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Extraction of *Eucalyptus globulus* Volatiles and Sensitivity Tests on Foodborne Pathogens

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Index

Abbreviations and Acronyms

SMs: Secondary metabolites

MVA: Mevalonic acid

MDA: Malondialdehyde

MDA-MB-231: Human Breast Carcinoma cell-line

MEP: Methylerythritol Phosphate

EO: Essential oil

HT: Hemiterpenes

UV: Ultraviolet

IPP: Isopentenyl pyrophosphate

DMAPP: Dimethylallyl pyrophosphate

TRCs: Compounds with a phenyl ring (C6) and a side chain (C3)

HIPVs: Herbivore-induced plant volatiles

VOCs: Volatile organic compounds

LPS: Lipopolysaccharides

IL -1β: Interleukin 1 beta

IL -4: Interleukin 4

IL -13:Interleukin 13

DMAPP: Dimethylallyl Pyrophosphate DNA: Desoxyribonucleic Acid

IPP: Isopentenyl Pyrophosphate

MDA-MB-231: Human Breast Carcinoma cell-line

MIC: Minimum Inhibitory Concentration

MV3: Human Melanoma cell-line MVA: Mevalonic Acid

SPCA-1: Human lung cancer cell line SW620: Colorectal Cancer cell-line

List of figures

List of tables

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Abstract

Food spoilage is one of the main concerns in the food industry, as about one third of food that is produced is wasted before it even reaches the consumers. Essential oils are known for their very strong antioxidant and antimicrobial activity. Little is known about the changes that essential oils undergo when subject to different biotic and abiotic stress. Still, essential oils have been used to preserve foods by using them as food additives, although the European legislation does not allow their use, due to potential toxicity regarding human consumption. Thus, given the high number of volatile molecules present in essential oils, the use of these airborne molecules could be an interesting prospect, namely using these extracts as volatile preserving molecules. This work intends to compare the bioactivities (antioxidant, antitumor, anti-inflammatory and antimicrobial) of *Eucalyptus globulus Labill* essential oils from leaves harvested in 2023, with the ones harvested from the same trees in 2022, both extracted through hydrodistillation. The essential oils were then screened against two foodborne fungi (*Aspergillus brasiliensis* and *Aspergilus fumigatus*) by placing these contaminants in airtight containers containing the *Eucalyptus* essential oils, to test if the airborne molecules present in the oils could inhibit the fungal growth. The findings show that overall, the changes in the bioactivities in the *Eucalyptus* essential oils in the two years is very low, with only some differences being found in the sensitivity of bacteria. The antifungal activity was maintained, as was the antioxidant activity for the DPPH analysis. In terms of inhibition of the fungi by the airborne molecules, both were inhibited at all stages of growth (immediately after plating, after 24 and 48 hours), although the essential oils seemed to inhibit to a better extent *Aspergillus fumigatus*. Overall, these results prove the antifungal activity of the volatile fraction of *Eucalyptus* essential oils, which could be used in the future as a volatile preservative for packed food.

Keywords: hydrodestillation, essential oils, *Eucalyptus globulus Labill*, antimicrobial, food preservative.

Resumo

A deterioração de alimentos é uma das principais preocupações na indústria alimentar, já que cerca de um terço dos alimentos produzidos é desperdiçado antes mesmo de chegar aos consumidores. Os óleos essenciais são conhecidos pela sua excelente atividade antioxidante e antimicrobiana. Pouco se sabe sobre as alterações que os óleos essenciais sofrem quando submetidos a diferentes estresses bióticos e abióticos. Ainda assim, os óleos essenciais têm sido usados para preservar alimentos, utilizando-os como aditivos alimentares, embora a legislação europeia não permita seu uso, devido à possível toxicidade em relação ao consumo humano. Assim, dada a alta quantidade de moléculas voláteis presentes nos óleos essenciais, o uso dessas moléculas em recipientes fechados poderia ser uma perspetiva interessante, especialmente usando esses extratos como moléculas voláteis conservantes. Este trabalho pretende comparar as bioatividades (antioxidante, antitumoral, anti-inflamatória e antimicrobiana) dos óleos essenciais de *Eucalyptus globulus Labill* de folhas colhidas em 2023, com aqueles obtidos das folhas das mesmas árvores em 2022, ambos extraídos por hidrodestilação. Os óleos essenciais foram testados contra dois fungos alimentares (*Aspergillus brasiliensis* e *Aspergilus fumigatus*), colocando esses contaminantes em recipientes herméticos contendo os óleos essenciais de eucalipto, para verificar se as moléculas voláteis presentes nos óleos poderiam inibir o crescimento fúngico. Os resultados mostram que, globalmente, as mudanças nas bioatividades nos óleos essenciais nos dois anos são muito baixas, com apenas algumas diferenças encontradas na sensibilidade de algumas bactérias. A atividade antifúngica foi mantida, assim como a atividade antioxidante através da análise de DPPH. Em termos de inibição dos fungos pelas moléculas no ar, ambos foram inibidos em todas as fases de crescimento (imediatamente após a sementeira, após 24 e 48 horas), embora os óleos essenciais parecessem inibir de maneira mais eficaz o *Aspergillus fumigatus*. Em geral, esses resultados comprovam a atividade antifúngica da fração volátil dos óleos essenciais de Eucalipto, que poderia ser utilizada no futuro como conservante volátil para alimentos embalados.

Palavras-chave: hidrodestilação, óleos essenciais, voláteis, *Eucalyptus globulus Labill.*, antimicrobiano, conservante alimentar.

I. Introduction

1. Secondary metabolites

Secondary metabolites (SMs) are natural products synthesized mainly by bacteria, fungi, and plants. They are low molecular weight molecules that have different chemical structures and biological activities (Enespa & Chandra, 2019). The name secondary metabolite comes from the original observation that their production is not necessary for the growth and reproduction of plants, unlike primary metabolites, which include lipids, amino acids, carbohydrates, and nucleic acids. However, SMs are anything but secondary, so the term "specialized metabolites" has come to be applied to them. It is now recognized that SMs play a key role in the survival of the organisms that produce them, as SMs determine interactions in their environment. Today, the production of SMs is an important area of research for organic chemists, molecular biologists, and bioinformaticians alike (Collemare et al., 2020). Secondary metabolites, which consist of terpenes, phenols, nitrogen and sulfur compounds, among others, protect plants from various biotic stresses, i.e., herbivores and pathogenic microorganisms, especially fungi, bacteria and other parasites. In addition to biotic stress management, they also provide a protective shield against certain abiotic stress factors.

1.1. Types of secondary metabolites

In general, secondary metabolites are the product of primary metabolites and are formed by modifications of biosynthesis, including methylation, glycosylation, and hydroxylation. Secondary metabolites are certainly more complex in their structural composition and side chains than primary metabolites. Depending on the biosynthetic pathway, three main classes of plant metabolites can be distinguished: (i) phenolic groups, (ii) terpenes and steroids (consisting mainly of carbon and hydrogen), and (iii) nitrogen-containing compounds (Twaij & Hasan, 2022).

1.1.1. Terpenes and Terpenoids

Terpenes are considered a structurally diverse and large group of metabolites, of which at least 35,000 different have been characterized to date. Terpenes consist of isoprene units that can be modified by cyclization reactions, making them easily recognizable. They are classified into different groups based on the number of isoprene units in their carbon skeleton. The biosynthesis of terpenes is involved in two main pathways in plants. One is the mevalonate pathway (MVA pathway), and the other is the 2-C-methyl-D-erythritol-4- phosphate pathway (MEP pathway), which exists in the cytoplasm and plastids, respectively (Dubey et al., 2003).

Terpenes are the main components of essential oils (EOs), whose molecular structure contains a carbon skeleton of 2-methylbuta-1, 3-diene (isoprene units) that can be converted into cyclic structures (Masyita et al., 2022)(Figure 1). The number of isoprene units is mainly responsible for the structural diversity of terpenes. Monoterpenes are the predominant constituents of EO's (90%), followed by sesquiterpenes (Stephane & Jules, 2020). Diterpenes, triterpenes, and tetraterpenes with their oxygenated derivatives are also detected in small amounts (Lv et al., 2022).

Hemiterpenes (HT) are a smaller portion of the terpenes found in EOs, they are formed from one isoprene unit (C5), monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20), triterpenes (C30), and tetraterpenes (C40) (Bhavaniramya, & al, 2019). The most prominent HT is the isoprene, which is given off by herb s and leaves of many trees such as conifers, oaks, poplars, and willows. Examples of HT include angelic acid, tiglic acid, isovaleric acid, and senecioic acid.Terpenoids, on the other hand, are another type of terpenes containing oxygen molecules formed by biochemical modifications (removal or addition of methyl groups). They are the largest class of natural products produced by many organisms, including plants, insects, and microorganisms. More than 80,000 different terpenoids are known, and they exhibit a wide structural diversity. Plant terpenoids have many biological functions, such as regulating plant growth and development and participating in plant defense (Lv et al., 2022). In flowering plants, terpenoids play an important role in attracting insect pollinators as floral scents. In addition, the industry has used terpenoids extensively as flavors, fragrances, and spices (Lv et al., 2022).

