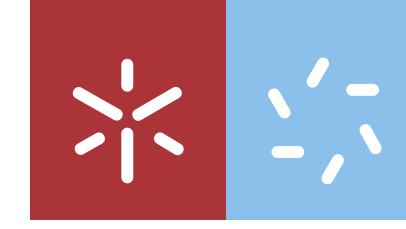**Universidade do Minho**
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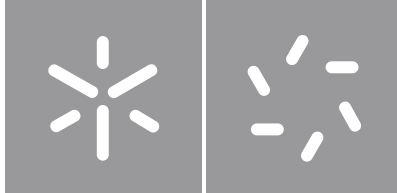**Universidade do Minho**
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Master Thesis
Plant Molecular Biology, Biotechnology and Bioentrepreneurship

Work performed under the guidance of
Professor Maria João Marques Ferreira Sousa Moreira

Dezembro de 2021

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AKNOWLEDGEMENTS

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University of Minho, 29/12/2021

Full name: Kelly Teixeira

TITLE: EVALUATION OF THE ACTIVITY OF *Satureja montana* AND *Thymus vulgaris*, FREE AND NANO-ENCAPSULATED ESSENTIAL OILS, AGAINST THE FOOD SPOILAGE YEAST, *Zygosaccharomyces parabailii*

ABSTRACT

Currently, the world losses of food products present values between 8% and 41%. This fact is related to post-harvest losses and also to the processing and packaging of goods, under the spoilage effects of microorganism, namely yeasts. The use of essential oils with antifungal and antimicrobial capacity has been widely studied by the scientific community, in different areas of interest. However, characteristics such as low stability and high volatility lead to loss of activity. The encapsulation of these compounds derived from the secondary metabolism of plants, can enhance activity and provide greater stability for the volatile compounds. In this study, the objective was to test different particle formulations, encapsulating the essential oils (EOs) of *Thymus vulgaris* and *Satureja montana,* against the food spoilage yeast *Zygosaccharomyces parabailii*, in view of their potential use as a biopesticide and food preservatives. In particularly zein, keratin and liposome particles were tested. The growth of *Z. parabailii* was reduced with all tested formulations for both EOs. Encapsulated *S. montana* essential oil (SMEO) originated a decrease in growth from 100 μg/mL, however, the essential oil of *T. vulgaris* (TVEO) did not inhibited growth significantly for this concentration. In over 60% of formulations examined, the MIC obtained for SMEO was 200 μg/mL. On the other hand, for TVEO a MIC of 200 μg/mL was only achieved with free essential oil. Regarding the assessment of plasma membrane integrity, at the concentration of 500 μg/mL the SMEO keratin particles had the most severe effect, reaching a maximum of 94% PI positive cells. Since this was also the condition for which CFUs were no more observed, the results suggest necrosis as a potential cell death mechanism. Fluorescence microscopy experiments with keratin SMEO particles labelled with FITC, showed that all green-labelled cells were also PI positive, again suggesting that the effect of the particles is directly related to membrane permeabilization. In summary, the essential oil *Satureja montana* demonstrated the best antifungal activity and the best efficacy was with the keratin particles formulation.

TÍTULO: EVALUATION OF THE ACTIVITY OF *Satureja montana* AND *Thymus vulgaris*, FREE AND NANO-ENCAPSULATED ESSENTIAL OILS, AGAINST THE FOOD SPOILAGE YEAST, *Zygosaccharomyces parabailii*

RESUMO

Atualmente, as perdas mundiais de produtos alimentícios apresentam valores entre 8% e 41%. Este facto está relacionado com as perdas pós-colheita e também com o processamento e embalamento de alimentos, sob os efeitos da deterioração dos microrganismos, nomeadamente as leveduras. O uso de óleos essenciais com capacidade antifúngica e antimicrobiana tem sido amplamente estudado pela comunidade científica, em diferentes áreas de interesse. No entanto, características como baixa estabilidade e alta volatilidade levam à perda de atividade. A encapsulação desses compostos derivados do metabolismo secundário das plantas, pode potencializar a atividade e proporcionar maior estabilidade aos compostos voláteis. Neste estudo, o objetivo foi testar diferentes formulações de partículas, encapsulando os óleos essenciais (EOs) de *Thymus vulgaris* e *Satureja montana*, contra a levedura de deterioração alimentar *Zygosaccharomyces parabailii*, tendo em vista seu potencial uso como biopesticida e conservante de alimentar. Em particular, partículas de zeína, queratina e lipossomas foram testadas. O crescimento de *Z. parabailii* foi reduzido com todas as formulações testadas para ambos os EOs. O óleo essencial de *S. montana* encapsulado (SMEO) ocasionou redução no crescimento a partir de 100 µg/mL, porém o óleo essencial de *T. vulgaris* (TVEO) não inibiu o crescimento significativamente para esta concentração. Em mais de 60% das formulações testadas, o MIC obtido para SMEO foi de 200 µg/mL. Por outro lado, para TVEO um MIC de 200 µg/mL, só foi alcançado com óleo essencial livre. Em relação à avaliação da integridade da membrana plasmática, na concentração de 500 µg/mL as partículas de queratina SMEO tiveram o efeito mais eficiente, atingindo um máximo de 94% de células PI positivas. Com esta concentração também não foram observados CFUs, os resultados esses que sugerem que ocorre necrose como mecanismo potencial de morte celular. Ensaios de microscopia de fluorescência com partículas de queratina SMEO marcadas com FITC, mostraram que todas as células marcadas com verde também eram PI positivas, sugerindo novamente que o efeito dos partículas está diretamente relacionado à permeabilização da membrana. Em resumo, o óleo essencial *Satureja montana* demonstrou a melhor atividade antifúngica e a melhor eficácia com a formulação de partículas de queratina.

INDEX

ABREVIATIONS

EO	Essential oil
CFU	Colony forming units
MIC	Minimum inhibitory concentration
OD	Optical density
SD	Standard deviation
PDI	Polydispersity index
PI	Propidium iodide
MFC	Minimum Fungicidal Concentration
SMEO	*Satureja montana* essential oil
TVEO	*Thymus vulgaris* essential oil

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INTRODUCTION

1. FOOD SPOILAGE

The world's population is currently 7.8 billion people, with a growth rate of 1.1 percent each year. Over the last three decades, population growth has resulted in an increase in the demand for food goods. It is estimated that one-third of the food produced for human consumption is wasted, amounting to around 1.3 billion tons (Bellú, 2017). Agriculture plays an important role in responding to the demand for fresh foods but is intensification has had detrimental environmental and health consequences due to the extensive use of synthetic fertilizer, pesticides and irrigation (Roberts *et al.*, 2021).

The major cause of food waste occurs in fresh food goods, mainly in vegetables and fruits, and is associated with field illnesses or post-harvest infections (Alghuthaymi *et al.*, 2021; De Oliveira *et al.*, 2018). Microbial spoilage of foods is influenced by the nutritional composition of the food (e.g. water, acidity, redox potential, available nutrients, natural antimicrobial substances) and the environmental factors of storage (e.g. temperature, humidity and atmosphere composition) (Blackburn, 2006).

Fundamental cultures for the development of human and animal species, are quite appealing for the proliferation of pathogens, as they are rich in carbohydrates. The yield of these products can present worldwide losses, approximately, in the order of 8% to 41% (Table 1.1) (Roberts *et al.*, 2021; Savary *et al.*, 2012).

Table 1.1. - Yield loss range of different plant cultures, globally, due to pest and pathogens (Roberts *et al.*, 2021)

Yield	Percentage (%)
wheat	10.1 – 28.1
rice	24.6 – 40.9
maize	19.5 – 41.1
potato	8.1 – 21.0
soybean	11.0 – 32.4

Microbiota development and various metabolic activities are influenced by physical conditions such as pH, temperature, and moisture. Chemical variables include the availability of nutrients in vegetables that microbes could proliferate. Finally, biological aspects such as the presence of a competitive microbiota and bacterial-plant interactions are important considerations (Luna-Guevara et al., 2019).

Preservation of food is currently controlled, above all, with physical and chemical treatments, or creating safe conditions of processing and storage, influencing mutual, synergistic or antagonistic parameters, such as the food organoleptic and associated beneficial microflora. (Blackburn, 2006; FAO, 2019)

About 20 to 40% of food spoilage, is caused by pathogens including the spoilage by fermentative yeasts. *Zygosaccharomyces parabailii* is a non- *Saccharomyces* budding spoilage yeast that is one of the most aggressive microorganism, due to its stress resistance capability (Kuanyshev , Giusy M. N. Adamo & Branduardi, 2017).

1.1.   Yeasts

Throughout the centuries, microorganisms were used in food processing, such as bread, beer, wine, cheeses, fermented meat and fermented dairy products. Fermentation processes, nowadays, rely on the awareness of the industrial microbiology, in the possibility of generating more diversification and new product quality (Barba *et al.*, 2018).

Approximately 1500 yeast species have been acknowledged thus far, representing 5-10% of the total fungal species. And about 12 species are quite used by the industry, and between 70 to 80 species have been recognized to present potential value in the future of biotechnology (Barba *et al.*, 2018; Dalton, 2017; Deak T., 2009).

The goal of the food and beverage industry's ongoing innovation is to produce food items that are both organoleptically fascinating and speedier with minimum and faster processing. This fact necessitates immediate action to prevent the prevalence of spoiled food and, as a result, spoilage yeasts (Blackburn, 2006). Among the many species of spoilage yeasts reported, about ten have been identified as the primary cause of degradation in processed and packaged foods, including: *Dekkera bruxellensis, Zygosaccharomyces bisporus, Schizosaccharomyces pombe,*

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Issatchenkia orientalis, Debaryomyces hansenii, Candida holmii, Pichia membranifaciens, Zygosaccharomyces bailii, Saccharomyces cerevisiae, Zygosaccharomyces rouxii, and *Kloeckera apiculata* (Barba *et al.*, 2018; De Oliveira *et al.*, 2018; Loureiro & Querol, 1999).

