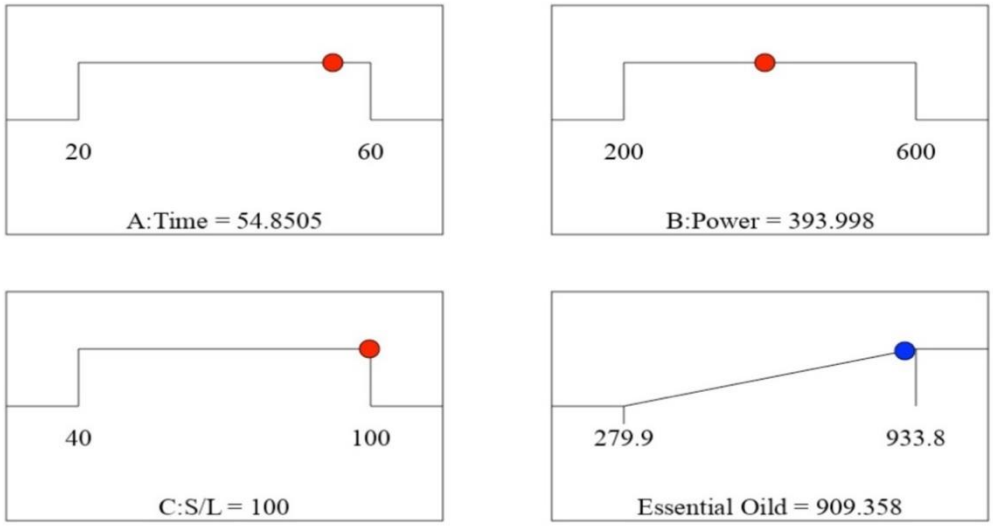

Figure 20. Graphical representation of the optimal point for the extraction of *E. globulus* E.O using MAHD

Figure 21 illustrates the 3D response surface plots for *M. piperita* E.O yield, using MAHD which rendered a quadratic function that showed an adequate fit and a non-significant lack. In this case (*M. piperita* MAHD), the 8th run was removed due to being an outlier. The coded quadratic equation was $R_{M.\ piperita}$ = 149.20 + 5.81A + 17.93B + 59.06C - 10.59AB - 0.04AC + 1.87BC - 18.53A^2 - 14.64B^2 - 3.27C^2, showing that once again the S/L ration showed the highest influence in the optimization model.

Considering the plots of Figure 21, the left one shows that power and time had a low influence as the red zones (highest yield) are homogenous throughout the surface, although being slightly promoted by higher power and intermediate time. The center plot shows a steep inclination towards higher S/L ratios and low influence of time, which was also observed in the right plot.

With all 3 factors set at their optimal values, the predicted yield in E.O would correspond to 211.729 mg, which is almost equal to the experimental yield in E.O obtained, being 212.3 mg (Figure 22) due to minor losses that occurred during E.O retrieval.

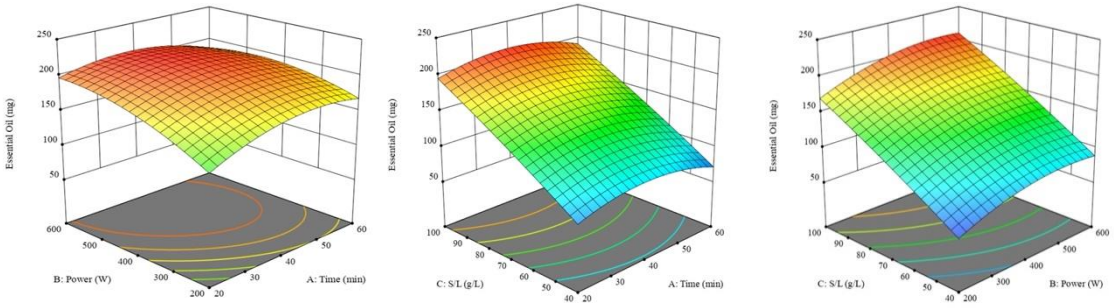

Figure 21. 3D response surface plots for the optimal point of *M. piperita* E.O extraction using MAHD. A-Time, B-Particle size and C-S/L ratio

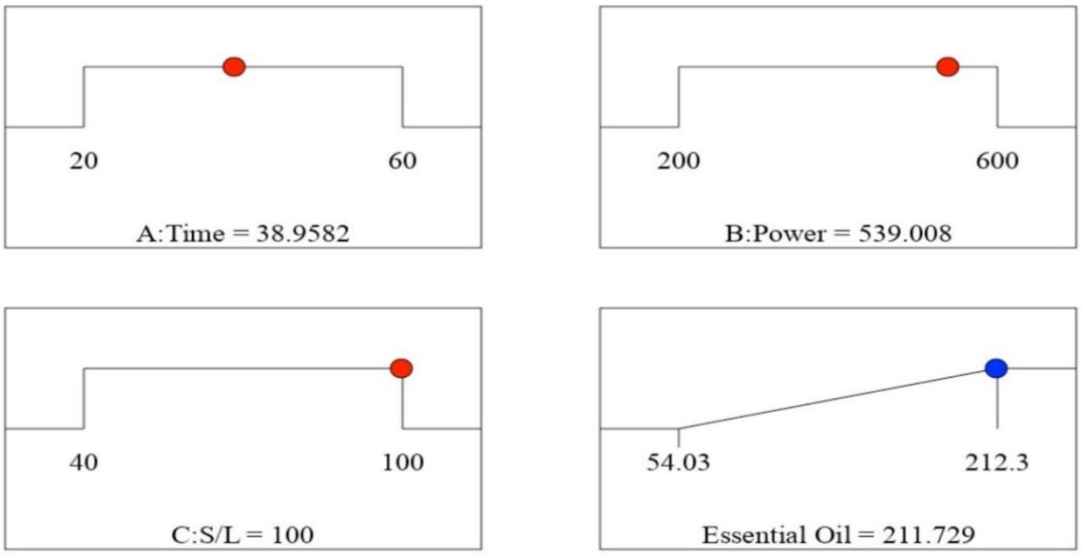

Figure 22. Graphical representation of the optimal point for the extraction of *M. piperita* E.O using MAHD

As presented in Figure 23, the 3D response surface plots show the results for *P. pinaster* E.O yield, using MAHD which were coded in a quadratic equation ($R_{P.\ pinaster}$ = 44.84 + 6.49A + 3.67B + 22.39C + 7.82AB + 1.25AC - 0.06BC - 5.29A^2 - 3.73B^2 + 3.8C^2) that displayed a non-significant lack of fit.

The left plot showed a similar behaviour to the plot of *M. piperita*, with a high yield over a large portion of the surface while the centre and right plots revealed that yield increase was linked to S/L ration, although at higher extraction times and power.