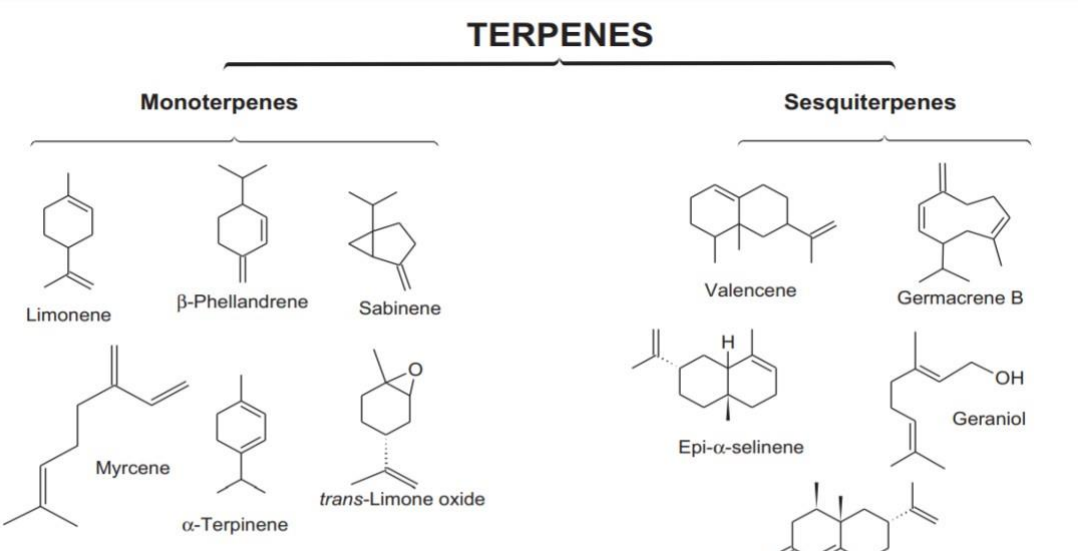


Figure 1: Examples of two classes of terpenes found in essential oils (Kon & Rai, 2016)

Terpenoids can be classified into alcohols, aldehydes, esters, ethers, epoxides, ketones, and phenols. Examples of terpenoids are: carvacrol, citronellal, geraniol, linalool, linalyl acetate, piperitone, menthol and thymol. These bioactive compounds confer various biological activities such as anticancer, antibacterial and antioxidant among others (Wang et al., 2018). Terpenes and terpenoids are synthesized via the mevalonic acid (MVA) pathway in the cytosol and the 2-C-Methyl-D-erythritol 4-phosphate (MEP) pathway in the plastid to form the precursors isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) (Masyita et al., 2022).

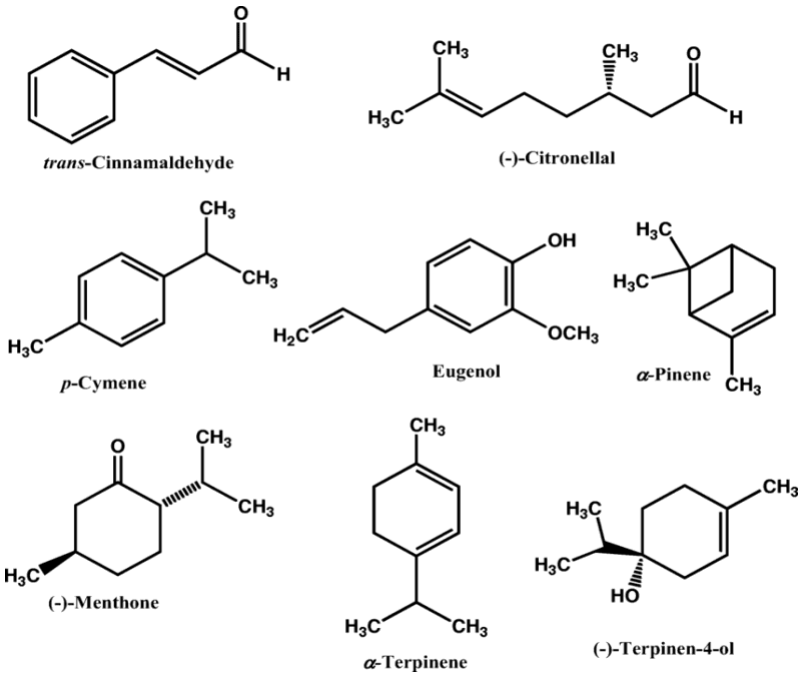


Figure 1: Chemical structure of phenylpropenes and monoterpenes (Saad et al, 2019)

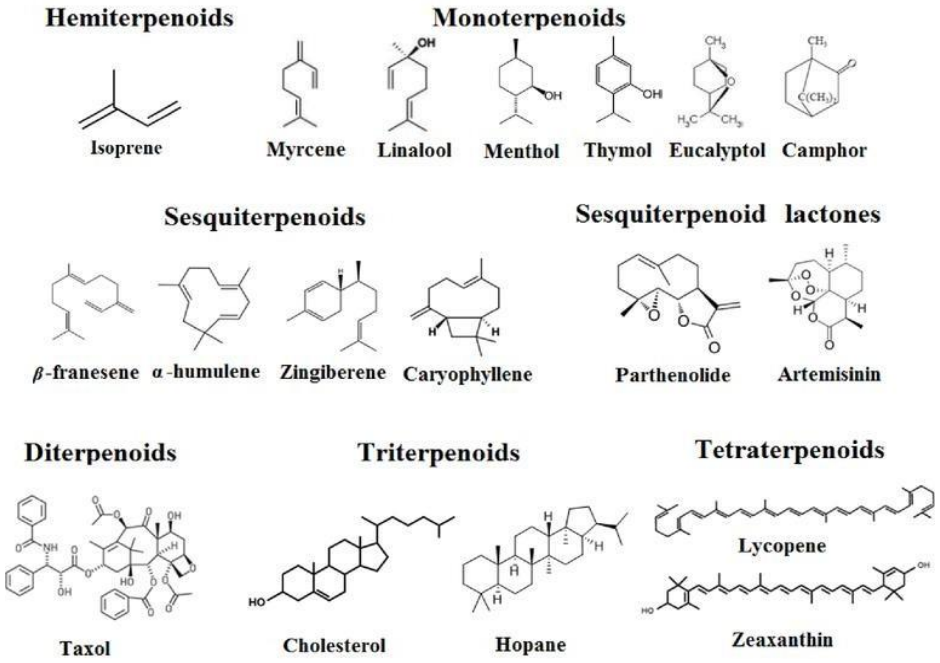


Figure 2: Examples of different classes of terpenoids (Abdallah & Quax, 2017)

Phenylpropenes are synthesized in plants from the amino acid precursor phenylalanine. Phenylpropenes take their name from the six-chain aromatic phenolic group and the three-chain propene tail of cinnamic acid formed in the initial phase of phenylpropanoid biosynthesis. The antibacterial activity of these compounds is attributed to their free hydroxyl groups. Phenylpropenes showed a broad spectrum of antibacterial activity (Nazzaro et al., 2013). Isoeugenol showed stronger activity against bacteria compared to eugenol, while both isoeugenol and eugenol were more active against Gram-negative bacteria than Gram-positive bacteria. (Lv et al., 2022).

1.1.2. Alkaloids

Alkaloids are a group of molecules that are relatively common in nature, formed from amino acids or by transamination. Alkaloids are classified by the amino acids that form their nitrogen atom and part of their skeleton. Similar alkaloids can have quite different biosynthetic pathways and different bioactivities. Alkaloids are derived from L-lysine, L-ornithine, L-tyrosine, L-tryptophan, L-histidine, L-phenylalanine, nicotinic acid, anthranilic acid, acetate, or amination and transamination reactions. The terpenoids, steroids and purine alkaloids are also important. Millions of humans around the world consume purine alkaloids daily, whether at the beginning of the day with a cup of coffee or in the afternoon with a cup of tea. They are vital for nutrition and the activation, aggression, and defense of various species (Dey et al., 2020) . The β-carboline alkaloids are a large group of natural and synthetic indole alkaloids that share a common tricyclic pyrido [3, 4-b] indole ring structure and are widely distributed in

nature, including various foods, plants, animals, and fungi and bacteria (Xiaozheng et al., 2023).

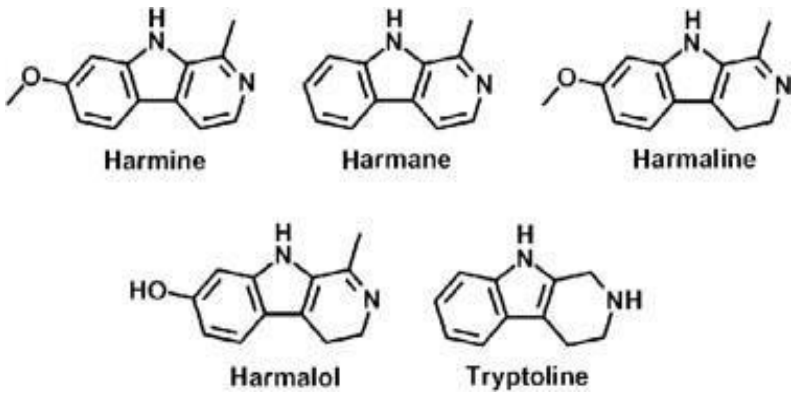


Figure 3: Examples of B- β-carboline alkaloids (Nafisi et al., 2010)

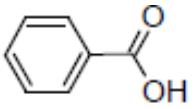
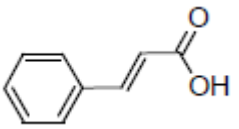
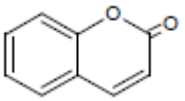
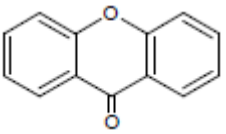
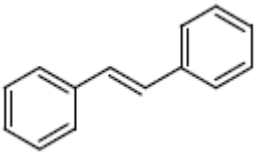
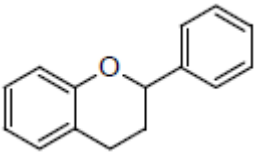
In 2020, Kwon and Chan reported that berberine (an alkaloid) could reduce the recurrence of colorectal adenomas and inhibit colorectal carcinogenesis for chemoprevention of colorectal adenomas and colorectal cancer (Kwon & Chan, 2020). Beyond this example, these compounds are of great interest due to their diverse bioactivities, such as anticonvulsant, antitumor, antiviral, antiparasitic, and antimicrobial activities. Studies have shown that harmaline has tremendous health benefits. Harmaline exhibits a number of pharmacological activities, such as vasodilatory, hypothermic, antimicrobial, antileishmanial, antiplasmodial, antiplatelet, and antitumor effects, which have been discussed in detail. Recently, it was shown to be effective against the carcinogenic enzyme CYP1A1. In addition, it is known to inhibit DNA excision repair and lead to enhancement of Ultraviolet (UV) or chemically induced damage (Khan et al., 2013).