The genera *Zygosaccharomyces* is characterized by colonizing and spoiling food and beverage products with high concentration of sugar and salt (Blackburn, 2006). These microorganisms, may also thrive in weak-acids, such as those that are often used as preventatives with a proven record of effectiveness, like sorbic, benzoic or acetic acids (Stratford *et al.*, 2013). In comparison to other spoilage species, this genera has the ability of high proliferation in harsh circumstances, making it a difficult species to regulate and avoid in food safety and security. (Blackburn, 2006).

1.1.1. *Zygosaccharomyces parabailli*

Spoilage yeasts constitute one of the most important challenges in food and drink preservation and also a mixed blessing. Several of these yeasts have appealing features in the fermentation of food products, mainly winery and beverage, due to their resilience towards certain conditions, but also have disadvantages when it comes to managing in food preservation conditions (Kuanyshev , Giusy M. N. Adamo & Branduardi, 2017).The species *Zygosaccharomyces bailii* (figure 1.1.) together with two recently discovered and phylogenetically related (hybrids), the *Z. parabailii* and *Z. pseudobailii.*are the most problematic yeast species (Kalli et al., 2022).
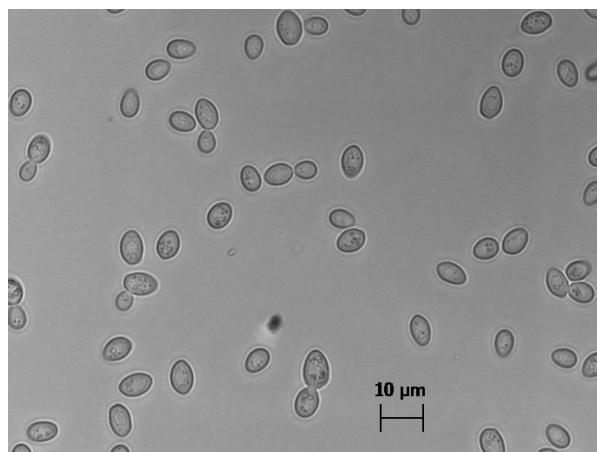
Figure 1.1. - Colonies of *Zygosaccharomyces bailii* by microscopy (Springfixer)*.*

In the beginning of twentieth century, Barker created the genus *Zygosaccharomyces*. This genus presented reproductive differences compared to the model yeast *Saccharomyces cerevisiae* (Solieri, 2021). Within the genus twelve main species were recognized, namely *Z. bisporus, Z. kombuchaensis, Z. lentus, Z. gambellarensis, Z. machadoi, Z. rouxii, Z. sapae, Z. siamensis, Z. mellis, Z. parabailii, Z. pseudobailii* and *Z. bailii*. (Kuanyshev , Giusy M. N. Adamo & Branduardi, 2017). Genetically *Z. parabailii* and *Z. pseudobailii* are hybrid species emerged from *Z. bailii* with two distinct mating events, having only 4–15% nucleotides of divergence compared to the parental lineages (Solieri, 2021).

Z. bailli is a well-known yeast include develops preferably in the presence of organic acids and low pH conditions (Kalli et al., 2022; Kuanyshev *et al.*, 2021; Pitt & Hocking, 2009). Due to its remarkable resistance to several stress conditions this genus is considered the most aggressive yeasts (Kuanyshev , Giusy M. N. Adamo & Branduardi, 2017; Pitt & Hocking, 2009), and causes a worldwide significant economic losses in the food industry, estimated in the order of millions of dollars per year (Pitt & Hocking, 2009). According to Kuanyshev et al (2017), the optimal growth temperature for *Z. bailii* is between 25 and 30 °C.

The presence of this spoilage yeast has been detected in various processed products such as: tomato sauce, mayonnaise, salad dressing, soft drinks including sports drinks, fruit juices and concentrates, ciders, wines (Pitt & Hocking, 2009; Stratford *et al.*, 2013), high-sugar syrups and acetic preserves (Stratford *et al.*, 2013).

Beneficial biological activities of the genera *Zygosaccharomyces* are mainly involved in fermentative processes of products, like the manufacture of miso, a fermented soybean paste and soy sauce, as well as Kombucha, a fermented tea-based beverage. They are also engaged in the manufacturing of honey vinegar and traditional balsamic vinegar. On the other hand, it has also the potential to become a new host for biotechnological processes; in particular, it is an appealing candidate for fermentation of lactate production, L-ascorbic acid (vitamin C), and vitamin B12 production (Sá-Correia et al., 2014).

Kalli *et al.* (2022) tested a wide variety of antifungal natural substances against *Z. parabailii*, such as fatty acids, sesquiterpenes, terpenoid alcohols and phenols, the most effective being the sesquiterpene polygodial, isolated from *Polygonum hydropiper*, with a minimum fungicidal concentration (MFC) of 50 µg/mL (213 µM).

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2. BIOPESTICIDES

Crop productions are expected to suffer because of global climate change, as well as the emergence of new pest and disease concerns. Disease management strategies must be consistent with sustainable and climate-resilient agricultural production methods in the future. In certain circumstances, precision agriculture will be used to minimize inputs, and biologically based technologies will be used to enhance soil health and manage soil erosion, as well as control diseases and pests. Although pesticides have shown to be effective in reducing pest populations, it is generally understood that their use can have detrimental consequences for the ecosystem and the crops themselves (Roberts *et al.*, 2021; Samada & Tambunan, 2020).

Currently, pesticides such as imazalil, pyrimethanil, thiabendazole, fludioxonil, and chloride-based compounds are being used to treat horticulture post-harvest infections. Despite their effectiveness, long-term usage of these fungicides has resulted in the emergence of resistant fungus species. Furthermore, for pesticides in general, active components are hazardous to both humans and the environment (Alghuthaymi *et al.*, 2021; Bellú, 2017; Essiedu *et al.*, 2020; Ni *et al.*, 2021; Thakur *et al.*, 2020; Werrie *et al.*, 2020).

A biopesticide, also known as a biological pesticide, is a natural product derived from living creatures such as plants, nematodes, and microorganisms such as bacteria, fungus, and viruses, that controls or decreases pest populations (Essiedu *et al.*, 2020; Samada & Tambunan, 2020; Thakur *et al.*, 2020). Because biological pesticides are effective, biodegradable, non-toxic, have several modes of action, and are made from readily available basic ingredients, their relevance has grown. Botanical, biochemical, and microbiological pesticides are among the subclasses of biopesticides. Botanical pesticides are plant derivatives that slow or destroy pests development (Essiedu *et al.*, 2020).

In terms of growth and acceptance, the main hurdles for innovative biopesticides are how to market or advertise them, as well as how to increase their stability and residual activity. Commercialization of botanical pesticides is heavily reliant on the availability of vast amounts of plant sources as well as plant culture. Until now, the source plants have been farmed for reasons other than food or medicinal (Essiedu *et al.*, 2020; Ni *et al.*, 2021; Samada & Tambunan, 2020; Sousa, 2016). With a market value of $3 billion, biopesticides account for just 5% of the overall crop protection industry globally. In comparison to Brazil, the United States, China, and India, the

European Union has a very limited number of biopesticides registered due to lengthy and difficult registration processes (Thakur *et al.*, 2020).

Essential oils and their main active components, which have a botanical origin, and showed considerable selectivity and complexity, were discovered as prospective agents in pest management employing agri-nanotechnology (De Oliveira *et al.*, 2018).

2.1. Essential oils

Essential oils (EOs) are complex mixtures of non-volatile and volatile, generally lipophilic, scarcely water-soluble compounds, produced by the secondary metabolism in different plant organs, including flowers, buds, leaves, stems, fruits, seeds, twigs, roots, bark or wood (Cui *et al.*, 2015; Donsì & Ferrari, 2016; Teixeira *et al.*, 2013). The secondary metabolites synthesized are mainly grouped in three large molecule classes such as: terpenoids, phenolic and alkaloid. There classification, in this three groups, is based on their biosynthetic pathways the isoprenoid pathway, shikimate pathway and polyketide pathway (Peyroteo Guedes, 2009).

This process occurs due to defense response to external stimuli like UV light, herbivores, insects and pathogens. EOs are stored in specific structures on the plant's surface, inside the cell organs secretory glandules, vacuoles, cavities, canals, epidermic cells or glandular trichomes, to offer an ideal protection and to prevent interaction with vital parts of the plant. Plants release them through mechanical means or changes in humidity (Donsì & Ferrari, 2016; Teixeira *et al.*, 2013; Zhu *et al.*, 2021).