With all 3 factors set at their optimal values, the predicted yield in E.O would correspond to 74.1773 mg, which is almost equal to the experimental yield in E.O obtained, being 74 mg (Figure 24).

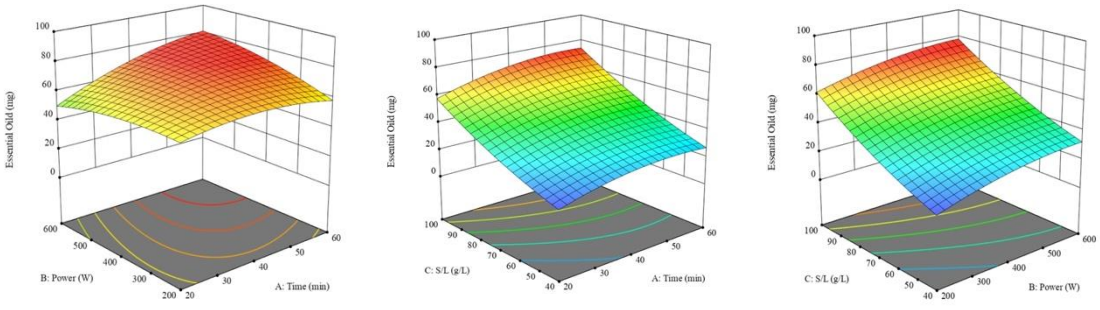

Figure 23. 3D response surface plots for the optimal point of *P. pinaster* E.O extraction using MAHD

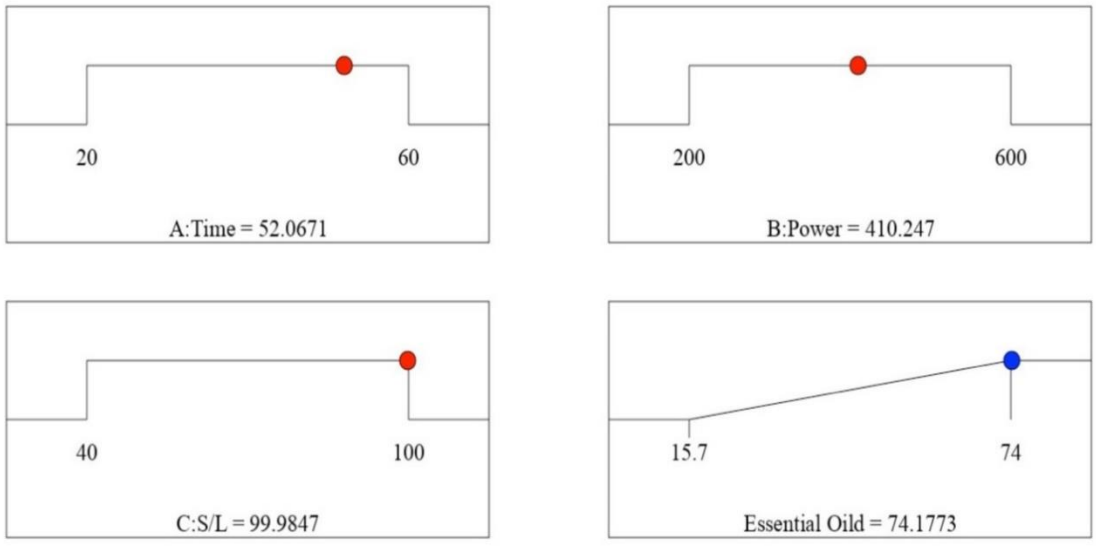

Figure 24. Graphical representation of the optimal point for the extraction of *P. pinaster* E.O using MAHD

The already-discussed results imply that S/L ratio (C) is the most impactful factor in terms of E.O yield, followed by time (A) and power (B) for the case of *E. globulus* and *P. pinaster*. However, for *M. piperita*, the most impactful factor is also S/L ratio (C), but it's followed by power (B) and time (A).

While both extraction methods (MAHD and HD) showed an overall similarity presented in the response surface plots for the 3 E.Os regarding their three-dimensional

profiles, however based on the yield results it is clear that the MAHD extraction method resulted in extracting higher yields in E.O from the 3 plants (*E. globulus*, *M. piperita*, *P. pinaster*) using less energy and done in less time comparing to the HD extraction method that demands a higher period of time and is energetically and water costly. Furthermore, longer extraction times could have an effect on the degradation of some bioactive compounds present in the E.Os. This interpretation is confirmed by Hashemi-Moghaddam et al., (2013) and Gupta et al., (2013). The variable S/L ratio (C) showed the highest influence on the extractability of all the E.Os using both extraction techniques, followed by time (A) and then particle size (B) or power (B) in HD and MAHD respectively, except for *M. piperita* E.O extracted using MAHD. To better understand the effects of the extraction techniques on each plant, the individual profile of essential oils was performed, followed by the analysis of bioactivities.

3.3 Chromatographic identification of volatile compounds using GC-MS

The profile in essential oils was analyzed using gas chromatography coupled to a mass spectrometer to confirm the identification of the different compounds. The different extracts were analyzed in their optimal conditions, except for MAHD of peppermint due to operational issues with the equipment. From the high number of molecules found only the major ones (30) were quantified, being their quantities expressed in the following tables.

As shown in Table 3 and Figure 25, the individual compounds of eucalyptus E.O are presented. For *E. globulus*, the extraction procedure showed a very strong influence, namely for the most abundant molecules. Eucalyptol, which was the most abundant in MAHD, standing out at 30%. It is a monoterpene that is known mainly for its antioxidant, antimicrobial, antiseptic, and anti-inflammatory benefits (Salehi et al., 2019), as well as a reported antiproliferative activity (Bhattacharjee and Chatterjee 2013). While in HD there was a more balanced distribution among the most abundant molecules (11 most abundant molecules in HD vs. 10 for MAHD) for MAHD, eucalyptol shoed a very high value. Furthermore, in terms of statistical differences among the extraction types, eucalyptol, as stated above, showed a statistically significant difference, with higher amounts being found in MAHD. Overall, the other compounds identified in both samples did not show significant differences. It seems like MAHD benefited the extraction of eucalyptol, which is a monoterpene.