1.1.3. Phenolic metabolites

Polyphenols 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 (Paliyath et al., 2018). In terms of structure, phenolic compounds have an aromatic ring with one or more hydroxyl substitutes, including their functional derivatives (Table 1) (Carocho & CFR Ferreira, 2013). They contribute to the protection of plants as well as to their sensory quality, tolerance to various abiotic and abiotic stresses (Paliyath et al., 2018). In fact, phenolic acids, flavonoids, and tannins represent the most common and abundant groups of phenolic metabolites.

These properties make phenolic relevant compounds in food preservatives, in human health management, in natural pesticides and in cosmetics (Paliyath et al., 2018;(Wu et al., 2021).

Table 1. Classification through number of carbons and basic structure of molecules within the phenolic compounds family (Carocho & Ferreira, 2013)

Number of Carbons	Classification	Example	Basic Structure
7	Hydroxybenzoic acids	Gallic acid	
9	Hydroxycinnamic acids	*p*-coumaric acid	
9	Coumarins	Esculetin	
13	Xanthones	Mangiferin	
14	Stilbenes	Resveratrol	
15	Flavonoids	Naringenin	

The bioactive properties of phenolic compounds are well known; there is countless research and review articles on this topic, which provide a thorough description of their biological and bioactive properties. In their review article, Quideau et al. (2011) provided an overview of the chemical and structural properties of numerous phenol families and related them to the biological properties and the way they are expressed after daily consumption of fruits, vegetables, beverages, red wine, and even chocolate. They concluded that despite their poor solubility and bioavailability, these compounds may have long-term health-promoting effects when consumed daily, and that chemical synthesis serving the academic and industrial communities could provide analogous compounds that could then be used in foods. In fact, phenolic compounds are among the most studied natural product families today because of

their bioactive properties, naturally produced by plants and their immense structural and chemical diversity (Salvatore et al., 2020).

Many studies still address the biological and chemical structure as well as the biosynthetic pathways (e.g., enzymes involved in their production, genetic pool, and proteins). For all these reasons, phenolic compounds became desirable targets for in vitro culture, which provides the necessary stress conditions for their production and increases the excretion of these secondary metabolites by plant tissues (Parr & Bolwell, 2000).

2. Importance of volatiles from aromatic plants

2.1. Essential oils and volatiles

Plants can synthesize tens to hundreds of thousands of metabolites with different functions and biological properties. The volatile organic compounds produced in both primary and secondary metabolic pathways account for about 1% of all plant secondary metabolites. There are approximately 1700 volatile compounds with a wide variety of chemical structures identified in over 90 gymnosperm and angiosperm plant families, and this number is expected to increase as more plants are discovered and analyzed using innovative detection methods. Plant volatiles are lipophilic liquids with low molecular weight and high vapor pressure that can penetrate membranes and disperse into the atmosphere or soil without a diffusion barrier. They are formed in plant tissues at developmental stages (flowering, maturity, or ripening) and in various plant organs, namely roots, stems, leaves, fruits, and seeds, with flowers being the part that contains the greatest amount and variety of volatiles (Muhlemann et al., 2014). Plants respond to insect predation by producing and releasing plant volatiles. These molecules are specific volatile organic compounds (VOCs) consisting of green leaf volatiles, terpenes, including mono-, hemi-, and sesquiterpenes, and some aromatic compounds. The Herbivore-Induced Plant Volatiles (HIPVs) serve different functions, thus HIPVs can have both direct and indirect effects on the attacking animal. Direct effects of HIPVs include repellency and toxicity, and indirect effects include attracting natural enemies of the aggressors. In addition, HIPVs can also serve as a communication signal between plants, identify healthy sections of the same plant and neighboring plants, and stimulate systemic resistance in the uninfected sections of the plant and neighboring plants. In general, insect herbivores are repelled by plant volatiles (Luo et al., 2022). Since the Middle Ages, essential oils have been widely used for bactericidal, virucidal, fungicidal, antiparasitic and insecticidal medicinal and cosmetic applications, especially nowadays in the pharmaceutical, sanitary, cosmetic, agricultural and food industries. Due to the method of extraction, usually by distillation (steam or hydrodistillation) from aromatic plants, they contain a variety of volatile molecules such as terpenes and terpenoids,

phenol-derived aromatic components, and aliphatic components. In vitro physicochemical studies identify most of them as antioxidants. EOs are part of volatile components of plants responsible for their aroma and biological effects, including antimicrobial activities, antioxidant, anti-inflammatory, anticancer, and antiparasitic activities (Jaouadi et al., 2021).

The Eucalyptus oil is a complex mixture of a variety of monoterpenes and sesquiterpenes, and aromatic phenols, oxides, ethers, alcohols, esters, aldehydes and ketones such as 1,8-cineole (Eucalyptol), citronellal, citronellol, citronellyl acetate, p-cymene, eucamalol, limonene, linalool, α-pinene, γ-terpinene, α-terpineol and aromadendrene .

Several bioactivities such as antioxidant, antimicrobial, insecticide, antibacterial and fungicide effects have also been observed for Essential Oils (EOs) produced by eucalyptus . Antimicrobial, acaricide, insecticide and herbicide activities associated with EOs from leaves of Eucalyptus have been reported in several articles every year, demonstrating the importance of this research field (Almas et al., 2021).

2.2. Mechanism of action of essential oils

2.2.1. Antimicrobial mechanism of action of essential oils

The hydrophobic nature of EOs allows them to combine with lipids in the cell membranes of bacteria and increase their permeability without destroying the cell structures. This in turn leads to bacterial death due to increased leakage of essential molecules and ions from the bacterial cell (Meenu et al., 2022). EOs are more active in Gram-positive bacteria due to the presence of a peptidoglycan layer outside of their outer membrane. The outer membrane of Gram-negative bacteria consists of a bilayer of phospholipids linked to the inner membrane via lipopolysaccharides (LPS). LPS consist of three main components: Lipid A, a core polysaccharide, and an O-side chain, which are responsible for the resistance of Gram-negative bacteria to EOs. However, the hydrophobic components of EOs can also penetrate their periplasm, due to the presence of porin proteins in their outer membrane (Meenu et al., 2022). In some cases, the combination of one or two EO's results in stronger antibacterial activity than individual actions. However, several studies have also reported that the use of whole essential oils has higher activity than the use of the main ingredients in combination. When an essential oil is combined with an antimicrobial agent, it also has a synergistic effect against multidrug-resistant bacteria, and in many cases the minimum inhibitory concentration (MIC) is significantly reduced (Meenu et al., 2022).

Overall, the biological activities of EOs are positively correlated with their volatile profile. As mentioned in previous studies, the phytochemistry of plant products is significantly

dependent on the species, location, and growing environment, and the composition of EOs is also significantly affected by the part of a plant from which it was extracted, the location of the plant, the growing conditions, and the harvest time of the plant part. In addition, drying time and extraction methods also affect the yield and composition of essential oils. Several reports linked the effects of essential oils from various plant parts against *Listeria* and *Salmonella* (Meenu et al., 2022). Currently, the most common and well-studied mechanisms of action of antimicrobial agents are related to a variety of bacterial targets and processes, such as inhibition of protein synthesis, inhibition of metabolic pathways, impairment of cell wall synthesis, inhibition of DNA and RNA synthesis, and lysis of the bacterial membrane, among others. On the other hand, the most common and well-studied mechanisms of bacterial resistance to antibacterial agents are modification of the antibiotic by enzymes, inactivation of the antibiotic, and expression of efflux pumps (Álvarez-Martínez et al., 2021). Cinnamaldehyde inhibits enzymes responsible for cell wall synthesis in *Saccharomyces cerevisiae* by acting as a non-competitive inhibitor of β-(1,3)-glucan synthase and as a mixed inhibitor of chitin synthase isozymes (Hyldgaard et al., 2012). In addition, the antifungal properties extend well into cell organelles to inhibit efflux pumps as well as mitochondrial membrane potential and mitochondrial respiratory activity through the accumulation of reactive oxygen species (ROS), leading to inhibition of sporulation and germination processes in fungi as well as destabilization of mycotoxine synthesis, ultimately leading to cell death (Nazzaro et al., 2017).

2.3. Aromatic Plants

2.3.1.Eucalyptus (*Eucalyptus globulus Labill.*)

Eucalyptus globulus Labill., or *Bluegum Eucalyptus*, also known as *Tasmanian Eucalyptus*, belongs to the *Myrtacea*e family, and is one of the most known eucalyptus tree species, reaching up to 60 m. Although its native to Oceania, in Europe it is considered an invasive species, still, it is widely used in several industries, specifically the wood industry as well as the paper (Adolphson, 2000).