The Food and Drug Administration (FDA) has recognized essential oils as safe substances, facilitating the process of I&D in food industry to produce innovative food antioxidant agents and preservatives against spoilage and pathogenic microorganisms using EOs (Ballester-Costa *et al.*, 2017; Cui *et al.*, 2015; Donsì & Ferrari, 2016; Ni *et al.*, 2021; Pinto *et al.*, 2021; Vahedikia *et al.*, 2019; Werrie *et al.*, 2020). EOs contain a wide range of biological effects, including growth inhibition of bacteria, yeasts, and fungus (Parris *et al.*, 2005; Zhang *et al.*, 2014). Antioxidant, Anti-inflammatory, Anticancer, Antidiabetic, Antimicrobial, Antiviral, Anti-hyperpigmentation, Cardioprotective, Hepatoprotective, and Neuroprotective (Figure 2.1) are just a few of the properties of essential oils (Jugreet *et al.*, 2020).
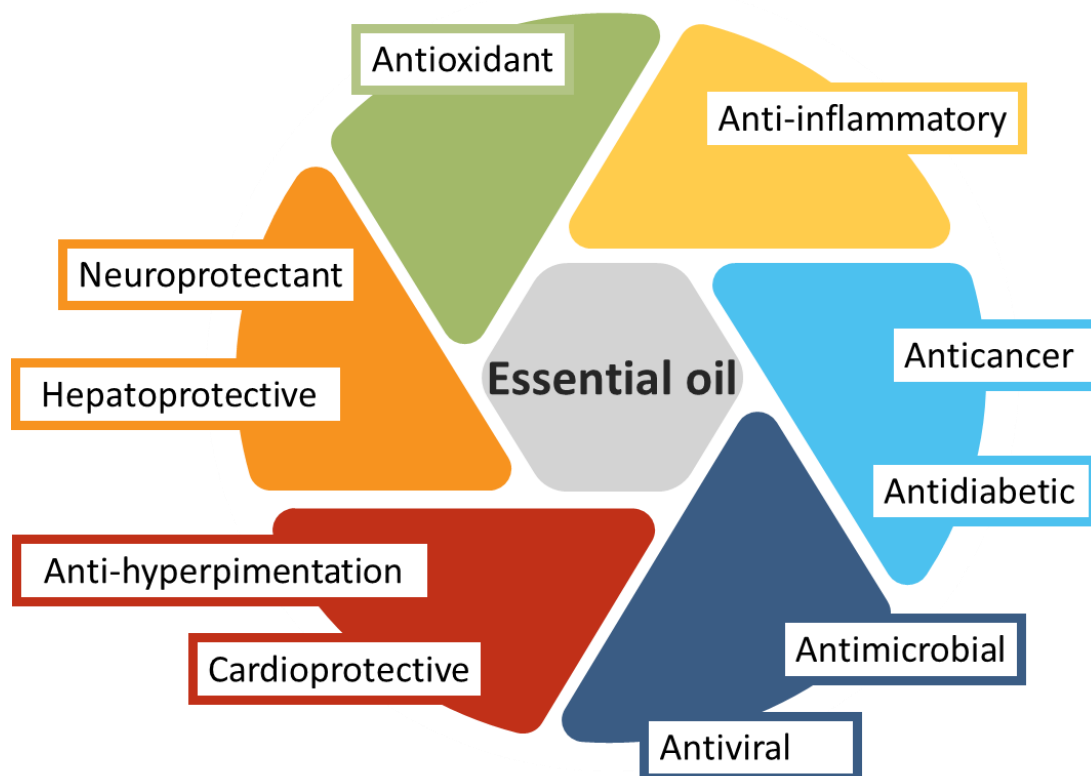
Figure 2.1. - Biological activities of EOs (Adapted from Jugreet et al., 2020).

There are around 3000 different types of essential oils, but only about 300 are used commercially, either in pharmaceuticals or cosmetics (Donsì & Ferrari, 2016). Currently there are about 3000 known essential oils, obtained from approximately 2000 plant species. Around 10% of these oils are economically relevant in the pharmaceutical, agricultural, food and beverage, agrochemicals, antimicrobial, flavoring, cosmetics, and fragrance industries (Gomes, 2017; Rota *et al.*, 2008; Sousa, 2016).

Despite their relevance, these chemicals have a low molecular mass and are very volatile. This unfavorable volatility characteristics of essential oils can be mitigated by encapsulating the essential oil in nanoparticles, which efficacy and durability have been demonstrated. The combination of nanoparticles with essential oils improves its effectiveness as a biopesticide. (Moghimi *et al.*, 2021; Ni *et al.*, 2021; Zhang *et al.*, 2014).

The great susceptibility of these free compounds to air, heat, illumination, and irradiation, humidity, temperature, and microbial degradation, creates limitations to their bioactivity, either when applied in agriculture or in the food industry (De Oliveira *et al.*, 2018; Zhu *et al.*, 2021). The

volatile elements of the EOs like monoterpene, sesquiterpene hydrocarbons, aliphatic aldehydes, alcohols, and esters constitute around 90–95% of their mass, while the non-volatile components such as aliphatic acids, sterol, carotene, coumarin, waxes, and flavones represent just 5–10% of the EOs contents (Zhu *et al.*, 2021).

Effectiveness in controlling fungal pathogens can be achieved using active principles such as monoterpenes, thymol and carvacrol (figure 2.2), which are the main constituents of the essential oils of the family *Lamiaceae*, which includes thyme (*Thymus vulgaris*) and savory (*Satureja montana*) (Santoro *et al.*, 2018).
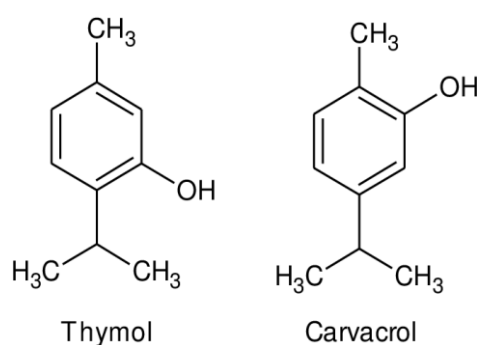
Figure 2.2. - Structure of thymol and carvacrol (Safwan Shiyab, 2012).

Nanodelivery systems can be developed to provide additional benefits, such as minimizing the influence on food items organoleptic qualities, increasing their bioactivity in agriculture and the food sector, and, thanks to subcellular size and better diffusion, contributing to the antimicrobial and anti-biofilm activities (Donsì & Ferrari, 2016; Zhu *et al.*, 2021).

2.1.1.*Satureja montana*

Around 200 species of aromatic and therapeutic herbs belong to the genus *Satureja*, which grow wild in the Middle East and Mediterranean European areas, as well as West Asia, North Africa, and South America. *Satureja montana* (figure 2.3.), a perennial tiny herb endemic to warm dry, sunny, and rocky environments, belongs to the *Lamiaceae* family and is widely known as winter or mountain savory. considered as one of the richest of the medicinal plants. Biologically active phytochemicals in *Satureja* essential oil (SMEO) offer the foundation for a wide range of biological and commercial uses with antibacterial, antioxidant, antifungal, analgesic, antiseptic, anti-

proliferative, anti-protozoal, anti-inflammatory, anti-nociceptive and digestive qualities (Gomes *et al.*, 2020; Maccelli *et al.*, 2020; Vitanza *et al.*, 2019).
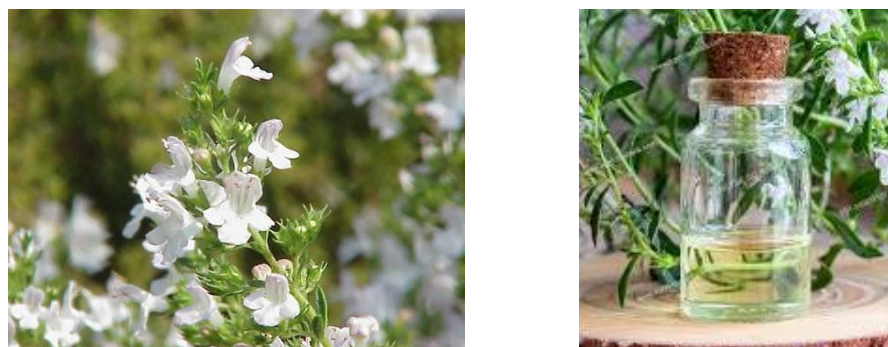
Figure 2.3. - *Satureja montana* flower and essential oil. (Caliban and Agefotostock)

The essential oils extracted from *Satureja* plants species have significant antibacterial properties to differing extents against pathogens that cause food spoilage and/or poisoning, such as *Salmonella*, *Listeria*, and *Staphylococcus* (Miladi *et al.*, 2013).

Oxygenated monoterpenes (e.g., thymol and carvacrol) and other chemicals (Maccelli *et al.*, 2020; Pateiro *et al.*, 2021; Šojić *et al.*, 2019) (table 2.1.) constitute most of the volatile fraction, with approximately 59% and sesquiterpenes hydrocarbons with approximately 6%. Both these compounds contribute to the cytotoxic bioactivity of this essential oil against pathogens (Miladi *et al.*, 2013).

Table 2.1. - Main constituents of the essential oil *S. montana* EO (Miladi *et al.*, 2013)

Constituents	Percentage (%)
thymol	0.89
p-cymene	13.03
carvacrol	53.35
α-Thujene	1.14
α-Pinene	0.73
Camphene	0.31
β-Myrcene	1.3

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α-terpinene	1.76
Borneol	1.14
y-Terpinene	13.54
Linalool	1.81
β-Bisabolene	1.3
Bomeol	1.14
β Caryophyllene	0.43

2.1.2. *Thymus vulgaris*

Thymus vulgaris (figure 2.4.) is an Aromatic and Medicinal Plant, commonly known as thyme. Belonging to the *Lamiaceae* family, the genus *Thymus* has 350 species worldwide. Due to their various distinguished composition with a large morphological and chemical variety, is widely studied in herbal medicine products (Aldosary *et al.*, 2021; Nikolić *et al.*, 2014). Particularities like anti-inflammatory, antibacterial, antioxidant, antifungal, sedative, antiaflatoxinogenic and antiviral activity, has instigate a lot of interest in the different species of the *Thymus* genus due to their potential. (Aldosary *et al.*, 2021).

Figure 2.4 - *Thymus vulgaris* plant and essential oil (Theindianmed and Citymall).

This plant is recognized for its therapeutic properties in various applications due to the presence of compounds (e.g. thymol, carvacrol) characterizing these volatile compounds with

interest in antifungal activity, antibacterial and antioxidant proprieties (Asprea *et al.*, 2017; Ballester-Costa *et al.*, 2017; Gedikoğlu *et al.*, 2019; Nikolić *et al.*, 2014; Rota *et al.*, 2008).

Analysis of *T. vulgaris* EO identified around 47 components (Table 2.2.), and the group of phenols was the most abundant compound group of the oil representing 61.8% (Rota *et al.*, 2008). The US Food and Drug Administration has accorded *T. vulgaris* GRAS designation, indicating that the plants are generally recognized as safe for human consumption with no restrictions on intake and are widely accepted by consumers (Mandal & DebMandal, 2016).