Table 3. Profile of the volatile compoinds present in *E. globulus* E.O, expressed in mg/mL, for both HD and MAHD extractions

E. globulus				**HD**	**MAHD**
Compounds	Retention time	Calculated LRI	Theoretical LRI	Mean ± SD	Mean ± SD
α-Pinene	13.93	925.71	932	7 ± 1	6.0 ± 0.4
Eucalyptol	19.07	1024.56	1026	8 ± 4*	**30 ± 3**
α-Terpineol	27.08	1182.71	1186	-	3.4 ± 0.4
α-Terpinyl acetate	34.46	1341.74	1346	7 ± 2	8.4 ± 0.4
Aromadendrene	38.45	1434.04	1439	10 ± 4	8.53 ± 0.1
Alloaromadendrene	39.33	1454.99	1458	4 ± 2	3.4 ± 0.04
Epiglobulol	43.31	1553.49	-	2 ± 1	-
Globulol	44.33	1579.50	1590	**11 ± 6**	9.1 ± 0.1
Viridiflorol	44.61	1586.37	1592	4 ± 2	4.1 ± 0.1
γ-eudesmol	46.045	1624.60	1630	3.2 ± 0.2	-
non identified	46.773	1643.88	-	6.2 ± 0.5	-
β-Eudesmol	46.79	1644.01	1649	-	3.6 ± 0.1
nonidentified	46.880	1646.95	-	4.8 ± 0.2	-
α-Eudesmol	46.89	1646.84	1652	-	2.62 ± 0.06

An asterisk in each line means statistically significant differences using a student's T-test and a significance of 0.05.

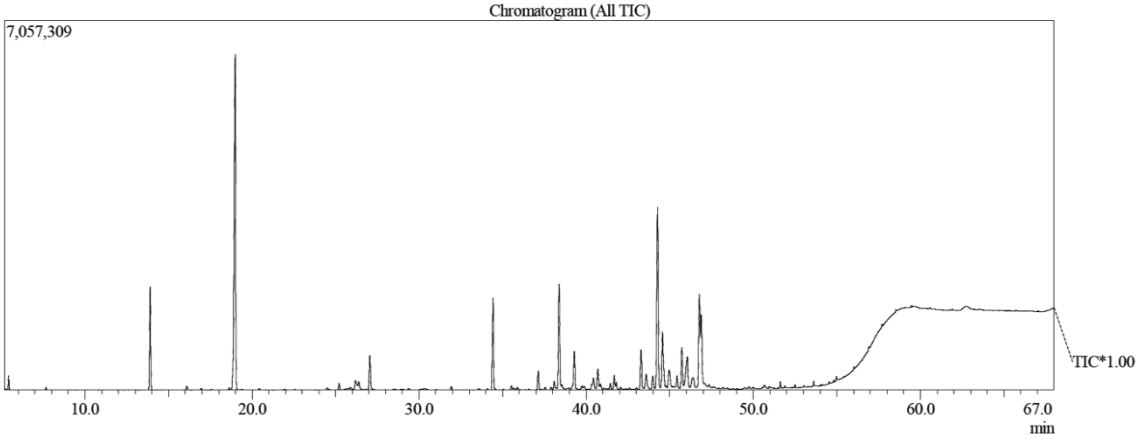

Figure 25. Chromatogram of *E. globulus* E.O extracted through MAHD

As for *M. piperita* E.O results shown in table 4 and figure 26, a dominating presence of menthol, with 30% ± 2, was found. This compound is famous for its ubiquitous employment in different products due to its coolness and refreshing aroma as well as its remarkable antibacterial, antifungal, and anti-inflammatory activities (Batool et al., 2018). Furthermore, high amounts of menthofuran and menthyl acetate were also found in the HD extraction among the 10 most abundant individual molecules. As stated above, no statistical analyses could be done for pine due not being possible to inject the MAHD samples in the GC.

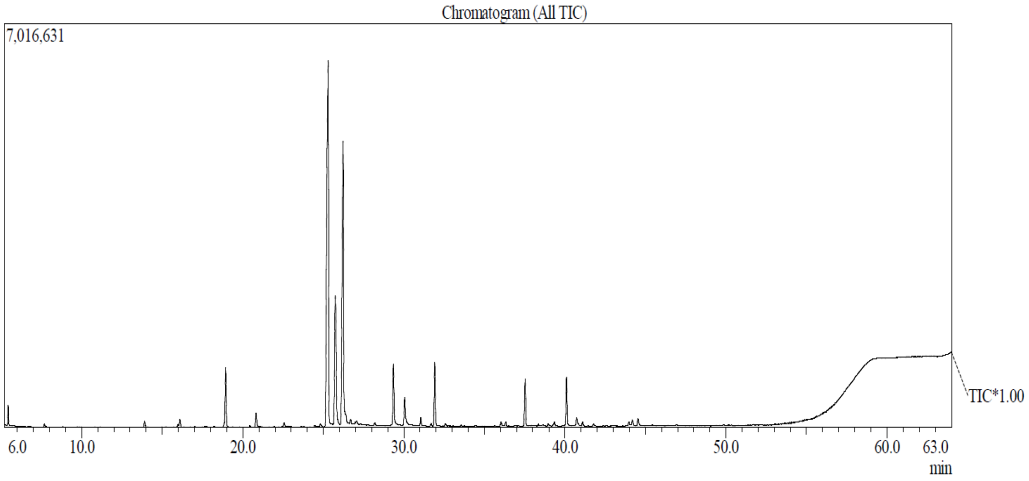

Figure 26. Chromatogram of *M. piperita* E.O extracted through HD

Table 4. Profile of the volatile compounds present in *M. piperita* E.O, expressed in mg/mL, for HD extraction

M. piperita				**HD**
Compounds	Retention time	Calculated LRI	Theoretical LRI	Mean ± SD
α-Pinene	13.92	926	932	0.39 ± 0.02
Eucalyptol	18.977	1023	1026	3.90 ± 0.02
Menthone	25.277	1146	1148	6.3 ± 0.3
Menthofuran + neo-Menthol	25.772	1156	1159	9.7 ± 0.2
Menthol	26.428	1170	1167	**30 ± 2**
Pulegone	29.409	1231	1233	2.86 ± 0.06
Piperitone	30.1	1246	-	2.25 ± 0.01
Menthyl acetate	31.997	1287	1294	9.2 ± 0.5
β-Caryophyllene	37.588	1413	1417	4.29 ± 0.02

Germacrene D	40.162	1475	1476	5.30 ± 0.07

As presented in Table 5 and figure 27, pine E.Os extracted using HD and MAHD were shown to have sesquiterpenes as the major compound type followed by diterpenes. germacrene-D represents the major compound detected (HD: 13.07 % ± 0.22; MAHD: 20.92 % ± 0.07), which confers antimicrobial and insecticidal properties to the E.O (Malik et al., 2019), followed by 7,13 – abietadiene (HD: 9.63 % ± 1.76; MAHD: 7.13 % ± 0.06), a diterpenic compound with no mentioned biological activities.

Comparing between the two extraction types, no statistical differences were sought between the compounds that were found in both, although MAHD seems to promote a higher amount of germacrene D.