Figure 4: *Eucalyptus globulus Labill* tree (https://www.invasoras.pt/en/invasive-plant/eucalyptus-globulus)

Eucalyptus characteristic odor is due to the essential oil produced and accumulated in the secretory cells (Barbosa et al., 2016). Eucalyptus oil is usually obtained by steam or hydrodistillation and is either a colorless or pale-yellow liquid (Bello et al., 2021). In addition, studies by Pan et al. (2020) and Palma et al. (2021) on leaves of *Eucalyptus globulus* have shown that *eucalyptus* essential oil is rich in monoterpenes, such as eucalyptol (1,8-cineole) as the main component (67.29%), which in some cases can exceed 80% (Barbosa et al., 2016), followed by pinocarvone (11.33%), pinocarveol (11.11%), α- pinene (9.80%), dihydrocarvone (5.15%), D-limonene (2.59%) and camphene (2.43%), among many others in lower concentrations (Pan et al., 2020). The oil also contains some sesquiterpenes such as β-caryophyllene (2.42%), δ-cadinene (1.52%) (Pan et al., 2020), virdiflorol (5.22%) (Palma et al., 2021), β-eudesmol, globulol, and spathulenol (Jinbiao et al., 2010), and some phenolic acids (2.93%) such as quinic acid (2.4%) and protocatechuic acid (0.15%), which is known for its antibacterial and antioxidant activity (Pan et al., 2020).

Figure 5: Leaves and fruits of *Eucalyptus globulus Labill*.
(https://www.invasoras.pt/en/invasive-plant/eucalyptus-globulus)

Eucalyptus essential oil has bacteriostatic, insecticidal, herbicidal, and antifungal activities (Zhang et al., 2010). For example, the study by Pan et al. (2020) showed results of antifeedant activity on Henosepilachna vigintioctopunctata (Fabricius) due to the repellent potential of protocatechuic acid and citral, while another study by Mareggiani et al., (2008) demonstrated insecticidal activity of *Eucalyptus globulus* essential oil against adult Aphis gossypii (Hemiptera, Aphididae). In general, it has been reported that the Gram-positive pathogenic bacteria *Staphylococcus aureus*, *Staphylococcus intermedius*, *Bacillus subtilis* and other *Staphylococcus* species, the Gram-negative *Escherichia coli*, *Shigella*, as well as some *Salmonella* species and the yeast species *Candida albicans* are sensitive to the essential oil of *Eucalyptus globulus* and other essential oils extracted from various *Eucalyptus* species (Barbosa et al., 2016). The antioxidant activity for which *Eucalyptus globulus Labill*. essential oil is also known for was studied by (Bello et al., 2021) to prove that the polyphenolic compounds that make up *Eucalyptus* essential oil, such as apigenin, luteolin, catechin, and quercetin, significantly inhibit lipid peroxidation, leading to a decrease in malondialdehyde. Beyond its antioxidant activity, *eucalyptus* essential oil possesses remarkable anti- inflammatory activity, which is why it has always been widely used to treat inflammatory symptoms and diseases (Nakamura et al., 2020). In fact, eucalyptol (1,8-cineole), one of the main constituents of *eucalyptus* oil, has been shown to block arachidonic acid metabolism in blood monocytes of asthma patients, inhibit lipopolysaccharide (LPS)-induced production of interleukin 1 beta (IL -1β) by human monocytes and suppresses the production of histamine, interleukin 4 (IL -4), and interleukin 13 (IL -13) (Nakamura et al., 2020). In addition, it has been praised for relieving and improving symptoms of skin irritation in patients with atopic dermatitis (Nakamura et al., 2020). α-terpineol, a

monoterpene found in *Eucalyptus* essential oil, is one of the known bioactive compounds with antitumor activity, reported by (Lahmadi et al., 2021) along with several other bioactive compounds such as trans-myrtanol, myrtenol, and decanoic acid, all of which were extracted from *Eucalyptus torquata Luehm* and *Eucalyptus salmonophloia F. Muell*. to investigate their cytotoxic activity against human colon cancer cell lines SW620 and MDA-MB-231. The study revealed a significant dose-dependent inhibition of the viability of the cancer cell lines, with the essential oils being more cytotoxic to SW620 colon carcinoma cells. The biological activities of eucalyptus essential oil are the reason it is included in numerous pharmaceutical products. In addition to its characteristic odor, the cosmetic and perfume industries have also used the essential oil in everyday products such as air fresheners, toothpastes, mouthwashes, detergents, disinfectants, soaps, skin cleansers, and moisturizers (Barbosa et al., 2016).

As with all other plants, *Eucalyptus* is also prone to changes in its biomolecules and bioactivities through differences in abiotic and biotic factors. Precipitation, temperature, droughts, and other abiotic factors are very important in the adaptation of the secondary metabolism of this tree. For this, when analyzing essential oils and bioactivities of *Eucalyptus* (and all other plants), it is important to analyze them over a course of several years, as their bioactivities tend to change from year to year (Nazari et al., 2023). For instance, a year with very high temperatures, low precipitation or even draughts increase the production of secondary metabolites, as the plants needs to react to these stress conditions.

3. Use of plant volatiles and essential oils against foodborne pathogens

3.1. Foodborne pathogens

From ancient times to the present, foodborne infections and poisonings have been considered an alarming threat to public health, the food industry, and the economy (Ji et al., 2021). Foodborne pathogens are pathogenic microorganisms (bacteria, viruses, fungi, and some parasites) that cause contamination of food by releasing their toxins and are consequently responsible for human infections and poisonings resulting from the consumption of contaminated food (Campini et al., 2021). Bacteria are the main cause of food contamination and human infectious diseases, accounting for 66% of them (Abebe et al., 2020). Symptoms of food poisoning are usually gastrointestinal in nature and are characterized by vomiting, diarrhea, nausea, abdominal cramps, loss of appetite, and sometimes severe complications. The most common strains of bacteria responsible for foodborne contamination and illness are *Staphylococcus aureus*, *Escherichia coli*, *Salmonella Campylobacter spp*., and *Listeria monocytogenes* (Abebe et al., 2020). Fungi such as *Aspergillus spp*, *Fusarium spp*, and *Penicillium spp*. are common and cause the spoilage of many foods and the release of their toxins,

which can distort organoleptic properties and potentially lead to food poisoning (Ji et al., 2021). In addition, the toxins released by these fungi, such as aflatoxins, are considered hazardous to health not only because of their infectivity but also because of their carcinogenic activity (Juárez et al., 2016). This work aims to compare the changes of *Eucalyptus* oils in two consecutive years, as well as to understand the effects this oil can have on the growth of two fungi strains, both in contact with them and through an airborne medium, by placing the fungi and the oils in a closed container.

II. Objectives

Main objective

The main objectives of this work were to use the volatile molecules within EO as airborne preservatives, in order to enhance food preservation and to understand if *Eucalyptus* essential oils can act as antifungals in closed headspace containers.

Specific objectives

- Extraction of essential oils through hydrodistillation
- Evaluation of antioxidant activity through DPPH and RP
- Evaluation of *in vitro* antiproliferative and anti-inflammatory activity
- Evaluation of antimicrobial activity through the microdilution method
- Comparison of the above bioactivities with extracts obtained in the previous year
- Evaluation of antifungal activity through the disk diffusion method
- Implementation of a preliminary airborne fungal sensitivity assay

III. Materials and Methods

3.1 Plant material collection and preparation

Eucalyptus globulus leaves were collected near Águeda, Portugal in October 2021. The samples were air dried in the shade and packed in a carboard box away from light and moisture at room temperature until further analysis. The leaves were reduced to a fine powder using a blender (Moulinex, La Moulinette 1, 2, 3 AD560120 800 W, New Borg El Arab City, Alexandria, Egypt). The samples were then stored in a cool, dry and away from light room.

3.2. Materials and reagents

All materials were purchased from scientific retailers, and were, at least of analysis purity.

3.3 Extraction method

The *Eucalyptus globulus* EO were obtained through conventional hydrodistillation. Hydrodistillation is a conventional method that uses water or steam to extract bioactive compounds, usually essential oils. This technique is usually performed with an apparatus known as the Clevenger. In this apparatus, the hydrated sample is heated to vaporize the volatiles, which are then condensed with a sold solvent back into an oil, that is collected at the end of a fine tube.

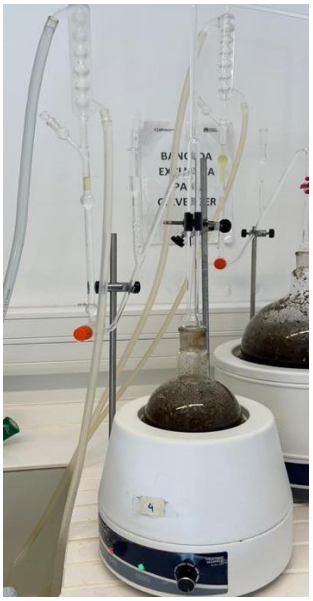


Figure 6: Clevenger apparatus

In the Clevenger apparatus, the hydrated sample is heated to evaporate volatile components, while in steam distillation, steam is passed through a bed containing the sample. Both methods produce two layers (aqueous and oil), and the oil can be further separated using separating funnels. From an economic point of view, this technique does not require organic solvents (Ashraf, et al., 2020), which makes it a desirable option when extraction costs are

important. The EOs were extracted using a Clavenger (Vilabo, Marinha Grande, Portugal), which, for water saving reasons was connected to a water cooling (Huber Minichiller 300 OLÉ, Huber kaeltemaschinenbau, Offenburg, Germany). 50 g of the dried sample, previously sieved to 1 mm particles were joined with 500 mL of distilled water, heated and left to extract for 4 hours and 26 minutes.