Table 2.2. - Main constituents of the essential oil *T. vulgaris*, analyzed by GC-MS and GC-FID (Pereira, 2021)

Constituents	Percentage (%)
thymol	45.85
o-cymene	17.32
carvacrol	3.22
α-Thujene	0.64
α-Pinene	1.12
Camphene	1.28
β-Myrcene	2.28
α-terpinene	1.89
1,8-Cineole	0.2
γ-Terpinene	11.7
Linalool	4.46
Camphor	1.31
Bomeol	1.18
Caryophyliene	2.62

3. NANOPARTICLES

Nanotechnology is the scientific manipulation and production of materials at the nanoscale (1-100 nm), allowing for the creation of varied surface-to-volume ratios with unique features

(Prasad, 2017). Nanomaterials of various sizes and shapes offer a wide range of uses in medical, environmental research, agriculture, and food processing (Shang *et al.*, 2019). Elements such as silver, gold, copper, iron, graphene, and silica, as well as polymers such as chitosan, PVC, PLGA, and other organic molecules, are used to create composites or nano formulations (Alghuthaymi *et al.*, 2021; Prasad, 2017).

Currently, the society requires more sustainable choices of food products, so agri-nanotechnology appears as an innovative field for increasing the search for nanotechnology-based solutions to a variety of agricultural and environmental problems. In the last decade, a wide range of applications have been discussed to improve some of the main problems (Figure 3.1), including controlled-release nanofertilizers that improve crop growth, yield, and productivity, nano-based delivery approaches (e.g. gene transfer) for crop improvement, the use of nanopesticides for efficient crop protection and food preservatives, that are the mainly applications investigated, and the development of nanosensors and computerized controls for precision farming, and the use of nanomaterials that promote plant stress tolerance and soil enhancement (Shang *et al.*, 2019; Singh *et al.*, 2018).
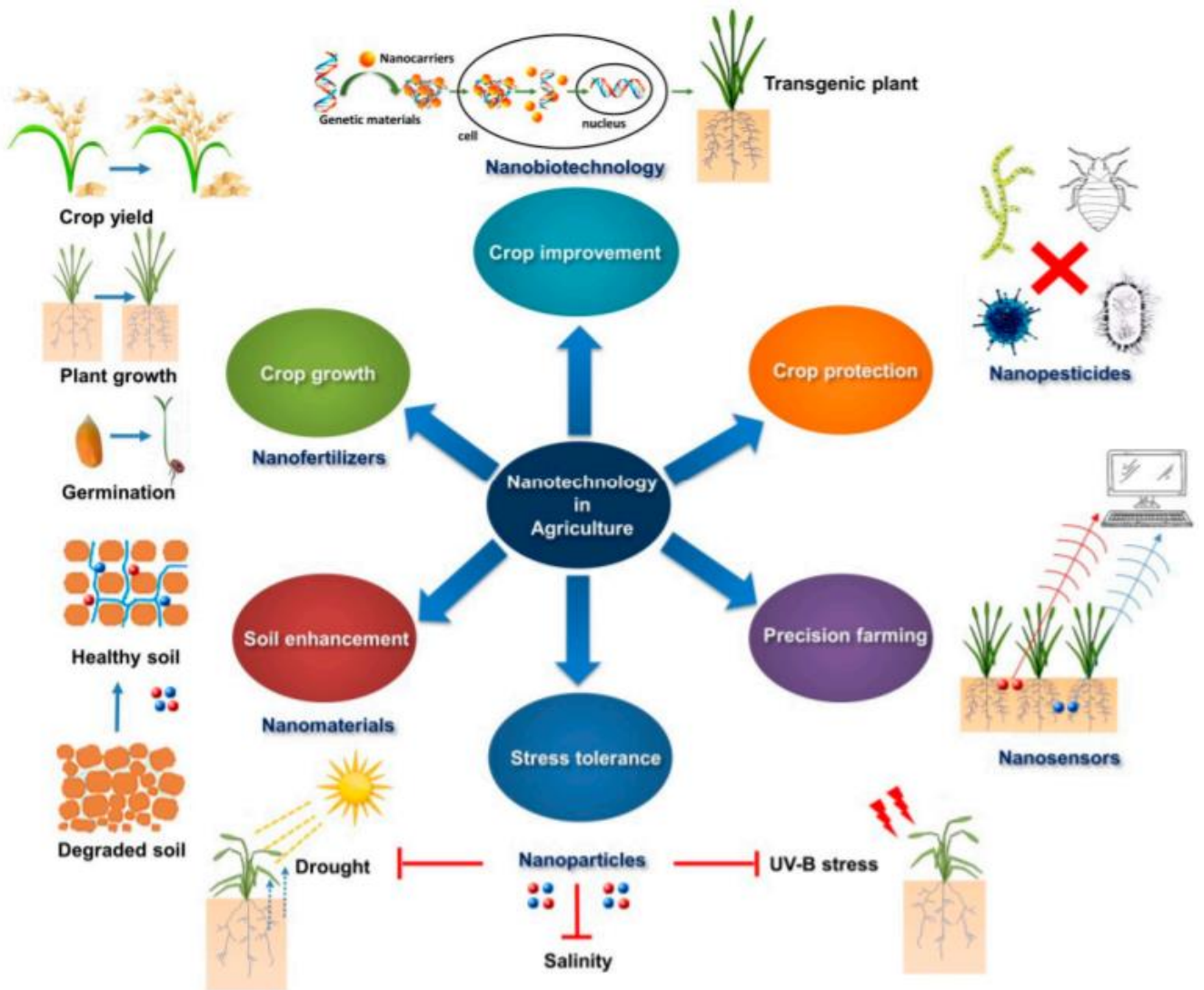
Figure 3.1. – Applications of nanotechnology in agriculture. (Shang *et al.*, 2019)

Nanoparticles with antifungal activity are thus critical for a synergistic approach to the treatment of fungal pathogens that cause post-harvest diseases, and also an environmentally sustainable alternative for better plant, animal, and human health. Antifungal nanoparticles are expected to meet the needs of growers, consumers and environmentalists, by providing quick, effective, and comparatively improved eco-safety features for managing production and preventing fungal phytopathogens from spoilage of fresh food and compromising food safety (Alghuthaymi *et al.*, 2021).

The the antifungal activity of thyme and savory essential oils, free and encapsulated in particles *against Zygosaccharomyces* spoilage yeast, on the other hand, has not been investigate.

3.1.   Zein-based particles

Zein is a key protein component in maize, a by-product of the agricultural industry, accounting for 45–50% of total protein, and is known for its high hydrophobicity due to its unique tertiary structure (Gonçalves *et al.*, 2020; Tinoco *et al.*, 2021). Due to its α-helix, which accounts for 50–60% of its structure, zein protein can self-assemble micro- and nanoparticles utilizing liquid–liquid dispersion or solvent evaporation processes. The isolated zein is biodegradable, biocompatible, and has a high coating capacity, ideal for application as a medication, enzyme, essential oil, or other component delivery system. (Martínez-López *et al.*, 2020; Tinoco *et al.*, 2021).

The use of zein, as potential material in food industry has been referred as sustainable and an excellent barrier to oxygen (Gonçalves *et al.*, 2020; Vahedikia *et al.*, 2019), and was approved as a generally recognized as safe (GRAS) excipient in 1985 by the Food and Drug Administration (FDA) (Martínez-López *et al.*, 2020).

Recently, the present issues tomato phytopathogen management were explored. the use of S. montana EO as nanocarriers and activity enhancers was found to be a promising alternative for integrating natural spoilage control products (Oliveira-pinto et al., 2021).

3.2.   Keratin-based particles

Keratins are fibrous and insoluble proteins which are the main ingredients in wool and feathers (Prasad, 2017). Keratin proteins are found in the cytoplasm of practically all differentiated eukaryote cells. Keratin is a complex mixture of proteins that can be classed as soft or hard keratins based on the presence of disulphide linkages. The stratum corneum and callus contain soft keratins with a low disulphide concentration, whereas epidermal appendages such as feathers, hair, nails, horns, and hoofs include hard keratins with a high disulphide content (Tinoco *et al.*, 2020, 2021).

Due to their inherent biocompatibility, biodegradability, and natural abundance, keratin-based biomaterials were prepared as films, hydrogels, dressings, and scaffolds that have been used in a variety of biomedical applications, including nerve regeneration, wound healing, bone regeneration and cell culture (Wang *et al.*, 2016). Keratins were also used in the establishment of controlled-release drug delivery systems due to their stability, low reactivity, and good mechanical

and structural characteristics, including the packaging and/or preserving goods (Tinoco *et al.*, 2020).

3.3.   Liposomes

Liposomes have been explored extensively as drug transporters for various therapeutic objectives since the 1960s (Gafur *et al.*, 2020). Liposomes are artificial membranes composed of phospholipid molecules that are segmented into hydrophilic and hydrophobic phases, allowing active compounds of lipophilic and hydrophilic substances (e.g. phytochemical oils) to be incorporated into a spherical particle with a diameter of 25–1000 nm.

Due to their biocompatibility and non-toxic effects, liposomes have been widely used for the targeted delivery of several hydrophobic medications in human organisms as a biomimetic encapsulating system (Gafur *et al.*, 2020; Zhu *et al.*, 2021). Liposomes are the best clinically established nanocarrier systems for drug delivery (Barbosa *et al.*, 2019). Lipid particles might also be synthesized to load different EOs for other purpose, since there were reported to be employed for medical purposes to improve the healing of skin lesions (Ni *et al.*, 2021).

4. MODE OF ACTION OF EOS

Few research were performed to evaluate the effects of encapsulated EO on yeast cells (Donsì & Ferrari, 2016), with the most common spoilage yeasts *Zygosaccharomyces bailii* and *Saccharomyces cerevisiae*. Essential oils antifungal effects have been extensively investigated for recent years, fueled by a desire to find natural alternatives to food preservatives and also other potential applications (Donsì & Ferrari, 2016).