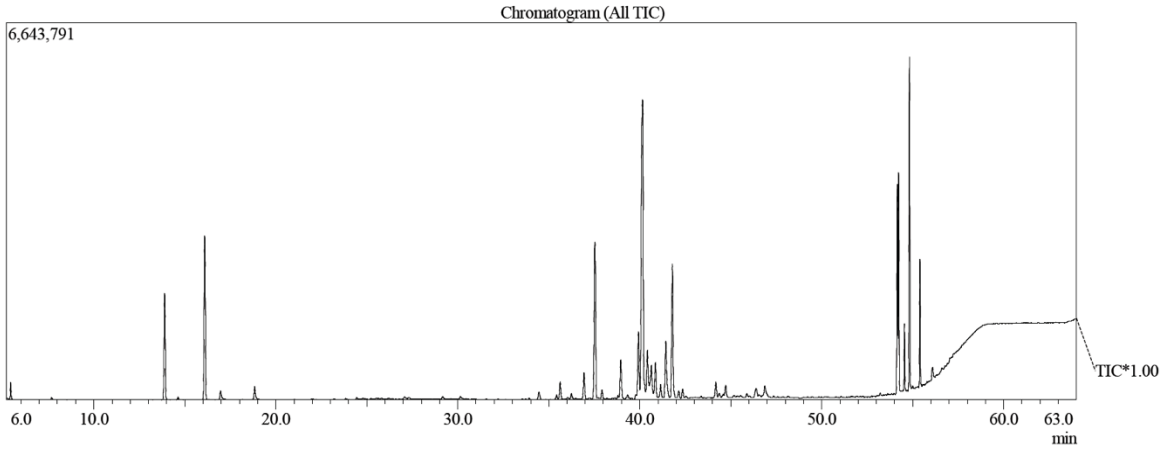

Figure 27. Chromatogram of *P. pinaster* E.O extracted through MAHD

Table 5. Profile of the volatile compounds present in *P. pinaster* E.O, expressed in mg/mL, for both HD and MAHD extractions

P. pinaster				**HD**	**MAHD**
Compounds	Retention time	Calculated LRI	Theoretical LRI	Mean ± SD	Mean ± SD
α-Pinene	13.89	925	932	-	4.35 ± 0.06
β-Pinene	16.10	967	974	2.18 ± 0.37*	7.13 ± 0.06
β-Caryophyllene	37.59	1413	1417	6.43 ± 0.65*	7.84 ± 0.04

γ-Muurolene	39.98	1471	1478	3.30 ± 0.46	3.31 ± 0.08
Germacrene D	40.20	1476	1484	**13.07 ± 0.22***	**20.92 ± 0.07**
γ-Cadinene	41.47	1507	1513	2.70 ± 0.13*	3.35 ± 0.09
δ-Cadinene	41.84	1516	1522	6.47 ± 0.22	6.46 ± 0.29
8,13-Abietadiene	54.19	2022	2035 (NIST)	6.98 ± 0.59*	5.52 ± 0.11
Levopimaradiene	54.26	2029	2040 (NIST)	5.76 ± 0.53	5.53 ± 0.21
7-Isopropyl(*)	54.57	2064	2074 (NIST)	3.61 ± 0.63*	1.74 ± 0.04
7,13-Abietadiene	54.86	2095	2087	9.63 ± 1.76	8.57 ± 0.32
Neoabietadiene	55.42	2167	2170 (NIST)	4.73 ± 1	3.10 ± 0.06

(*)7-Isopropyl-1,1,4a-trimethyl-1,2,3,4,4a,9,10,10a-octahydrophenanthrene; An asterisk means statistically significant differences using a student's T-test using a significance of 0.05, if no asterisk is present, there are no statistically significant differences; NIST: National Institute of Standards and Technology

Overall, HD seems to promote the extractability of higher amounts of volatile compounds seen its longer extraction time and the absence of high-powered magnetic waves that could either influence the compounds' structure making them partially or even unidentifiable, or more likely have an affinity to other compounds such as the polarized ones more than hydrocarbons that are less absorbed by magnetic waves and are consequently less likely to be separated and identified (Tran et al., 2020).

3.4 Evaluation of bioactive properties of the essential oils at their optimal points

The evaluation of the bioactive properties of the E.Os extracted using HD and MAHD was performed on a total of five E.Os at their optimal points, in which three of them were extracted using HD (*E. Globulus, M. piperita, P. pinaster*) and only two of them were extracted using MAHD (*E. Globulus, M. piperita*) due to the fact that *P. pinaster* E.O extracted using MAHD rendered low yields, which means that the extraction process needs to be run a multitude of times in order to give a volume that can be worked with in order to be able to assess the biological activities of this specific E.O, especially considering the repetitions needed to have reproducibility, therefore it was excluded.

3.4.1 Antioxidant activity

The antioxidant activity of the E.Os was analysed using three different *in vitro* assays: 2,2-diphenyl-1-dicrylhydrazyl (DPPH), reducing power (RP), and cellular antioxidant activity (CAA) in order to provide a comparative thorough study of the antioxidant capacity of each E.O, seen that each method is based on a different mechanism in different environments.

3.4.1.1 DPPH and RP assays

The DPPH and RP assays are two chemical-based assays that were employed to evaluate the antioxidant activity of a total of five E.Os at their optimal points. For the case of DPPH, the results are expressed in EC_{50} (mg/mL), whereas for RP, the results and expressed in $EC_{0.5}$ (mg/mL). Final results are displayed in Table 3.

Considering the antioxidant activity assay evaluated using DPPH, *M. piperita* E.O extracted using HD showed the lowest EC_{50} value of 8.94 ± 0.76 (mg/mL). Whereas the highest EC_{50} values of 42.71 ± 0.75 (mg/mL) and 47.55 ± 4.27 (mg/mL) were attributed to *E globulus* E.O extracted using MAHD and HD respectively. These results were considered higher comparing to the standard used for this assay, being Trolox at 41 μg/mL.

As for the evaluation of the antioxidant activity using RP, *P. pinaster* E.O extracted through HD showed the lowest $EC_{0.5}$ value of 0.36 ± 0.065 (mg/mL). The highest was attributed to *E. globulus* E.O extracted using MAHD (1.76 ± 0.08 mg/mL) followed by *M. piperita* E.O extracted using HD (1.8 ± 0.1 mg/mL). These results were proven to be higher in comparison with the standard used for this assay, which is Trolox at 41 μg/mL.

Table 6. Assessment of antioxidant activity using DPPH and RP assays expressed in mg/mL

	Hydro-distillation			**Microwave-assisted hydro distillation**		**Standard** (μg/mL)
	E. globulus	*M. piperita*	*P. pinaster*	*E. globulus*	*M. piperita*	Trolox
DPPH (EC_{50} mg/mL)	47.55 ± 4.27c	8.94 ± 0.76a	11.05 ± 0.63b	42.71 ± 0.75*	35.15 ± 0.72	41
RP ($EC_{0.5}$ mg/mL)	1.76 ± 0.08c	1.33 ± 0.23b	0.36 ± 0.065a	1.22 ± 0.32*	1.8 ± 0.1	41

For HD extraction, the letters in each line mean significant statistical differences using a Tukey's test while for the MAHD, an asterisk means statistically significant differences using a student's T-test and a significance of 0.05.