The conditions for extraction were based on an optimization previously done within the research group, that defined the optimal extraction yield to be obtained at a solid/liquid ratio of 48.6g/500 mL, a particle size of 1mm and an extraction time of 4 hours and 26 minutes, obtaining about 679.4 mg of essential oil (Hached, 2022).

3.4 Bioactive properties

In terms of the bioactivities of the EOs of *Eucalyptus*, the antioxidant activity was analyzed, as well as the antiproliferative, anti-inflammatory and antimicrobial.

3.4.1 Antioxidant activity

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

The antioxidant activity of the EOs was determined based on the ability of the bioactive molecules to donate hydrogen and decolorize a methanol solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals. DPPH produces a purple color in methanol solution, which changes to yellow in the presence of antioxidants (Fernandes et al., 2023). Thus, 100 mg of the EOs were mixed in 1 mL of methanol to prepare the mother solution with a concentration of 100 mg/mL., 60 µL of the mother solution was poured into each of the first three wells of a 96-well plate, followed by the addition of 30 µL of methanol, and successive dilutions were pipetted in the remaining wells. Then, 270 µL of a methanolic solution of DPPH with a concentration of 6×10-5 mol/mL was added to all wells. The plate was stored in the dark for 60 min to trigger the reaction, and the absorbance was measured at 515 nm using a microtiter plate reader (SpectroStar nano, Labtech, Ortenberg, Germany). The analysis was performed in triplicate, and the EC_{50} was calculated by interpolation using Trolox as the positive control. The EC_{50} is an antioxidant representation of concentration where 50% of radicals are quenched by the antioxidant solution.

3.4.1.2 Reducing Power

Another method used to determine the antioxidant activity was the reducing power, which is based on the on the ability of the bioactive molecules do reduce Fe3+ to Fe2+ Martins et al. (2015). In an Eppendorf tube, 20 mg of EO was weighed to perform successive dilutions with methanol, starting with 20 mg/mL down to 0.0195 mg/mL. After addition of 0.5 mL of sodium

phosphate buffer solution (200 mmol/L; pH=6.6), 0.5 mL potassium ferricyanide (1% w/v) was also added. The mixtures were incubated at 50 °C for 20 minutes to promote the reaction. Then, 0.5 mL of trichloroacetic acid (10% w/v) was added to stop the reaction. The mixture was poured into a 48-well plate, and the procedure was performed in duplicate with 0.6 mL of distilled water and 120 µL of ferric chloride (FeCl3) (0.1% w/v), with the last row of wells being the negative control. The absorbance was measured at 690 nm using a microplate reader (SpectroStar nano, Labtech, Ortenberg, Germany). The antioxidant activity of the essential oils was determined in comparison to Trolox. The results were obtained by determining the EC_{50}.

3.4.2 Antiproliferative Activity

Although not intended for this purpose, the EO were also screened for their potential as antitumor extracts. The cell lines used to evaluate the cytotoxic potential were human cancer cell lines, namely AGS (gastric adenocarcinoma), CaCo2 (colon adenocarcinoma), HeLa (cervical cancer cells), MCF-7 (breast adenocarcinoma), and NCI-H460 (large cell lung carcinoma). AGS and CaCo2 cells were purchased from the European Collection of Authenticated Cell Cultures, while MCF-7 and NCI-H460 cells were provided by the Leibniz Institute DSMZ. Concomitantly, the toxicity to normal cells was assayed using porcine liver cell lines, which were established in the laboratory. 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). Culture flasks were incubated at 37 °C and 5% CO2 in a humid atmosphere to mimic physiological conditions. When confluence reached 70–80%, the cells could be used for further analysis. To obtain stock solutions with a concentration of 8 mg/mL, 8 mg of the extracts were dissolved in 1 mL of distilled water. From this, successive dilutions were made to obtain the concentrations of interest (0.125 - 8 mg/mL). 10 µL of the previously prepared extractions were added to 190 µL of the cell suspension of the tested cell lines in 96-well microplates after ensuring that the cells were well adherent. The microplates were incubated for 72 hours at 37 °C and 5% CO2 in a humidified atmosphere. All cell lines were tested at a concentration of 10,000 cells/well.

After the incubation period, previously cooled trichloroacetic acid (TCA) (10% w/v; 100 µL) was added to stop the reaction, and the plates were incubated at 4 °C for 1 hour. They were then washed with distilled water and dried. Then, sulforhodamine B (SRB) solution (0.057%, w/v; 100 µL) was added, and the plates were allowed to stand at room temperature for 30 minutes to ensure the adhesion of SRB to the tumor cell lines. To remove the non-adherent SRB, the plates were washed three times with an acetic acid solution (1% v/v) and laid out to dry. Finally, the adherent SRB was dissolved with Tris (10 mM, 200 µL) and the absorbance

was read in the previously mentioned microplate reader at 540 nm. Results were expressed as concentration of extract inhibiting cell growth by 50% (GI_{50}). The positive control was ellipticin. To rule out cytotoxicity of the EO towards "normal" cells, two cell lines were tested with them, namely PLP2 and VERO cell lines. The PLP2 cell lines are hepatocytes from the liver of pigs, while the VERO cell line pertains to the epithelial cells of the kidney of African green monkeys. Both assays followed the same procedure of the tumor cell lines.

3.4.3 Anti-inflammatory activity

As for the antiproliferative assay, anti-inflammatory activity was also tested. 8 mg of the extracts were dissolved in 1 mL of water to obtain a final concentration of 8 mg/mL, from which successive dilutions were made to obtain 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 ℃ and 5% CO2 and under humid atmosphere. Macrophages were separated to prepare a cell suspension, of which an aliquot (300 µL) with a cell density of 5 x 105 cells/mL and a percentage of dead cells below 5% was added to each well according to the trypan blue exclusion test. To ensure high confluence and sufficient proliferation of cells, the microtiter plate was incubated for 24 hours at 37 ℃ and 5% CO2 and in a humid atmosphere. After 24 hours, the cells were treated with the different concentrations of the samples (15 µL, 0.125 - 8 mg/mL), followed by a one-hour incubation, where the range of tested concentrations was 6.25 - 400 µg/mL. To stimulate the anti-inflammatory response in the cells, 30 µL of liposaccharide solution - LPS (1 mL/mL) was added, and the microtiter plates were placed in the incubator for 37 additional 24 hours. Dexamethasone (50 mM) was used as a positive control and samples in the absence of LPS as a negative control. Nitric oxide (NO) was quantified using a Griess reagent system (nitrophenamide, ethylenediamine, and nitrite solutions) and the nitrite calibration curve (100 mM sodium nitrite at 1.6 mM) prepared in a 96-well plate. By reading the absorbance values at 540 nm and comparing with the standard calibration line, the amount produced was calculated from NO. The results were calculated by graphing the percentage inhibition of nitric oxide production as a function of sample concentration and expressed in terms of the concentration of each of the extracts that causes 50% inhibition of nitric oxide production - IC_{50}.

3.4.4 Antimicrobial activity

3.4.4.1 Antibacterial activity – Microdilution method

The extracts were tested against a total of eight food contaminants (bacteria). Five Gram-negative bacteria, namely *Enterobacter cloacae* (ATCC 49741), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 9027), *Salmonella enterica* (ATCC 13076), *Yersinia enterocolitica* (ATCC 8610), and three Gram-positive bacteria, namely *Bacillus cereus* (ATCC 11778), *Listeria monocytogenes* (ATCC 19111), and *Staphylococcus aureus* (ATCC 25923) were assayed against the *Eucalyptus* EOs. All microorganisms are purchased from Frilabo, Porto, Portugal, incubated at 37 ºC for 24 hours in appropriate fresh medium prior to analysis to preserve the exponential growth phase. Following the procedure described by Pires et al. (2018), 50 mL of Muller-Hinton-Broth medium (MHB) and 250 µL of Tween 80 (MHB with 0.5% Tween 80) were combined in a sterile Schott flask to prepare the culture medium. Thirty mL of MHB medium was added to a sterile Schott bottle, followed by 200 µL of inoculum (standardized to 1.5 x 10^6 colony-forming units (CFU)/mL). Concentrations of 2.5%, 1.25%, 0.625%, 0.313%, 0.156%, 0.078%, 0.03%, and 0.01% were used to prepare sample dilutions. To the first well of a 96-well microplate, 190 µL of MHB medium with Tween 80 and 10 µL of the sample were added in duplicate to the remaining wells, 90 µL of MHB medium with Tween 80 was added. Samples were then serially diluted to obtain concentration ranging from 2.5% to 0.01%. 100 µL of the inoculum (standardized to 1.5 x 10^5 colony-forming units (CFU/mL) was poured into the wells. Two negative controls, one with MHB and Tween 80 and another 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. Ampicillin and streptomycin were used for all bacteria tested, and methicillin was used for Staphylococcus aureus. To determine the minimum inhibitory concentration (MIC) of all bacteria, the microdilution method and the dye p-iodonitrotetrazolium chloride (INT) were used, allowing colorimetric measurement as described by Pires et al. (2018) with some adjustments. For this purpose, 40 µL 0.2 mg/mL p-iodonitrotetrazolium chloride (INT), an indicator dye used as a microbial growth indicator, was added to the wells and the microtiter plates were then incubated at 37 ºC for 30 min. The minimum inhibitory concentration (MIC) of the samples was defined as the lowest concentration required to inhibit bacterial growth, indicated by a color change from yellow to pink upon two-electron capture, when the microorganisms are viable (Milagres de Almeida et al., 2023). To determine the minimum bactericidal concentration (MBC), 10 µL of liquid from each well that showed no color change was applied to a solid medium, blood agar (7% sheep blood), and incubated for 24 hours at 37 ºC. The lowest concentration at which no growth occurred determined the MBC, defined as the lowest concentration required to kill bacteria.