Rather than relying on a single method, EOs interact with the yeast cell membrane through several sites. It is also widely established that their effects are based on their molecular hydrophobicity (Donsì & Ferrari, 2016). The phenolic components, create a dynamic interaction with cell membrane lipids, increasing membrane permeability, disrupting original cell structure, disrupting homeostasis, inducing mitochondrial dysfunction and reactive oxygen species accumulation, decreasing ATPase and dehydrogenase activities, as well as nuclear and cytoplasmic mutagenicity (Donsì & Ferrari, 2016; Sousa, 2016; Zhu *et al.*, 2021). Encapsulation the essential oils, while boosting physicochemical stability and dispersibility, causes a substantial

impact on their interaction with pathogenic microorganisms, as well as improvement of their biological activity (Donsì & Ferrari, 2016).

The improvement accessibility to microbial cells by passive interaction with the cytoplasmic membranes (Figure 4.1 (A)) could be due to the increased surface area and passive transport across the cell surface membrane. The EOs can be carried to the cell membrane surface, improving accessibility to microbial cells, and allowing cell membrane disruption, possibly by modifying the phospholipid bilayer integrity or disrupting with active transport proteins incorporated in the phospholipid bilayer.

The merge with the cell membrane phospholipid bilayer (Figure 4.1 (B)) likely aids in the targeted release of the EOs at the desired locations. Furthermore, unique interactions between the EOs and cell membranes have been observed to boost the antibacterial effectiveness of EO.

The activity of EOs can be prolonged by the persistent release of EOs from particles over time (Figure 4.1 (C)), which might be caused by EO partition between the encapsulated oil and the aqueous phase, in a state of dynamic equilibrium between the dispersed oil and aqueous phases.

The electrostatic interaction (Figure 4.1 (D)) between positively charged particles and negatively charged microbial cell walls raises the concentration of EOs at the action site. Due of the complex structure of cell walls, containing substances with diverse charges, the encapsulation effects on charge particles might impair or boost antibacterial action (Donsì & Ferrari, 2016).
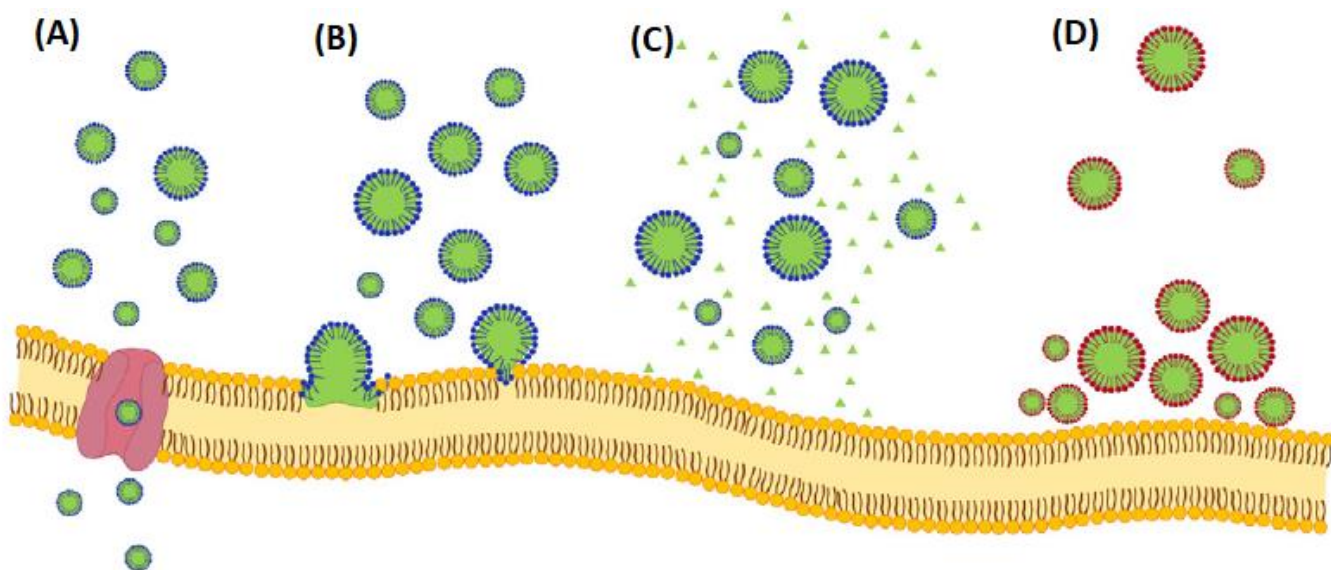
Figure 4.1. - Schematic representation of the possible several paths of events between the essential oil derivates with microbial cell membranes in case of antimicrobial synergism. **(A)** Passive transport through cell membrane; **(B)** Fusion with phospholipid bilayer; **(C)** Partition in aqueous phase; **(D)** Electrostatic interaction with cell membrane (Adapted from Donsì & Ferrari, 2016).

5. AIM

In this work, we aimed to study the antifungal encapsulated essential oils of *Satureja montana* and *Thymus vulgaris* to evaluate their potential use as a biopesticide in agriculture and, simultaneously, as food preservative. Overall, most studies on the antimicrobial activities of essential oils have reported loss of antimicrobial activity due to the high volatility of is active compounds, so its urgent to develop methodologies that can increase EO stability, namely by employment of encapsulation as a novel green nanotechnological approach.

Therefore, the objectives of the present study were: (i) to investigate the inhibitory activity against the food spoilage yeast *Zygosaccharomyces parabailii* of the free essential oils of *Satureja montana* and *Thymus vulgaris* and compare it with different encapsulated EO formulations, namely zein-based, keratin-based and liposome particles (ii) to study cell death and loss of plasma membrane integrity induced by the essential oils *Satureja montana* and *Thymus vulgaris*, free and encapsulated in keratin-based particles; (iii) to determine the most effective formulation of EO particles; (iv) to determine minimum inhibitory concentration and minimum lethal concentration of the formulations studied.

6. MATERIALS

6.1.   Essential oils

The essential oils used were from *Satureja montana* and *Thymus vulgaris*. They were supplied by Florihana (France) and were extracted from flowering tops with origin in Albania/Spain and flowering tops with origin in Spain, respectively, for *S. montana* and *T. vulgaris*.

6.2.   Particles

Zein-based, keratin-based particles and liposomes were developed by Dr. Artur Ribeiro.

Zein-based particles were prepared using an anti-solvent precipitation method with ethanol 70%, as described in Oliveira-pinto *et al*. (2021). 500 mg of zein (Sigma, Madrid, Spain) were dissolved in 70% ethanol and stirred constantly until the solution turned transparent. To this solution were then added 500 mg of S. montana EO and it was swirled for 5 minutes at 250 rpm to make EO-Zein particles. The EO-Zein solution was rapidly distributed into deionized water at high speeds until a single phase was produced. The procedure was only adapted due to the respective changes in the concentration of essential oils of *Satureja montana* (1 mg/mL and 2 mg/mL) and *Thymus vulgaris* (2 mg/mL), and in the different tested protein concentrations (1 mg/mL, 2 mg/mL and 10 mg/mL).

The develop the keratin-based particles was adapted from Tinoco et al. (2021). The procedure was only adapted due to the respective changes in the concentration. Hair keratin previous treated was used to develop the particles. A keratin solution was used and the particles solution was stirred at 500 rpm for 30 min, at room temperature. The essential oils (prepared in ethanol 70 % HPLC-grade) were dissolved and added to 22.5 mL of keratin solution. The particles formulation with the EO were then stirred at 500 rpm for 30 min, at room temperature. The final keratin concentration was of 6.25 mg/mL keratin per 12.04 mg/mL of SMEO and 6.25 mg/mL keratin per 12.07 mg/mL of TVEO. In these formulations was also added green Fluorescein Isothiocyanate (FITC), for flow cytometry and microscope fluorescence analysis.

Liposomes were produced by pre-concentration ethanol injection method described in Guimarães *et al*. (2020). Liposomes composed of DOPE or EPC/cholesterol/DSPE-mPEG were

produced by the ethanol injection method. Lipids were weighted at the initial molar ratio of 54:36:10. MTX disodium salt (soluble in aqueous buffer) was prepared by adding two NaOH molar equivalents to phosphate-buffered saline (PBS) containing commercial MTX. After the complete solubilization of MTX, the pH was adjusted to pH 7.4. The procedure was only adapted due to the incorporation of the essential oils of *S. montana* and *T. vulgaris* at the concentrations 1.193 mg/mL, 2.15 mg/mL and 1.040 mg/mL, 1.25 mg/mL, respectively.

6.3. Strain and culture conditions

Antimicrobial activity was evaluated against *Zygosaccharomyces parabailii* (ISA 1307). The yeast cells were grown at 30 °C for 48 hours and maintained for a week at 4 °C on yeast extract peptone dextrose agar (YPDA) medium plates with the following composition: 2% (w/v) glucose, 2% (w/v) bactopeptone, 1% (w/v) yeast extract and 2% (w/v) agar. This process was performed every week using cell stocks stored at - 80°C.

7. METHODS

7.1. Antimicrobial Activity

To perform the antimicrobial activity assay, the cells were incubated overnight at 30 °C to early exponential phase at $OD_{640\,nm}$= 0.5-0.6 in liquid YPD media. Centrifugation at 5000 rpm for a period of 5 minutes was then performed, followed by resuspension with sterile dH_2O and centrifugation under the same conditions. Fresh YPD medium was added to the cell suspension to obtain $OD_{640\,nm}$=0.1 to complete a final volume of 5mL. A previously determined volume of OE, Zein particles, Keratin particles and liposomes was subsequently added, depending on the concentrations of EO loaded, in order to obtain final concentrations of: 100, 200 and 500 µg/mL of EO. In the assay was also considered the cells control without added particles and control with particles without EO in their composition. The assay was performed tree times, each one, in duplicate. The cell with the different conditions were incubated at 26 °C, in order to simulate ambient temperature, and 160 rpm, and $OD_{640\,nm}$ readings were taken at 24 hours and 48 hours.