The E.Os extracted using HD showed lower EC_{50} values comparing to the E.Os extracted using MAHD, meaning that the E.Os extracted using HD, mainly *M. piperita* followed by *P. pinaster* E.Os, have a higher antioxidant activity that is expressed by a high scavenging capacity. This could be due to the high concentration in monoterpenes, mainly Menthol, contained in peppermint E.O extracted using HD. Overall, for HD, eucalyptus showed the lowest statistically significant activity, while mint showed the statistically highest for DPPH, but pine the highest for RP. Interestingly, for MAHD, mint was the most antioxidant in RP while eucalyptus the most antioxidant in RP. The presented statements are confirmed by the study conducted by Ruas et al. (2022) on the antioxidant property of *P. pinaster* E.O using DPPH, which showed an IC_{50} value of 55.2 ± 0.9 mg/mL which is about 5 times higher than these results. Whereas Wu et al. (2019) studied the scavenging activity of several mint species' E.Os including peppermint E.O, using DPPH, which resulted in an EC_{50} value of 70.29 ± 4.59 mg/mL which is almost 10 times higher than the EC_{50} value found in these results.

Specifically for RP assay, the difference between the results is not clear, which makes it perplexing to assume which extraction method gives a higher antioxidant capacity measured through RP. This is due to the different antioxidant mechanism that this method is based on.

Comparing to literature, *P. pinaster* needles' E.O was shown to have a reducing capacity of 18.31% when tested using the ferric reducing antioxidant power (FRAP) assay (Alonso-Esteban et al., 2022). For the case of *M. piperita* E.O, Wu et al. (2019) reported an EC_{50} value of 22.7 ± 1.66 mg/mL which is higher than our result. Limam et al. (2020) have also confirmed an antioxidant activity in *E. globulus* E.O with an EC_{50} value that is higher than the one found in this work: 18.79 ± 0.70 (mg/ mL).

The difference in these results comparing to the literature results are normal since the E.Os' antioxidant capacity differs with the environment, the period of samples' collection, the extraction methods' artifacts, or even the storage conditions.

3.4.1.2 Cellular antioxidant activity (CAA)

In order to further confirm the antioxidant activity results acquired through DPPH and RP assays, CAA assay was employed as it mimics the mechanism of free radicals' reduction inside the macrophage cell culture, which provides a more accurate simulation to the expected results *in vivo* (Wolfe et al., 2008).

The assessment of the CAA was performed on a total of four E.Os at their optimal points, in which three of them were extracted using HD (*E. globulus, M. piperita, P. pinaster*) and one of them was extracted using MAHD (*E. globulus*). The reason for using only four E.Os in this test is due to the unavailability of the RAW 264.7 cells to perform the assay on the peppermint E.O extracted using MAHD. Final results are expressed in percentage (%) of inhibition, and are displayed in Table 4.

The four E.Os displayed antioxidant capacity for the maximum tested concentration (2000 μM). Considering the HD distillation, the best antioxidant activity was recorded for *P. pinaster* (60% ± 7.54) which was statistically higher than the other two plants, on which no statistically significant differences could be sought.

Table 7. Assessment of antioxidant activity using Cellular Antioxidant Assay (CAA) expressed in % inhibition

	Hydro distillation			**Microwave-assisted hydro distillation**	**Positive control** (%)
	E. globulus	*M. piperita*	*P. pinaster*	*E. globulus*	Quercetin
Max [ ] tested (μM)	2000	2000	2000	2000	0.3
CAA (%)	48 ± 3.64a	49 ± 3.68a	59 ± 3.65b	60 ± 7.54	95 ± 5

For HD extraction, the letters in each line mean significant statistical differences using a Tukey's test and a significance of 0.05.

Overall, this assay's results confirm to a certain degree those obtained that were measured through the RP assay. Nevertheless, due to the missing E.O that would have made a difference however slight or important it might be, it is unsensible to compare which method promises a higher antioxidant activity when measured using CAA.

Throughout the DPPH, RP, and CAA assays, it is possible to indicate that *P. pinaster* and *M. piperita* E.Os extracted using HD as well as *E. globulus* E.O extracted using MAHD show a better antioxidant capacity, which is also confirmed by Assaggaf et al. (2022), Fkiri et al. (2019), and Pavlić et al. (2021). Both oils are capable of being effective when applied in a food preservative formula. It is necessary to bear in mind that the three antioxidant *in vitro* tests are based on different mechanisms that are sensitive to some molecules more than some others, which could be the reason behind the conflicting results. Moreover, the molecules in the E.Os differ from one extraction technique to the other.

3.4.2 Antimicrobial activity

The antimicrobial activity was evaluated using the Microdilution method that permits the determination of the minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) as well as minimum fungicidal concentration (MFC), on a total of five E.Os at their optimal points. In this assay, different bacterial strains were used, between clinical and alimentary bacterial strains, as well as two fungal species, in order to better assess the antimicrobial potential of the tested E.Os. The results are displayed in Table 5, 6, and 7.

3.4.2.1 Antibacterial activity

Considering Table 6, all of the tested E.Os revealed an antibacterial capacity against all of the clinical bacterial strains except the Gram-negative bacteria *Pseudomonas aeruginosa*. For the Gram-negative bacterial strains, all of the tested E.Os showed the highest inhibition capacity against *Proteus mirabilis* seeing that the lowest MIC values for all of the E.Os (0.6 - 1.25 mg/mL) were registered against this specific Gram-negative bacteria.

E. globulus and *M. piperita* E.Os extracted using HD both showed the lowest MIC values of 0.6 mg/mL against another Gram-negative bacterial strain *Klebsiella pneumoniae*. On the other hand, the highest MIC value studied (2.5 mg/mL) against Gram-negative bacteria was assigned to all of the tested E.Os against *Escherichia coli.*

As for Gram-positive bacterial strains, the lowest MIC value of 0.6 mg/mL was attributed to *M. piperita* E.O extracted using HD when tested against *Enterococcus faecalis*. Inversly, all of the tested E.Os showed high MIC values of 2.5 mg/mL against *L. monocytogenes.* The highest MIC value of 5 mg/mL was associated with eucalyptus E.O extracted through HD against Methicillin-resistant *Staphylococcus aureus* (MRSA). It is important to highlight that all of the tested E.Os presented notable inhibition of MRSA, which is a multi-resistant bacteria, with MIC values ranging between 1.25 - 5 mg/mL.

Table 7 presents antibacterial results against alimentary bacterial strains. The tested E.Os were able to inhibit the growth of the majority of the bacterial strains, except *P. aeruginosa.* In terms of Gram-negative bacterial strains, the lowest MIC value of 0.6 mg/mL was observed for eucalyptus E.O extracted through HD against *Salmonella enterica*. Whereas the highest MIC values ranging between 2.5 - 5 mg/mL were noticed for all of the E.Os against *Yersinia enterocolitica*.