3.4.4.2 Antifungal activity – Microdilution method

The antifungal activity was performed as described by Heleno et al. (2013) with some rearrangements. The fungal strains used were obtained from Frilabo, Porto, Portugal, namely *Aspergillus fumigatus* (ATCC 204305) and *Aspergillus brasiliensis* (ATCC 16404). The fungi were maintained on malt agar and the cultures were stored at 4 ℃ before being transferred to a new medium and incubated at 25 ℃ for 72 hours. For the antifungal activity assay, 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 105 to a final volume of 100 µL per well. Samples were first dissolved in Mueller-Hinton agar (MHA) medium containing Tween 80. Next, 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. 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 in 96-well microplates. Minimum fungicidal concentrations (MFCs) were determined by serially subculturing 2 µL of the extracts dissolved in the medium and inoculating them into microplates containing 100 µL of MEB per well for 72 hours, followed by incubation at 26 °C for 72 hours. The minimum fungicidal concentration was defined as MFC, indicating 99.5% kill of the original inoculum. The commercial fungicide ketoconazole (Frilabo, Porto, Portugal) was used as a positive control.

3.4.5 Agar disk diffusion methodology

The agar disk diffusion method is the established procedure used in many clinical microbiology laboratories for performing routine antimicrobial susceptibility testing. In the case of this work, it was used to understand if the EO could inhibit the growth of the fungi through physical contact. The used fungi were the ones available in the laboratory, namely the ones used in the microdilution method, *Aspergillus fumigatus* (ATCC 204305) and *Aspergillus brasiliensis*. The Clinical and Laboratory Standards Institute (CLSI) publishes a variety of recognized and sanctioned standards for the testing of bacteria and yeasts (CLSI et al.,2019) (CLSI et al.,2023). Muller-Hinton agar plates are inoculated with a standardized concentration of about 1–2×10^8 CFU/mL on the surface of a large agar dish (with a diameter of 55 mm) containing the test microorganism (Xavier et al.,2023). Still, in this assay, lower concentrations were chosen due to the preliminary nature of the whole study, and the fact that the inhibition would occur through airborne molecules, rather than through physical contact, which is, expectedly, more effective. Thus, concentrations of 1×10^7, 1×10^6 and 1×10^5 CFU/mL were used, and the results compared in terms of the inhibition halo. Subsequently, filter paper discs,

approximately 6 mm in diameter, containing the test compound (EO), are carefully positioned onto the center agar surface. A filter paper disc without any extract was used as a negative control. Petri dishes then were subject to culturing at 25 ºC for 72 hours. For the preparation of Muller-Hinton Agar, according to the manufacturer's instructions the petri dishes had a depth of 4.0 ± 0.5 mm.

3.5 Sensitivity assay

The sensitivity assays are two assay that have been preliminary implemented in the research group following the work carried out in this dissertation. They are a means of testing the sensitivity of fungi to volatile molecules within a closed headspace, and without physical contact between the microorganism and the extract, being the growth inhibition achieved through airborne (volatile) molecules present in the extracts.

3.5.1 Culture start inhibition

The culture start inhibition assay was used to evaluate the inception of fungal growth, by placing EOs immediately after plating the fungi on the petri dishes. Thus, the above-mentioned fungi were suspended and plated in a petri dish with malt agar containing 1-2×10^8 CFU/mL. The petri dishes were immediately introduced in airtight containers with 18x11x6 cm (length, depth, height), approximately 1 L of volume. A container with no essential oil was used as a negative control. The assay was carried out at least in duplicate. Four batches were prepared, two for each fungus. Two batches (one for each fungus) used 1 mL of *Eucalyptus* EO, while the other two batch used 3 mL. After incubation at 25 ºC for 72 hours the containers were opened, and the fungal growth was examined.

3.5.2 Growth inhibition

The growth inhibition assay is quite similar to the culture start assay, although the addition of the EO is done in the growth phase of the fungi. Thus, the same fungal species were plated in petri dishes containing malt agar at the same concentration (1-2×10^8 CFU/mL). Four sets were prepared for each fungus in this analysis: Set A – Two petri dishes inoculated with the fungus and incubated for 72 hours (negative control). Set B – Two petri dishes inoculated with the fungus, with 1 mL of EO being added after 24 of fungal growth. Set C - Two petri dishes inoculated with the fungus, with 1 mL of EO being added after 48 of fungal growth. Set D – Two petri dishes inoculated with the fungus with 1 mL of EO, incubated for 72 hours. All sets were incubated at 25 ºC. After 72 hours, the containers were opened, and the growth of the fungi analyzed.

3.6 Statistics

Throughout the whole document, all data was expressed as mean ± standard deviation (SD). An analysis of variance (ANOVA) was used to analyze the samples, relying on a Student's T-test for classification. For all statistical analysis the significance was set at 0.05.

IV. Results and Discussion

The results obtained for the evaluation of *Eucalyptus* EOs is shown below, including comparison with the results obtained in assays performed last year (2022) on the leaves of the same trees.

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

4.1.1 Antioxidant activity

The antioxidant activity of the EOs was analyzed using two different in vitro assays, DPPH and RP, 2,2-diphenyl-1-dicrylhydrazyl and reducing power, respectively. Regarding the DPPH assay, the EC_{50} (concentration that quenches 50% of the DPPH radical) showed an activity of 50.9 mg/mL (Table 2), which compared to the previous year, 47.55±4.27 mg/mL was a very similar result, without any significant changes (Hached, 2022). Still, further comparing the results with other works of literature a work by Spadi et al. (20211) showed an EC_{50} of *Eucalyptus* EO of 145.5±0.7 mg/mL. Overall, these results show some interesting results, firstly the non-significant variation in antioxidant activity from 2022 to 2023, but a great statistical difference between the results obtained in this work when compared to the one from Spadi et al. (2021). The authors (Spadi et al., 2021) did not disclose the origin of the *Eucalyptus* leaves, the state of maturation of the plant they were extracted from, only disclosing the length of extraction with the clavenger, 3h. This highlights the importance of extraction optimization, whose optimal points were used in this work. Still, even though the EO extract used in this work showed quite interesting activity against DPPH assay it did not come close to activity of Trolox, the positive control, with an EC_{50} of 41 μg/mL.

Table 2: Assessment of antioxidant activity using DPPH assay

	Hydro distillation (mg/mL)	Standard (μg/mL)
	E. globulus	Trolox
DPPH	50.9±0.6	41±3

With respect to RP, the *Eucalyptus* EO did not yield any type of results, as the successive dilutions did no show linear behavior, thus, due to time constraints, the repetition of this assay could not be concluded in time to be included in this work.

4.1.2 Antiproliferative activity

The effects of the extracted EOs on the growth of six human tumor cell lines were evaluated according to the National Cancer Institute (NCI) in vitro screening of anticancer

drugs procedure, which uses the sulforhodamine B (SRB) assay to assess cell growth inhibition. The results are reported as GI50 (concentrations of extract that produces 50% inhibition of cell growth). This study was performed on five human tumor cell lines selected as representative of lung, breast, colon, gastric, and cervical cancers, and on a two non-tumor cell line PLP2 and VERO to exclude possible toxicity to normal cells. From the results shown in Table 3, *Eucalyptus* EO showed the best results for the gastric cancer cell line AGS (GI_{50} = 239.47 μg/mL), and the lowest for MCF-7 and NCI-H460 in *ex aequo* at 318 μg/mL. Overall, compared to the positive control ellipticine, the EO did not come close to being antitumor extracts. Comparing the obtained results with the ones obtained in the samples of 2022, there are significant differences, namely a better activity for AGS (gastric), CaCo2 (colorectal) NCI-H460 (lung) cell lines in 2023, while the extracts from 2022 showed a better result for MCF-7 (breast). In 2022, neither the HeLa cell lines, or VERO were tested. Comparing these results to Spadi et al. (2021), the authors only showed results for gastric cell lines, in which their results showed a GI_{50} of 73±5 μg/mL, revealing a better antitumor activity. These results are also similar to Abiri et al. (2022), who highlighted the antitumor activity of Eucalyptus essential oil, showing that experimental and clinical studies demonstrated the cytotoxicity on human carcinoma cells (A549) and cervical cancer cells (HeLa) (Molodinashvili et al., 2019).

Overall, the idea of EOs as antitumor extracts is a longshot, and thus, the most important results shown in Table 3 are the cytotoxic effects on PLP2 and VERO cells. These results are inspiring as well as interesting as the *Eucalyptus* EOs did not show any toxicity to the PLP2 cells, showing a value over 400 μg/mL, while the VERO cell GI_{50} was set at 377 μg/mL. This result, despite being below the safety threshold is considered and excellent cytotoxic result, as VERO cells are very sensitive to any type of natural extract. Thus, toxicity of *Eucalyptus* can be ruled out in terms of in vitro assays.

Table 3: Evaluation of the antitumor and cytotoxic of the EOs against five cancer cell lines (AGS, CaCo2, MCF-7, NCI-H460 and HeLa) and against two normal cell lines (VERO and PLP2), all expressed as GI_{50} (μg/mL)

	Hydro distillation	Standard
	E. globulus	Ellipticine
Antitumor potential (GI50 μg/mL)		
AGS	239±16	1.23±0.03
CaCo2	275±10	1.21±0.02
MCF-7	318±5	1.02±0.02
NCI-H460	318±5	1.01±0.01
HeLa	281±13	1.40±0.06
Cytotoxic activity (GI50 μg/mL)		
VERO	377 RAW 264.714	1.41±0.06

PLP2	>400	1.4±0.1

4.1.3 Anti-inflammatory activity

The anti-inflammatory activity of the EO is shown in Table 4, obtained by measuring the inhibition of NO production. According to the results, *Eucalyptus* showed a reasonable ability to inhibit inflammatory responses, scoring 319±8 μg/mL. These results are statistically higher than the ones found for the sample of 2022, of 41±3 μg/mL (Hached, 2022). This is first assay in which the changes from one extract to the other are so drastic.