The minimum inhibitory concentration (MIC) was defined as the lowest concentration of EO or particles at which the microorganism did not demonstrate significant growth, inhibiting the growth of a certain pathogen in 80% (Leitão, 2015; Pinto *et al.*, 2007), compared to controls. The minimum lethal concentration (MLC) is described to the lowest antifungal concentration required to totally inhibition fungal growth and proliferation (Pinto *et al.*, 2007).

7.2.   Colony forming units (CFU)

The colony forming units were determined for the minimum inhibitory concentrations (MIC) obtained in each assay. This assay was performed to confirm MIC values and lethality at 48 hours.

An aliquot of 50 μL was removed from each culture tube and serially diluted in sterile water. Five aliquots of 40 μL each were removed and plated on YPDA plates. The dilution factor was chosen to ensure 50-250 colonies per drop could be counted. Colony counts were determined after incubation of the plates at 30 °C for 24-48 h.

The formula bellow was used for more accurate representation of CFU results after 48 hours of assays with *Zygosaccharomyces parabailli* yeast (Adapted from Annesi *et al.*, 2021).

$$\log_{10}(CFU/mL) = \log_{10}\left(\frac{n^{\underline{o}} \ of \ colonies \ x \ dilution \ factor}{Volume \ of \ the \ culture \ spread}\right)$$

7.3.   Cell membrane integrity

In order to detect the membrane integrity of *Zygosaccharomyces parabailii* yeast cells treated with particles, a sample of the culture was stained with propidium iodide (PI). PI is a fluorescent probe, in the red spectrum, that only stain cells that present compromised membrane integrity, due to severe lesions. To observe permeabilization, flow cytometry and fluorescence microscopy was used.

Z. parabailii suspensions were grown overnight in YPD medium at 30 °C, in an incubator at 200 rpm, until an $OD_{640 \ nm}$ of 0.5-0.6 was reached. The suspensions were centrifuged, washed and the OD_{640nm} was adjusted at 0.1 with fresh medium. Different concentrations of particles (200 and 500 μg/mL) were then added and different cell controls were performed (cells without particles, cells with empty particles).

At predetermined time points (0, 1, 5, 24 and 48 hours), a volume of culture was collected to obtain a final $OD_{640 nm} = 0.1$ in 400 µL of PBS. The sample was centrifuged, the pellets were washed in 1 mL of PBS and then resuspended in 400 µL of PBS. After, 2 µg/ml of PI were added, the cell suspension was incubated for 10 min at room temperature in the dark and then analyze by flow cytometry and by fluorescent microscopy. For the staining control, all the samples were analyzed without staining (autofluorescence). For the PI positive control, cells were submitted to a heat treatment (95 °C 5 min.) before staining. For the microscopy observation the suspension of stained cell was centrifuged, concentrated 10 times, and with red and green fluorescence filter.

7.3.1. Epifluorescence microscopy and flow cytometry

Fluorescence microscopy analysis was carried out at time points 24 and 48 hours, using a conventional fluorescence Leica DM500 + CTR500 + ebq100 Microscope, equipped with phase contrast objectives and camera. The specimen was observed with green and red filters, due to the use of PI experiments and FITC content in the particles of keratin.

Flow citometry samples were analyzed by Beckman Coulter CytoFLEX System V 2.4.0.28, with optical configuration of Blue Laser: 525/40 BP; 585/42 BP; 610/20 BP; 690/50 BP. The data was obtained on SSC-A, FSC-A, FITC-A and PE-A. Twenty thousand cells were analyzed per sample at low flow rate.

7.4.   Data and statistical analysis

Antimicrobial activity and CFU experiments were repeated in triplicate (n≥3) with 2 replicates each. The results obtained are represented by mean and standard deviation (± SD) values and analyzed using two-way ANOVA analysis of variance. Statistical analyses were carried out using GraphPad Prism Software v 9.0.0. (GraphPad Software, California, USA). P-values lower than 0.05 were assumed to represent a significant difference.

8. RESULTS AND DISCUSSION

8.1.   Characterization of the particles

The characterization of particles consists of evaluating the stability of particles size, surface charge and polydispersion index (PDI) by Dynamic light scattering (DLS) (Gonçalves *et al.*, 2020).

Dynamic light scattering resorts to translational diffusion coefficient and, as a result, the hydrodynamic diameter is calculated by measuring time-dependent changes in scattering intensity. This method also provides data of polydispersity index (PDI) of the particle and the solution zeta potential. If the PDI value is less than 1, then the particle is considered as monodispersed. The degree of aggregation of particles is determined by the solution zeta potential (i.e. the higher the zeta potential, the less aggregation) (Singh *et al.*, 2018).

During the experiments, three types of particles were used, zein, keratin and liposome. These particles were characterized by Dr. Artur Ribeiro and the data provided is present in table 8.1. to 8.3, in the sections below.

8.1.1. Size

Three different types of particles (zein-based, keratin-based, and liposomes) were produced to improve the antifungal activity of the essential oils *Satureja montana* and *Thymus vulgaris*, and their size properties were analyzed to be tested in a later phase of the study. The zein particles ranged between 120.0 and 388.4 nm with the EOs of *S. montana* and *T. vulgaris*, respectively, while the empty particles ranged between 92.0 and 209.5 nm.

The average size of empty zein-based particles is between 100 and 400 nanometers. The size can be influenced by the pH, ethanol concentration or shearing rate conditions (Martínez-López *et al.*, 2020). Meanwhile, in the present study it is possible to observe that the size of particles encapsulating EO is superior to that of the empty particles, which is not verified in the literature. According to Gonçalves da Rosa *et al.* (2020), particles developed with zein from maize and by the method of two phases, the particles loaded with *T. vulgaris* EO and their empty control particles obtained the size of 138.2 and 153.9 nm, respectively. Also, in Zhang *et al.* (2014), with particles with 97% of protein content (zein) using liquid–liquid dispersion method, the particles loaded with

thymol (i.e. main compound of *S. montana* EO and *T. vulgaris* EO) displayed a size of 118.30 ± 0.42 nm.

Table 8.1. Values of the concentration, particle size, polydispersity index (PDI) and surface charge regarding of Zein particles with *Satureja montana* and *Thymus vulgaris* EOs

Particles	Essential Oil	[EO/ZEIN] (mg/mL)	Size (nm)	Zeta Potential (mV)	PDI
Zein	*Satureja montana*	1/1	139.2±5.4	24.7±0.4	0.252±0.019
		Empty	99.2±2.2	25.1±0.2	0.201±0.035
		2/2	120.0±0.9	29.1±0.6	0.267±0.008
		Empty	92.0±1.7	28.2±0.2	0.243±0.021
		1/10	388.4±9.7	17.4±0.5	0.136±0.028
		Empty	209.5±3.2	20.4±0.7	0.145±0.071
	Thymus vulgaris	2/2	134.0±2.3	30.2±2.3	0.231±0.006
		Empty	92.0±1.0	28.2±0.2	0.243±0.021

The SMEO keratin-based particles used were at the concentration of 12.4/6.25 mg/mL and measured 370 nm. Although the TVEO keratin-based particles present a higher concentration of EO for the same amount of keratin, 12.7/6.25 mg/mL, the particle size decreases, thus measuring 350 nm. The control empty keratin particles presented the largest size, measuring 440 nm.

Keratin-based particles developed from human hair are described in literature with different size ranges. According to Tinoco *et al.* (2018) and Gonçalves (2017) they can obtained a size distribution between 150 to 250 nm. Meanwhile, according to Wang *et al.* (2016) empty particles with concentration of 7.5 mg/mL of keratin from chicken feather obtained a size of 195.0 ± 1.7 nm, suggesting that different protein supply sources do not have a direct impact on the size of the particles. The encapsulation of essential oil can justify the larger size of the particles used in this study compared to the literature. Encapsulation can produce a superior particle size to maintain its stability.

Table 8.2. - Values of the concentration, particle size, polydispersity index (PDI) and surface charge regarding Keratin particles with *Satureja montana* and *Thymus vulgaris* EOs

Particles	OIL	[EO/KERATIN] (mg/mL)	Size (nm)	Zeta Potential (mV)	PDI
Keratin	*Satureja montana*	12.04/6.25	370.0±4.4	-37.0±0.8	0.190±0.017
		Empty	440.0±6.9	-39.0±0.3	0.235±0.052
	Thymus vulgaris	12.07/6.25	350.0±2.3	-33.8±0.2	0.195±0.005
		Empty	440.0±6.9	-39.0±0.3	0.235±0.052

The SMEO liposomes reached a size 314.1 to 399.2 nm, while the TVEO liposomes ranged between 208.9 to 268.5 nm. The empty liposomes particles ranged 160.4 to 199.4 nm. Asprea *et al.* (2017) obtained size values of 200.0 ± 4.3 nm with liposomes loaded with 1 mg/ml of *Thymus*, being in agreement with the result obtained for thyme. Liposomes larger than 200 nm can effectively encapsulate and stabilize active compounds, although other variables such as surface charge and PDI are also important for dispersibility and stability (Gafur *et al.*, 2020). Thymol and carvacrol have also been shown, in the literature, to reduce the size of liposomes by causing the lipid structure to increase its surface curvature (Asprea *et al.*, 2017).