As per Gram-positive bacterial strains, all of the tested E.Os exhibited a growth inhibition ability against all of the tested strains, with the lowest MIC value of 2.5 mg/mL noticed for all of the studied E.Os against *L. monocytogenes* and *S. aureus* equally. However, the highest MIC value of 5% was perceived for all of the tested E.Os against *B. cereus*.

Table 8. Evaluation of antibacterial capacity of the E.Os against clinical bacterial strains

	Hydro distillation						**Microwave-assisted hydro distillation**				**Positive control**					
	E. globulus		*M. piperita*		*P. pinaster*		*E. globulus*		*M. piperita*		Ampicillin (10mg/mL)		Imipenem (1mg/mL)		Vancomycin (1mg/mL)	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
Gram-negative bacteria																
Escherichia coli	2.5	>5	2.5	>5	2.5	>5	2.5	>5	2.5	>5	<0.15	<0.15	<0.0078	<0.0078	n.t.	n.t.
Klebsiella pneumoniae	0.6	>5	0.6	>5	2.5	>5	2.5	>5	5	>5	10	20	<0.0078	<0.0078	n.t.	n.t.
Morganella morganii	1.25	>5	1.25	>5	1.25	>5	1.25	>5	1.25	<5	20	>20	<0.0078	<0.0078	n.t.	n.t.
Proteus mirabilis	0.6	>5	0.6	>5	1.25	>5	1.25	>5	0.6	>5	<015	<0.15	<0.0078	<0.0078	n.t.	n.t.
Pseudomonas aeruginosa	>5	>5	>5	>5	>5	>5	>5	>5	>5	>5	>20	>20	0.5	1	n.t.	n.t.

Gram-positive bacteria																
Enterococcus faecalis	2.5	>5	0.6	>5	2.5	>5	5	>5	1.25	>5	<0.15	<0.15	n.t.	n.t.	<0.0078	<0.0078
Listeria monocytogenes	2.5	>5	2.5	>5	2.5	>5	2.5	>5	2.5	>5	<0.15	<0.15	<0.0078	<0.0078	n.t.	n.t.
MRSA	5	>5	1.25	>5	1.25	>5	5	>5	2.5	>5	<0.15	<0.15	n.t.	n.t.	0.25	0.5

The results are presented in percentage % (V/V). The maximum tested concentration: 5%. n.t: not tested.

Table 9. Evaluation of antibacterial capacity of the tested E.Os against food bacterial strains

	Hydro distillation						**Microwave-assisted hydro distillation**				**Positive control**					
	E. globulus		*M. piperita*		*P. pinaster*		*E. globulus*		*M. piperita*		Streptomycin 1mg/mL		Methicillin 1mg/mL		Ampicillin 10mg/mL	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
Gram-negative bacteria																
Enterobacter Cloacae	1.25	>5	1.25	>5	1.25	>5	1.25	>5	2.5	>5	0.007	0.007	n.t.	n.t	0.15	0.15
Escherichia coli	>5	>5	1.25	>5	1.25	>5	>5	>5	5	>5	0.01	0.01	n.t.	n.t.	0.15	0.15
Pseudomonas aeruginosa	>5	>5	>5	>5	>5	>5	>5	>5	>5	>5	0.06	0.06	n.t.	n.t.	0.63	0.63
Salmonella enterica	0.6	>5	1.25	>5	1.25	>5	2.5	>5	2.5	>5	0.007	0.007	n.t.	n.t.	0.15	0.15
Yersinia enterocolitica	2.5	>5	5	>5	5	>5	5	>5	5	>5	0.007	0.007	n.t.	n.t.	0.15	0.15
Gram-positive bacteria																
Bacillus cereus	5	>5	5	>5	2.5	>5	5	>5	5	>5	0.007	0.007	n.t.	n.t.	n.t.	n.t.
Listeria monocytogenes	2.5	>5	2.5	>5	2.5	>5	2.5	>5	2.5	>5	0.007	0.007	n.t.	n.t.	0.15	0.15
Staphylococcus aureus	2.5	>5	2.5	>5	>5	>5	2.5	>5	2.5	>5	0.007	0.007	0.007	0.007	0.15	0.15

The results are presented in percentage % (V/V). The maximum tested concentration: 5%. n.t: not tested.

Overall, eucalyptus and peppermint E.Os extracted using HD showed a higher antibacterial activity against Gram-negative bacterial strains. While peppermint E.O extracted using HD was proven to have a better bacterial inhibition against Gram-positive bacterial strains. They also demonstrated a higher inhibition capacity against Gram-negative and Gram-positive food bacterial strains. With peppermint and pine E.Os having the same effect at the same concentrations, while eucalyptus E.O performing at half concentrations of the previously-mentioned E.Os, but lacking an inhibition aptitude against *E. coli*.

Thus, it is possible to say that, generally, the E.Os extracted using HD have a promising inhibiting capacity against clinical and food bacterial strains and are more potent as antibacterial substances, making them a considerable option for food-preserving purposes.

The reason that eucalyptus, peppermint, and pine E.Os were shown to be potent against different bacterial strains can be explained by the fact that these oils contain high amounts of monoterpenes, sesquiterpenes and diterpenes that collaborate together to disrupt vital microbial components such as the cell wall. Many studies have investigated the antibacterial capacity of the before-mentioned E.Os, including Fkiri et al. (2019) who highlighted an antibacterial capacity of two varieties of *P. pinaster* E.O against *S. aureus*. and *B. cereus*. While Mahboubi and Kazempour (2014) were able to study the high inhibitory effect of peppermint E.O against numerous Gram-positive and Gram-negative strains. Tyagi and Malik (2011) have also focused on determining the antibacterial activity of *E. globulus*, which resulted in an inhibitory capacity against various Gram negative (*E. coli* αDH5, *E. coli* ATCC 25922, *P. aeruginosa*, *P. fluorescens*) and Gram positive (*B. subtilis* and *S. aureus*) bacterial strains.

3.4.2.2 Antifungal activity

The antifungal activity of the five E.Os was assessed based on their inhibition capacity against two most common opportunistic fungal pathogens: *Aspergillus brasiliensis* and *Aspergillus fumigatus*.

As shown in Table 7, all of the tested E.Os exhibited remarkable antifungal activity against both fungal species. In fact, the lowest MIC value of 0.3 mg/mL was noted for the inhibition capacity of both peppermint E.O extracted using HD and eucalyptus E.O extracted using MAHD against *A. brasiliensis.* The highest MIC value of 1.25 mg/mL reported against the same species was noticed for pine E.O extracted using HD.

In terms of MFC values, the lowest (0.6 mg/mL) was reported for peppermint E.O extracted through HD as well as eucalyptus E.O extracted using MAHD. The highest MFC of 1.25 mg/mL was noted for eucalyptus E.O extracted through HD along with peppermint E.O extracted using MAHD. The only E.O on which the MFC wasn't achieved is pine E.O extracted through HD.