Table 4:Evaluation of the anti-inflammatory activity the EOs on RAW 264.7 murine macrophage cells expressed in (μg/mL)

	Hydro distillation	Positive control
	E. globulus	Dexamethasone
RAW 264.7	319±8	6.3±0.4

Numerous pre-clinical studies have reported the anti-inflammatory effects of *Eucalyptus* EO in in vitro and in vivo models (Chandorkar et al. 2021; Ho et al. 2020; Lin et al. 2018; Shao et al. 2020). Essential oils (EOs) exhibit promising potential as anti-inflammatory agents for various applications, as indicated by the findings of Arooj et al. (2023). They reported a maximum anti-inflammatory effect of 40.96 ± 1 μg/mL , investigating the anti-inflammatory mechanisms of Eucalyptus globulus EO, particularly rich in eucalyptol, both individually and in combination with flurbiprofen.

4.1.4 Antimicrobial activity – Microdilution method

Antimicrobial activity of the EO was evaluated using the microdilution method for both the bacteria and fungi, which allows the determination of minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC). Eight foodborne pathogenic bacterial strains as well as two fungal species were used to evaluate the antimicrobial potential of the EO, and the results are shown in Table 5 and 6.

4.1.4.1 Antibacterial activity

The antibacterial activity of the EOs is expressed in Table 5, exhibiting efficacy against five Gram-negative bacteria and three Gram-positive strains commonly associated with foodborne infections. Among the Gram-negative strains, *Yersinia enterocolitica* displayed the

highest inhibitory activity with the lowest MIC value (0.625 %), whereas other bacteria showed MIC values of either 2.5% or higher. The MBC was consistently above 2.5%. for all strains. Considering Gram-positive bacteria, *Staphylococcus aureus* showed the most inhibition at 0.15 %, followed by *Listeria monocytogenes* at 0.31%. The MBC was equal to that of Gram-negative bacteria, at higher concentration of 2.5%. Overall, the EO demonstrated greater effectiveness against Gram-positive bacteria. The inhibition observed for *Staphylococcus aureus* is quite surprising, as it's the same as the value found for the positive control, while for *Listeria monocytogenes*, the MIC of the EO is only double the value found for the positive control.

Table 5: MIC and MBC of the Eucalyptus EO

	Eucalyptus		Streptomycin		Methicillin		Ampicillin	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Gram-negative Bacteria								
Enterobacter cloacae	>2.5	>2.5	0.007	0.007	n.t	n.t	0.15	0.15
Escherichia coli	2.5	>2.5	0.01	0.01	n.t	n.t	0.15	0.15
Pseudomonas aeruginosa	>2.5	>2.5	0.06	0.06	n.t	n.t	0.63	0.63
Salmonella enterica	2.5	>2.5	0.007	0.007	n.t	n.t	0.15	0.15
Yersinia enterocolitica	0.625	>2.5	0.007	0.007	n.t	n.t	0.15	0.15
Gram-positive Bacteria								
Bacillus cereus	2.5	>2.5	0.007	0.007	n.t	n.t	n.t	n.t
Listeria monocytogenes	0.31	>2.5	0.007	0.007	n.t	n.t	0.15	0.15
Staphylococcus aureus	0.15	>2.5	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. The positive control are presented in 1mg/mL (for Methicillin and Streptomycin) and in 10mg/ mL (for Ampicillin).

Upon comparing the findings of this study with those of Hached (2022), who investigated the same species of *Eucalyptus globulus* last year, several distinctions become evident. While the MIC and MBC's are similar in terms of being quite high, over 2.5 %, still, the best result for Hached's work was found for *Salmonella enterica* and *Enterobacter cloacae* (0.6% and 1.25%), both Gram-negative bacteria. This result can be linked with differences in the *Eucalyptus globulus* EO resulting from abiotic factors, in which specific conditions promoted the over or underproduction of a specific compound that one bacterial species is especially susceptible to, thus justifying these slight differences. Many studies have studied the antibacterial capacity of Eucalyptus EO, including Pino et al. (2021) who highlighted an antibacterial capacity of against *Escherichia coli* and *Staphylococcus aureus* reported a MIC value of 1.90% and 2.87% respectively.

4.1.4.2 Antifungal activity

The antifungal activity was assessed against two foodborne fungi, namely *Aspergillus brasiliensis* and *Aspergillus fumigatus*, which are cultured routinely in the laboratory. The found MIC are quite remarkable, in which they are 0.07% for *Aspergillus brasiliensis* and 0.6%

for *Aspergillus fumigatus*, respectively. These results inspired the prosecution of assays into the realm of airborne inhibition with the EO. The results obtained for Hached, (2022), with *Eucalyptus globulus* EO obtained from the previous year show a very similar result, namely a MIC of 0.07% for *Aspergillus brasiliensis* and a MIC of 0.6 % for *Aspergillus fumigatus*. This, firstly shows that the antifungal activity is quite remarkably preserved in the two years of analysis. Furthermore, it also inspired the use of these specific EO to continue the analysis with sensitivity tests. In summary, the *Eucalyptus globulus* essential oil has demonstrated reliable and consistent antifungal activity over two years, indicating its potential for future applications.

Table 6: MIC and MFC of the Eucalyptus EO

	Eucalyptus		Ketoconazole	
	MIC	MFC	MIC	MFC
Aspergillus brasiliensis	0.07	2.5	0.06	0.125
Aspergillus fumigatus	0.6	2.5	0.5	1

4.1.5 Disk diffusion assay

The disk diffusion test is used to evaluate the susceptibility of a microorganism to an antimicrobial drug. In this work, due to the remarkable MIC obtained for the two fungi, they were both subjected to this test using different spore concentrations against the *Eucalyptus globulus* EO, impregnated in a 6 mm disk, placed in the center of the petri dish. Figure 8 shows the results obtained for this assay.

Figure 7: Petri dishes with the different inhibition halos at different concentrations for the two fungi species.

The disk diffusion assay showed that both fungi are susceptible to the *Eucalyptus* EO, even at the highest concentration of CFU (1×10^7 CFU/mL). In terms of intrasample variance, it was hardly noticeable, and thus, the comparison was done without any statistical treatment. Still, *Aspergillus brasiliensis* seems to be more susceptible to the EO as the inhibition halos showed a considerably larger diameter, which showed a variation under 0.1 mm between the different concentrations. *Aspergillus fumigatus* reduced the inhibition halos with the increasing concentration. The biggest inhibition halo, with 10 mm of diameter was sought for 1×10^5 CFU/mL, thus reducing to 7 mm at 1×10^6 CFU/mL and finally only 5 mm at 1×10^7 CFU/mL. This second assay to assess the antifungal activity through contact with the disk further confirmed the expectations that *Eucalyptus* EO would have some kind of inhibition at an airborne level and thus, the next step implied the execution of those assays.

4.2 Sensitivity test

4.2.1 Culture start inhibition

The first assay to determine the inhibition of the 2 fungi species was performed at two different volumes of EO. Due to the most sensitive interval of a culture being when it establishes itself, the first assay was to determine what volume of EO would be enough to inhibit the growth of the fungi. For this, batches of the fungi were made, adding to one of them 1 mL of the EO, and to the other 3 mL, and left to grow at 25 ºC for 72 hours. The results of this assay are shown in Figure 9. A control batch was also performed, where no EO was added, being the rest of the conditions the safe. The fungi were plated at a concentration of 1×10^8 CFU/mL, following the Clinical and Laboratory Standards Institute, due to the confirmation in the previous assay that at lower concentrations the fungi were completely inhibited.

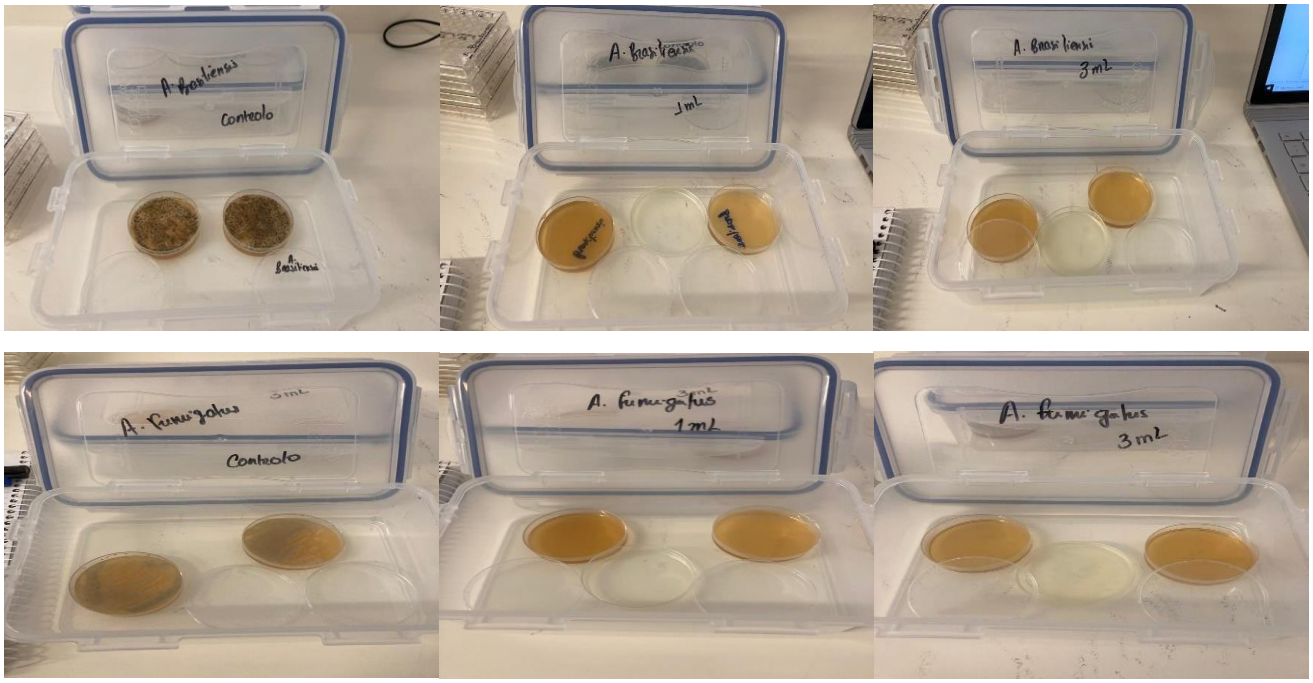


Figure 8: Petri dishes showing the culture star inhibition with 1 and 3 mL of EO for both fungi