Table 8.3. - Values of the concentration, particle size, polydispersity index (PDI) and surface charge regarding Liposome particles with *Satureja montana* and *Thymus vulgaris* EOs

Particles	OIL	[EO] (mg/mL)	Size (nm)	Zeta Potential (mV)	PDI
Liposome	*Satureja montana*	1.193	314.1 ± 3.16	- 3.150 ± 0.580	0.259 ± 0.015
		2.15	399.2 ± 4.96	- 0.636 ± 0.109	0.367 ± 0.069
		Empty	160.4 ± 0.93	- 0.719 ± 0.494	0.102 ± 0.017
			199.4 ± 1.75	- 0.818 ± 0.851	0.158 ± 0.015
		1.040	208.9 ± 2.25	- 0.952 ± 0.206	0.215 ± 0.007

Thymus	1.25	268.5 ± 7.11	- 2.130 ± 0.420	0.298 ± 0.027
vulgaris	Empty	199.4 ± 1.75	- 0.818 ± 0.851	0.158 ±0.015

8.1.2.Zeta Potential and polydispersity index (PDI)

To improve the knowledge of the antifungal activity of the encapsulating essential oils *Satureja montana* and *Thymus vulgaris* formulations, were also analyzed their electrical charges properties and polydispersity index. The zeta potential values for zein particles in this work ranged from +17.4 to +30.2 mV. As shown by Li *et al.* (2013), their electrical charges can be influenced also by the thymol-to-zein ratios. Values in the range of +30.0 mV, have colloidal stability maintaining by electrostatic repulsion (Cho *et al.*, 2013; Gonçalves, 2017).

The potential Zeta values for keratin-based particles range from -33.8 to -37.0 mV, which are clearly in agreement with the literature, as values found between -8.9 and -30.3 mV are in agreement for various keratin particles formulations. It's also been proven in the literature that the higher the particles charge, the more stable it is (Cho *et al.*, 2013; Gonçalves, 2017).

In liposome formulations, the zeta potential and PDI were analyzed (Table 8.3). The zeta potential of the particles tested was all negative, ranging from -3.150 to -0.636 mV with the SMEO and -2.130 to -0.818 mV with the TVEO. In general, the liposomes had a charge very close to electrical neutrality. According to Asprea *et al.* (2017) liposomes with *Thymus* presented values of zeta potential -32.0 mV with a similar concentration of 1 mg/mL, and empty - 37.5 mV. However, because the surface charge is important and determines a particle interaction with its surroundings, particles with charges less than -30.0 mV present low colloidal stability and exhibit low electrostatic repulsion (Cho *et al.*, 2013; Gonçalves, 2017). Nonetheless, liposomes should be neutral to minimize non-specific absorption, adsorption to proteins, and the little negative charge may also be acceptable (Maritim et al., 2021).

All formulations obtained PDI values that were compromised between 0.102 to 0.367, very similar to values obtained in Asprea *et al.* (2017), Li *et al.* (2013) and Gonçalves (2017). PDI values lower than 0.5 present a broad size distribution (Cho *et al.*, 2013; Gonçalves, 2017), and the oscillation of values can be explained by comparisons of empty particles to loaded with essential oil (Li *et al.*, 2013).

8.2.   Antimicrobial activity of the free and encapsulated EO of *Satureja montana* and *Thymus vulgaris*

As described in previous studies, the use of the essential oil *Satureja montana* and *Thymus vulgaris* has highly antimicrobial activity against spoilage microorganisms (Miladi *et al.*, 2013; Ballester-Costa *et al.*, 2017; Gedikoğlu *et al.*, 2019). Furthermore, the use of particles encapsulating it, can reduce the disadvantages of the low stability of the metabolites (Moghimi *et al.*, 2021; Ni *et al.*, 2021; Zhang *et al.*, 2014).

Antimicrobial activity was assessed for the free EOs and three formulations of particles, two proteins based, with zein and keratin, and the third lipid based, the EO being incorporated in liposomes. For zein particles, the ratios of zein and EO were optimized out of three different concentrations, 1 mg/mL EO per 1 mg/mL zein, 2 mg/mL OE per 2 mg/mL zein and 1 mg/mL EO per 10 mg/mL zein, using only the EO of *S. montana*.

In the figures 8.1 and 8.2 are represented the activity of the free essential oils of *S. montana* and *T. vulgaris*, respectively. The activity against *Z. parabailii* of zein-based formulations with both EOs are shown in the figures 8.3 to 8.6, the keratin-based particles in figures 8.7 and 8.8, and liposomes in figures 8.9 and 8.10. For a better analysis of the combined results, the minimum inhibitory concentrations (MIC values) of the antimicrobial activity of *S. montana* and *T. vulgaris*, free or encapsulated EO are shown in table 8.4. Also, in figure 8.11 and 8.12, CFU assessment complementing previous data, are presented.

8.2.1. Free essential oil

To assess antifungal activity were tested of free EO from *Satureja montana*, for 100, 200 and 500 µg/mL final concentrations were tested against *Zygosaccharomyces parabailii*. A partial inhibition of growth of *Z. parabailii* was observed already at 100 µg/mL and it increased for the concentrations of 200 and 500 µg/mL (p≤0.0001 compared to the control) (Figure 8.1). The MIC was reached at 200 µg/mL, the same outcome being observed both at 24 and 48 hours.
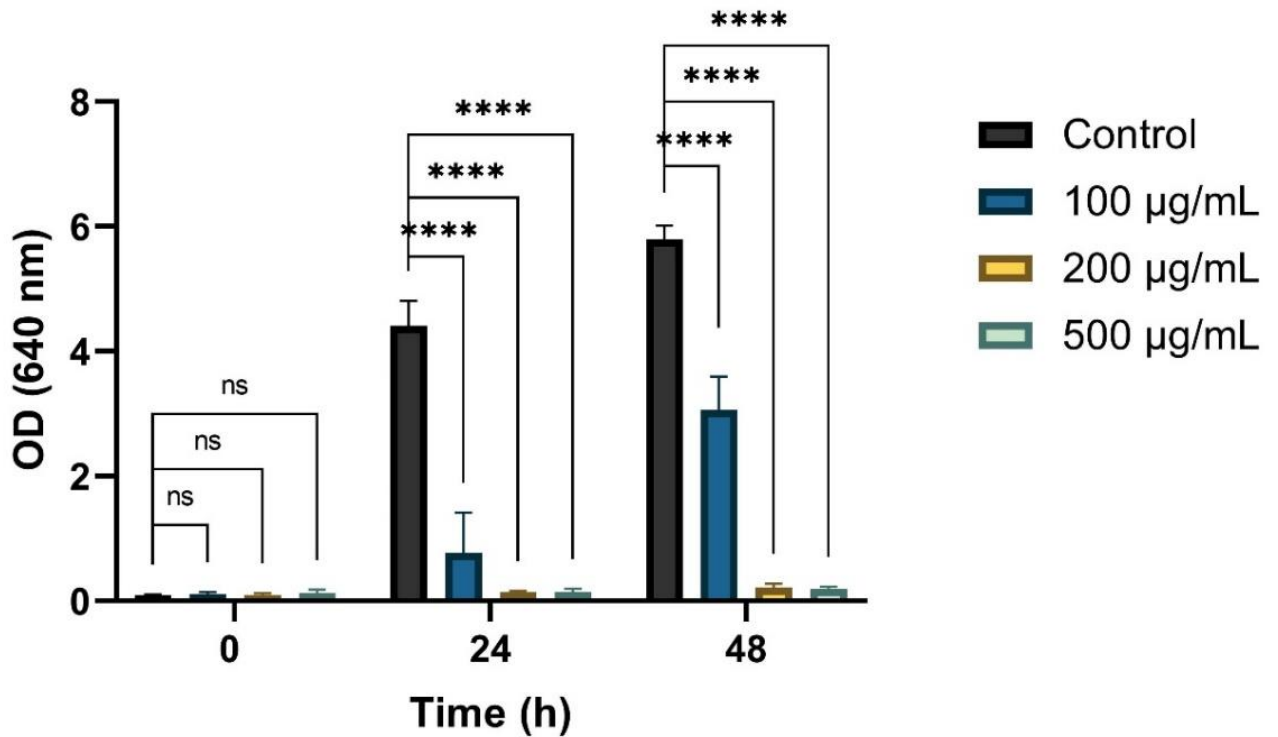
Figure 8.1. Antimicrobial activity of *Satureja montana* free EO against *Z. parabailii*. Optical density was measured at 640 nm. The reported values are means ± SD (n≥3). Statistical analysis was performed by the two-way ANOVA. **** and ns indicate p≤0.0001 and not significant respectively, compared with cells control.

To assess antifungal activity of free essential oil from *Thymus vulgaris* (TVEO), 100, 200 and 500 µg/mL final concentrations of EO were tested. For this essential oil (TVEO), an inhibition of growth of *Z. parabailii* was observed at 100 µg/mL at 24 hours of experiment (Figure 8.2), the yeast recovering its growth from 24 to 48 hours. On other hand, the inhibition was higher for the concentrations of 200 and 500 µg/mL, and maintained the trend of significant inhibition (p≤0.0001 compared to the control) for both 24 and 48 hours of assay. The MIC, read at 48 hours, was obtained for the concentration of 200 µg/mL.