As for inhibiting the growth of *A. fumigatus*, MIC values were reported lower than those needed to kill *A. brasiliensis*. For instance, the lowest MIC value of 0.15 mg/mL was noticed for peppermint E.O extracted using HD as well as eucalyptus and peppermint E.Os extracted through MAHD, followed by a MIC value of 0.3 mg/mL for eucalyptus E.O extracted using HD. These results are lower than the MIC value registered for the control Ketoconazole, being 0.5 mg/mL.

Regarding MFC values, the results were also lower than those needed to kill *A. brasiliensis*, and all of the E.Os showed interesting results. In fact, the lowest MFC value was registered as 0.15 mg/mL, and it was the concentration needed for peppermint E.O extracted through HD to kill the fungus, followed by 0.3 mg/mL for peppermint E.O extracted using MAHD as well as eucalyptus E.O extracted through HD and MAHD, equally. These MFC values are lower than that of the control Ketoconazole, being 1 mg/mL.

Table 10. Evaluation of the antifungal activity of the tested E.Os against two fungal species (*A. brasiliensis* and *A. fumigatus)*, expressed in mg/mL

	Hydro distillation						**Microwave-assisted hydro distillation**				**Positive control** (mg/mL)	
	E. globulus		*M. piperita*		*P. pinaster*		*E. globulus*		*M. piperita*		Ketoconazole	
	MIC	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**
Aspergillus brasiliensis	0.6	1.25	0.3	0.6	1.25	>5	0.3	0.6	0.6	1.25	0.06	0.125
Aspergillus fumigatus	0.3	0.3	0.15	0.15	1.25	2.5	0.15	0.3	0.15	0.3	0.5	1

The maximum tested concentration: 5%

Altogether, the E.Os extracted using both extraction techniques have a more or less similar inhibition and fungicidal capacities on the two fungi, while being slightly more effective on *A. fumigatus*. It is sensible to highlight the antifungal activity of peppermint extracted using HD and eucalyptus E.O extracted through MAHD, which makes them better candidates as food-preserving agents. This was also highlighted by Bansod and Rai (2008) that studied the inhibition capacity of peppermint and eucalyptus E.Os on *A. fumigatus*.

3.4.3 Antiproliferative activity

The effects of the EOs extracted on the growth of four human tumor cell lines were evaluated, according to the procedure adopted in the National Cancer Institute's (NCI) *in vitro* anticancer drug screening which uses sulforhodamine B (SRB) assay to assess cell growth inhibition. This was carried out for five E.Os . Results are expressed as GI_{50} (concentrations of extracts that cause 50% cell growth inhibition). This study was conducted on four human tumor cells lines, selected as being representative of lung, breast, colon and gastric cancer, and a non-tumor cell line PLP2 to rule out potential toxicity to normal cells.

From the presented results in Table 9, out of all the cell lines, eucalyptus E.O extracted using MAHD was able to inhibit 50% of the tumoral cell growth of AGS cell lines with the lowest GI_{50} value of 136 ± 2 µg/mL. Considering the HD, pine E.O showed the statistically best inhibition for AGS, CaCo2 and MCF7, although none of which were close to the positive control. This was quite expected as the E.Os are not antiproliferative-focused, albeit being quite interesting, results from E. globulus and M. piperita E.Os showed statistically lower values. None showed activity for NCI-460 cell line. These findings are supported by Moteki et al., (2002) that highlighted the ability of eucalyptol to cause DNA fragmentation inducing the apoptosis in known-to-be resistant cell lines such as leukaemia HL-60 and MOLT-4 cells. Zhang et al. (2015) have also reported an antiproliferative activity of pine E.O throughout the action of α-Pinene and β-pinene against non-small-cell lung carcinoma (NSCLC).

Table 11. Evaluation of the antiproliferative and the hepatotoxic properties of the five E.Os against four cancer cell lines (AGS, CaCo2, MCF-7, and NCI-H460) and against one normal cell line (PLP2) expressed in GI_{50} (µg/mL)

	Hydro distillation			**Microwave-assisted hydro distillation**		**Standard** (µg/mL)
	E. globulus	*M. piperita*	*P. pinaster*	*E. globulus*	*M. piperita*	Ellipticine
Antiproliferative activity						
AGS	286 ± 7b	215 ± 3a	215 ± 10b	136 ± 2*	279 ± 29	1.23 ± 0.03
CaCo2	303 ± 10b	304 ± 17b	205 ± 18a	376 ± 3	>400	1.21 ± 0.02
MCF-7	301 ± 15b	339 ± 3c	197 ± 5a	>400	>400	1.02 ± 0.02
NCI-H460	>400	368 ± 32	>400	>400	>400	1.01 ± 0.01
Hepatotoxic activity						
PLP2	>400	>400	>400	>400	>400	1.4 ± 0.1

For HD extraction, the letters in each line mean significant statistical differences using a Tukey's test while for the MAHD, an asterisk means statistically significant differences using a student's T-test. Both analyses were performed using a significance of 0.05.

Generally, peppermint E.O extracted using HD showed a reasonable ability to inhibit the tumoral cell growth on the studied cell lines, which is also supported by studies conducted by Lu et al. (2007) that proved an inhibitory activity of menthol on murine leukaemia WEHI-3 cell line at both concentrations of 1 or 10 mg/kg, and by Dolghi et al. (2022) that highlighted a cytotoxic activity of menthol against human colorectal carcinoma cell line (HCT 116) at a maximum concentration tested 500 µg/mL. Nonetheless, pine E.O was able to inhibit the tumoral growth at lower concentrations while not being effective on NCI-H460 cell line. Thus, it is evident from our results that the E.Os extracted through HD performed better in terms of tumoral cell growth inhibition and have a remarkable antiproliferative ability.

The most important result from table 9 is the fact that no toxicity was sought for any of the extracts in a normal cell line (PLP2) which rules out any harm to hepatic cells. This is an encouraging aspect on the use of essential oils for food preservation.

3.4.4 Anti-inflammatory activity

The anti-inflammatory activity of a total of five E.Os at their optimal points, in which three of them were extracted using HD (*E. globulus*, *M. piperita*, and *P. pinaster*) and two using MAHD (*E. Globulus* and *M. piperita*) was assessed by measuring the inhibition of NO production in order to evaluate their aptitude as a potential source to reduce or even inhibit inflammatory responses. The results are expressed in IC_{50} (µg/mL) and are presented in Table 9.

According to the results, considering MAHD, peppermint E.O showed the lowest IC_{50} value of 38 ± 1 (µg/mL), Regarding HD, the lowest value was recorder for eucalyptus, followed by pine, both with statistically significant differences. Peppermint was the least anti-inflammatory plant.