Considering *Aspergillus brasiliensis*, it is clear that the EO inhibited the growth of the fungi, especially when comparing the petri dishes with the control batch, in which the fungi completely covered the surface of the dish. The same was recorded for *Aspergillus fumigatus*, in which, for the control sample, the fungi completely covered the surface, which is clear in both the samples with 1 and 3 mL of *Eucalyptus* EO. Overall, this preliminary assay showed that the EO could completely inhibit the growth of the fungi though airborne molecules present in the oil, although the exposure of the fungi to the oils was immediately after planting them, which is an especially vulnerable phase for fungi. Overall, 1 mL of EO is enough to inhibit the

growth of 2 petri dishes inoculated with the two fungi, plated at a concentration of 1×10^8 CFU/mL

4.2.2 Growth inhibition

The final assay performed in terms of airborne inhibition of the two fungi was performed at different stages of the growth phase. Due to the previous confirmation that the EO inhibited the growth in an early stage, in this assay, the addition of the oils was done after 24 hours of microbial growth for one batch, at 48 hours for another and left to grow until a maximum of 72 hours. As the previous assay allowed to infer, 1 mL was enough to inhibit the fungi at the early stages, and thus, only 1 mL was used to try and inhibit the growth in the more resistant phases. The growth phase, when the fungi is completely established is the strongest phase, where it is more resistant to inhibitory extracts, and this assay was able to show if these two specific fungi were susceptible to the oils in these stages. The results are shown in Figure 10.

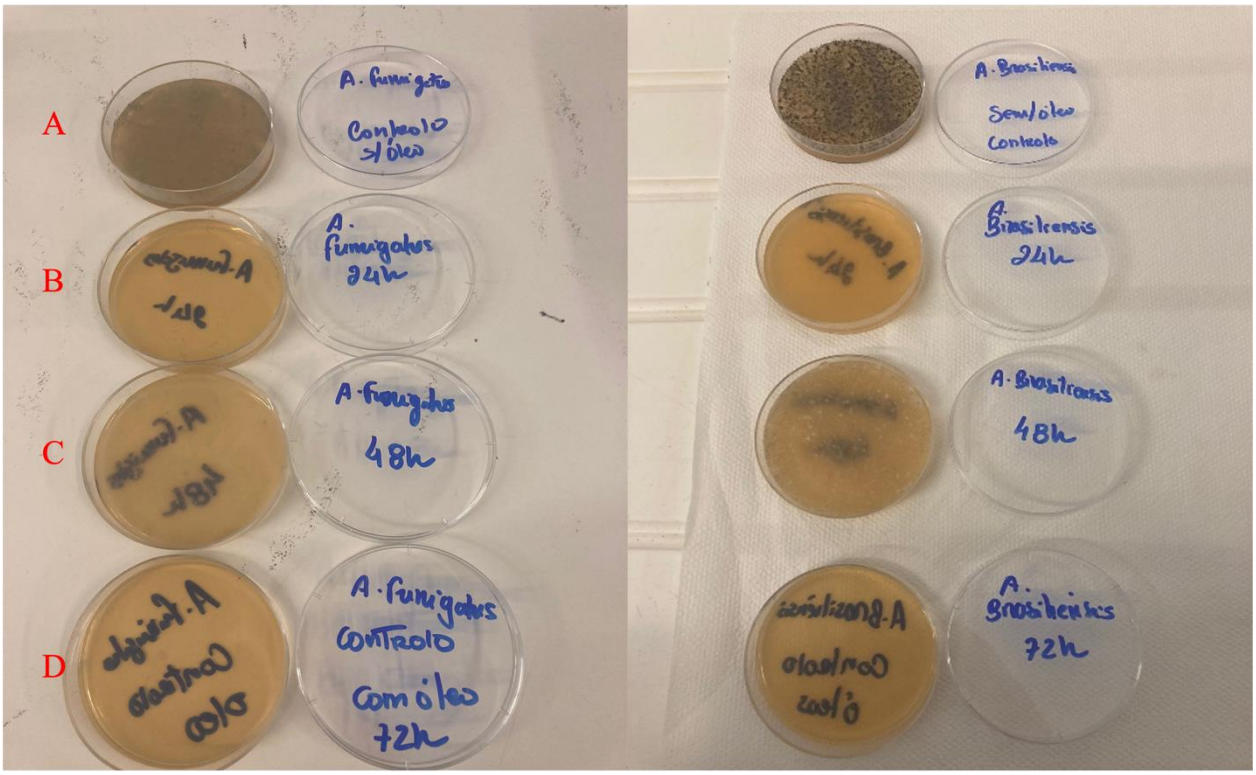


Figure 9: Petri dishes showing the inhibition of the two fungi at different points of addition of the EO. A -negative control, no EO; B - EO added after 24h of fungal growth; C - EO added after 48h of fungal growth; D – EO added at the beginning (positive control)

Considering the results of the growth inhibition assay, it is clear that in the negative control (set A), both the fungi grew quite vigorously, and where completely inhibited in the positive control, where they did not even start growing, as the medium is completely transparent, further corroborating the claim that *Eucalyptus* EO completely inhibit the growth

of these two *Aspergillus* species. The second set (B), in which the EO were added after 24h of free growth of the fungi, in the case of *Aspergillus fumigatus*, the plate was almost completely transparent, showing an almost completely inhibition, while small spots were found in the corresponding petri dish for *Aspergillus brasiliensis*. In the case of the addition of EO after 48 hours, the same trend was sought, although in this phase, where the growth was quite vigorous, especially for *Aspergillus brasiliensis*, there is still some growth, although a high growth hindering activity can be observed. It seems that *Eucalyptus* EO inhibited quite well both *Aspergillus* species, even at the most resistant stages of growth, in a medium that favors the growth of these microrganisms. Furthermore, it seems that *Aspergillus fumigatus* is the most susceptible fungus.

V. Conclusions and Future Perspectives

The work contained in this document is integrated in a bigger ongoing project that aims to uncover how volatile compounds can reduce microbial load in fresh produce. Thus, the main objectives of this work were to understand the stability of Eucalyptus EO over two years, being the leaves picked from the same trees, both in 2022 and 2023. Furthermore, it also aimed at implementing preliminary assays of sensitivity of fungi to the volatile compounds of EO. Regarding the stability of bioactivities of EO over the two years, the DPPH antioxidant activity showed relatively the same EC_{50} values, while the RP in 2023 showed erroneous values and was discarded. In terms of antitumor activity, while there were variations in the GI50, none of the extracts showed any real perspective as an antitumor extract. Still, more important than the antitumor activity is the cytotoxicity of the extracts which in both years was deemed not cytotoxic. In the 2023 samples the very sensitive VERO cells were testes and even in those cells no cytotoxicity was sough, which is quite satisfying in terms of the security of using these EO. In terms of the antibacterial activity, the EO of 2022 showed higher effectiveness against other bacterial strains when compared to the ones of 2023, although for the antifungal activity the MIC's of both fungi were almost the same. Overall, the EO of 2022 and 2023 showed slight differences, but overall, in terms of the bioactivity they were maintained from one year to the other. Still, further research should be carried out, namely the identification of the individual molecules, to further understand the differences in some bioactive properties.

The second objective was the implementation of new sensitivity assays for volatiles to inhibit fungi in a closed container. In this case, both Aspergillus brasiliensis and Aspergillus fumigatus were inhibited by the EO in all stages of growth, namely at the plating stage, after 24 and 48 hours. This, in a food industry perspective is quite promising, as EO can be used to either protect foods from microbial spoilage in closed contained during transport, or even hinder their growth after contamination has been started. Still, these assays are preliminary, and this project can still be improved in several aspects. The EO should be tested in bigger containers, at lower EO volume and higher microbial load and against other foodborne fungi, and even with a larger contaminated surface. The tested fungi were not the ideal ones, as there are others more ubiquitous in food contamination, but at the time of writing this thesis, they were not implemented in the laboratory. In terms of the next stage for this work, food will be used as a substrate for fungal contamination, as in, a food will be deliberately contaminated and allowed to be in a closed container with the EO to understand if there is any type of inhibition.

Still, the results achieved with this work are both promising and reassuring. Promising due to allowing knowledge of airborne fungicidal extract to be uncovered and reassuring for showing that in two consecutive years the efficacy of Eucalyptus EO do not change considerably.

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