Table 12. Evaluation of the anti-inflammatory activity of five E.Os on RAW 264.7 murine macrophage cells expressed in (µg/mL)

RAW 264.7	**Hydro distillation**			**Microwave-assisted hydro distillation**		Positive control
	E. globulus	*M. piperita*	*P. pinaster*	*E. globulus*	*M. piperita*	Dexamethasone
Anti-inflammatory activity (IC_{50} µg/mL)	41 ± 3a	114 ± 9b	60 ± 3c	144 ± 5*	38 ± 1	6.3 ± 0.4

For HD extraction, the letters in each line mean significant statistical differences using a Tukey's test while for the MAHD, an asterisk means statistically significant differences using a student's T-test. Both analyses were performed using a significance of 0.05.

Although the difference between the extraction techniques in terms of which method offers a higher inhibition of inflammation cannot be significantly palpable seen the conflicting results, the difference in the performance of E.Os is tangible. Peppermint E.O extracted using

MAHD as well as eucalyptus E.O extracted using HD, followed by pine E.O seem to be potential anti-inflammatory substances in different applications, as supported by Hejna et al. (2021) that studied the dose-dependent inhibition of the production of tumor necrosis factor α (TNF-α) in porcine alveolar macrophages in different increasing concentrations of peppermint E.O (0, 25, 50, 100, 200 μg/mL). Belkhodja et al. (2022) have also highlighted the anti-inflammatory capacity of eucalyptus E.O through the inhibition of albumin denaturation at a concentration of 250 μg/mL. While Tümen et al. (2018) showed a 30.3% inhibition of inflammation of paw oedema in the Whittle method using carrageenan.

4. Conclusion

The primary goal of this thesis was to identify alternative natural molecules present in essential oils that could be used for food preservation purposes as a way to respond to a global need for clean label food products that respect both human health guidelines and environmental regulations. In addition, this work aimed at promoting circular economy of the studied plants based on their economic and forestry importance in Portugal, by using leftover vegetable materials.

The essential oils (E.Os) studied in this work were obtained from three aromatic plants (*E. globulus*, *M. piperita*, and *P. pinaster*) seen their antioxidant, antimicrobial, antiproliferative, and anti-inflammatory properties, using two different extraction techniques: hydro distillation (HD), which is a conventional and traditional extraction technique, and microwave-assisted hydro distillation (MAHD), which is a modern and sustainable extraction method.

A comparative study in terms of yields in E.O as well as biological activities of the volatile molecules was conducted in the attempt to highlight which extraction technique could be potentially used to extract higher yields in volatiles with more important bioactive properties.

As a result of the optimization designs done using response surface methodology, E.Os extracted using MAHD generally rendered higher yields than the ones extracted using HD. For instance, *E. globulus* E.O that had the highest yield of 933.8 mg using MAHD. However, HD resulted in a higher yield in *M. piperita*. And overall, the solid/liquid ratio factor was the most influencing factor comparing to time, particle size, and power in both extraction techniques.

The identified volatile compounds using GC-MS on the extracted E.Os were mainly monoterpenes and sesquiterpenes. These compounds were the reason these E.Os showed interesting bioactive characteristics mainly in the oils extracted through HD. *E. globulus* and *M. piperita* E.Os extracted through MAHD have also resulted in some interesting biological activities such as antioxidant, antimicrobial, and anti-inflammatory activities.

The tested E.Os performed well as antioxidant substances both on chemical and cellular levels. This was also highlighted by their antibacterial capacity on all of the studied bacterial strains, specifically on Methicillin-resistant *Staphylococcus aureus* (MRSA) which is a multi-resistant bacteria, except an absence of antibacterial effect on *Pseudomonas aeruginosa*. As

well as an interesting antifungal activity on two fungal strains (*Aspergillus brasiliensis* and *Aspergillus fumigatus*). In fact, *E. globulus* and *M. piperita* E.Os extracted using HD, along with *E. globulu*s E.O extracted through MAHD showed a fungicidal capacity against the two tested fungi species with concentrations lower than the control's (ketoconazole).

In terms of antiproliferative activity, all of the studied E.Os inhibited the growth of the majority of the tested cancer cell lines (AGS, MCF-7, and CaCo2), with *P. pinaster* E.O extracted through HD performing well with the lowest concentrations. *M. piperita* E.O extracted using HD was the only E.O to report a tumoral cell growth inhibition against all of the tested cancer cell lines (AGS, MCF-7, CaCo2, and NCI-H460). No cytotoxicity on normal cell line PLP2 was reported, meaning that these E.Os could potentially be safe to use as food preservatives as well as potential antitumoral drugs.

An anti-inflammatory study was also conducted to evaluate the E.Os' capacity to inhibit the production of nitric oxide (NO). All of the tested E.Os were reported to show anti-inflammatory responses, with *E. globulus* E.O extracted using HD as well as *M. piperita* E.O extracted through MAHD inhibiting the production of NO with less concentrations than the other E.Os. These results could be rather interesting for pharmaceutical and nutraceutical applications.

Some research is still needed to refine some aspects if this work, namely the extraction of higher yields of *P. pinaster* extracts using MAHD to test it in the bioactivity assays, as well as the determination of a complete E.O profile of *M. piperita* extracted with MAHD.

Overall, the results of this study could be highly interesting for several industrial applications such as in food, cosmetic, nutraceutical, and pharmaceutical.

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Published work and conference participations

1. Article under submission

Hached, H., Heleno, S., Amaral, J., Barros, L., Rezouga, F., Vaz, J., Carocho, M. (2022). Optimization of Essential Oils Extraction: Case studies with peppermint, pine and eucalyptus leaves extracted with microwaves and hydro distillation.

2. Conference participation as oral communication

Hanine Hached, Mariana C. Pedrosa, Sandrina Heleno, Lillian Barros, Josiana Vaz, Márcio Carocho*. Response Surface Methodology Applied to Essential Oil Extraction of Eucalyptus Leaves. March 2022. Transcolab Summit: Trends in grain-based foods. 23-25 March 2022, Instituto Politécnico de Bragança, Portugal.

Hanine Hached, Mariana C. Pedrosa, Sandrina Heleno, Lillian Barros, Josiana Vaz, Márcio Carocho*. Extraction optimization of essential oils from three aromatic plants using hydrodistillation and microwave assisted extraction. Novermber 2022. Encontro de Jovens Investigadores - STEAM. 22-24 November 2022, Instituto Politécnico de Bragança, Portugal.

3. Book chapter

Hached, H., Cassani, L., Zbiss, Y., Fraga-Corral, M., Oliveira, I., Pereira, A. G., Prieto, M. A., Heleno, S., Carocho, M. (2022). Non-Alkaloid Nitrogen Containing Compounds from Fungi. In Carocho, M., Heleno, S., Barros, L. (Eds), *Natural Secondary Metabolites: From Nature, Through Science, to Industry*. New York. Springer Nature International Publishing. ISBN-13: 9783031185861.