27
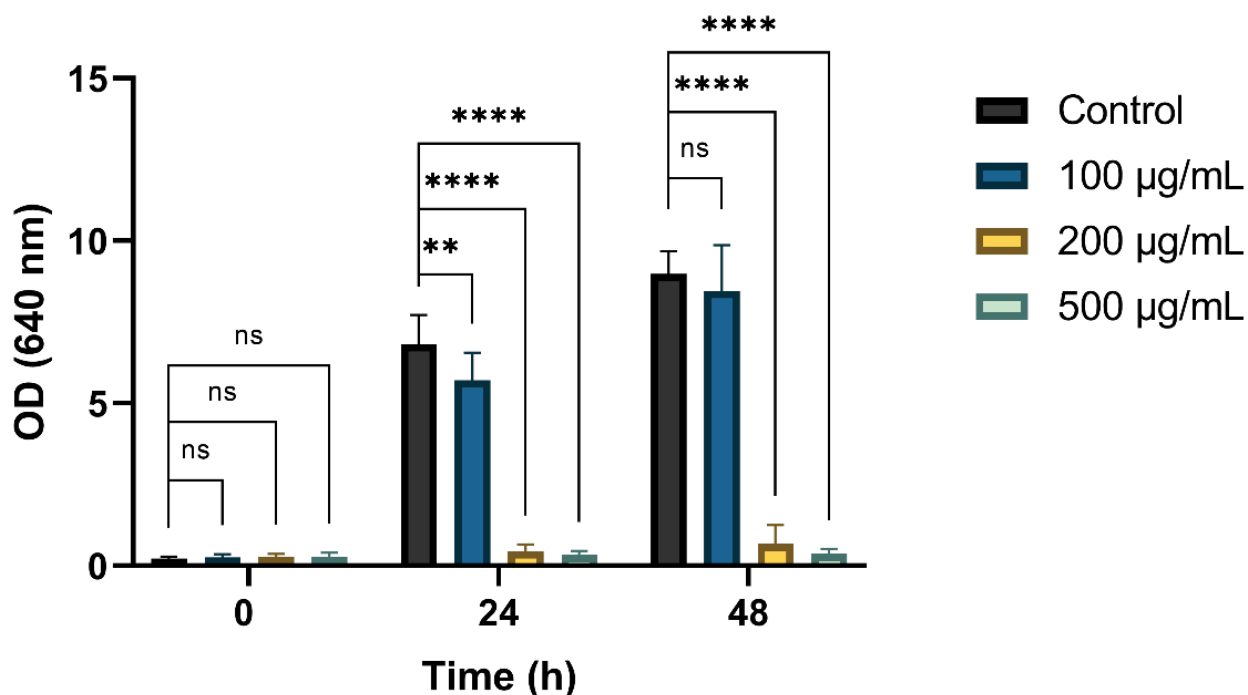
Figure 8.2. - Antimicrobial activity of *Thymus vulgaris* free EO against *Z. parabailii*. Optical density was measured at 640 nm. The reported values are means ± SD (n≥3). Statistical analysis was performed by the two-way ANOVA. ns, ** and **** indicate not significant, p≤0.01, and p≤0.0001, respectively, compared with cells control.

8.2.2.Zein-based particles

For assessing the activity of the *Satureja montana* EO zein particles, first particles were prepared at a concentration of 1 mg/mL of zein to 1 mg/mL of EO, and they were then used to test 100, 200 and 500 µg/mL final concentrations of EO. At the concentrations of 100 and 200 µg/mL of the SMEO, the zein particles demonstrate some antifungal activity, with p≤0.01 and p≤0.001, respectively (Figure 8.3). Yet, for these concentrations there was still growth, and the minimum inhibitory concentration was reached only at 500 µg/mL. Regarding the zein control (500 µg/mL), microbial growth appears to be considerably (p≤0.0001) reduce compared to the control, suggesting the positive anti-proliferative effect on yeast and potentiating the use of this formulation as antifungal.

At beginning of the assay (time zero) an increase in absorbance is observed over the concentrations, mainly between the control without EO and the zein control with a significance of

p≤0.001. This fact was due to the dispersed particles of zein that influences the absorbance, and at the time 24 and 48 hours, due to their precipitation.
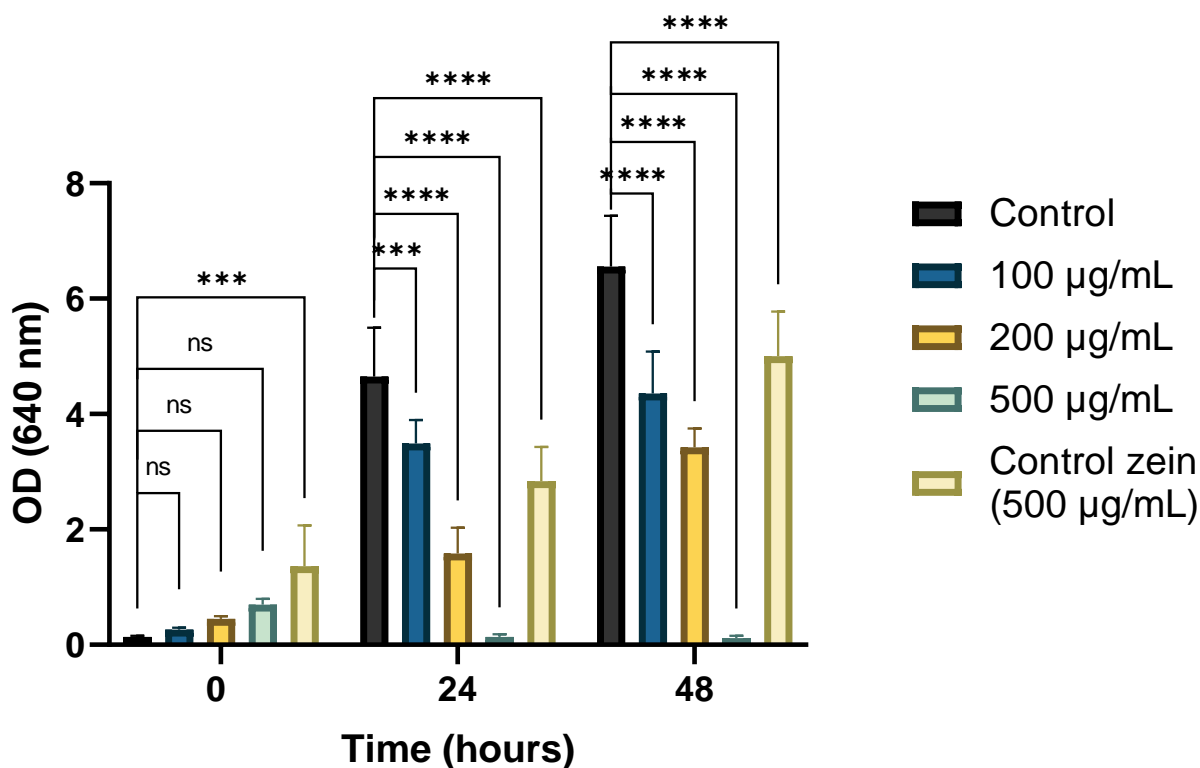
Figure 8.3. - Antimicrobial activity of zein particles with *Satureja montana* EO against *Z. parabailii*. The particles were at concentration 1 mg/mL zein to 1 mg/mL of EO. Control zein were at concentration 500 μg/mL of zein. Optical density was measured at 640 nm. The reported values are means ± SD (n≥3). Statistical analysis was performed by the two-way ANOVA. ***, **** and ns indicate p≤0.001, p≤0.0001 and not significant, respectively, compared with cells control.

To determine the antifungal activity of the *Satureja montana* EO zein particles, a second formulation of particles that were prepared at a concentration of 2 mg/mL of zein to 2 mg/mL of EO was used, and the particles were then used to tested at 100, 200 and 500 μg/mL final concentrations of EO. For this formulation of zein particles prepared with 2 mg/ml of zein to 2 mg/mL of TVEO, the results showed antimicrobial activity from 100 μg/mL (Figure 8.4) and the minimum inhibitory concentration was observed at 200 μg/mL with a significance of p≤0.0001 compared to the control at 48 hours of exposure to particles. In the figure 8.4, it is also possible to observe that the zein control (500 μg/mL) appears to significantly (p≤0.0001) decrease microbial growth when compared to the control.

Figure 8.4. - Antimicrobial activity of zein particles with *Satureja montana* EO against *Z. parabailii.* The particles were at concentration 2 mg/mL zein to 2 mg/mL EO. Control zein were at concentration 500 µg/mL. Optical density was measured at 640 nm. The reported values are means ± SD (n≥3). Statistical analysis was performed by the two-way ANOVA. **, ***, **** and ns indicate, $p \leq 0.01$, $p \leq 0.001$, $p \leq 0.0001$ and not significant respectively, compared with cells control.

To determine the antifungal activity of the *Satureja montana* EO zein particles, a third formulation of particles that were prepared at a concentration of 10 mg/mL of zein to 1 mg/mL of EO was used, and the particles were then used to tested at 100, 200 and 500 µg/mL final concentrations of EO. For this formulation of 10 mg/mL of zein to 1 mg/mL EO, a decrease in growth of *Zygosaccharomyces parabailii* was obtained from 200 µg/mL EO (Figure 8.5) at 48 hours. Although the highest concentration (500 µg/mL) was more effective and had significant differences ($p \leq 0.0001$), the MIC of the formulation couldn't be established within the concentrations used in this study. This results show that this formulation compared with the other two zein-based encapsulated SMEO particles, was the least efficient against *Z. parabailii*.

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Figure 8.5. - Antimicrobial activity of zein particles with *Satureja montana* EO against *Z. parabailii.* The particles were at concentration 10 mg/mL zein to 1 mg/mL EO. Control zein were at concentration 100 μg/mL and 200 μg/mL. Optical density was measured at 640 nm. The reported values are means ± SD (n≥3). Statistical analysis was performed by the two-way ANOVA. *, **, *** and **** indicate p≤0.05, p≤0.01, p≤0.001 and p≤0.0001, respectively, compared with cells control.

After optimization the antifungal activity of zein particles, the formulation that gave the lowest MIC with *S. montana*, was used for preparing *T. vulgaris* EO particles. So, the particles were prepared with a concentration of 2 mg/mL of zein to 2 mg/mL of EO, and then used to test 100, 200 and 500 μg/mL final concentrations of TVEO. Antifungal activity of TVEO zein-based particles against *Z. parabailii* was observed from 100 μg/mL EO (Figure 8.6), compared to control both for 24 and 48 hours of assay. However, the minimum inhibitory concentration was reached only at 500 μg/mL, the inhibition observed at 24 h being maintained for 48 h of assay.

Again, at the beginning of the assay, there is a significant increase in absorbance with increasing concentrations, and between the control and the zein control.

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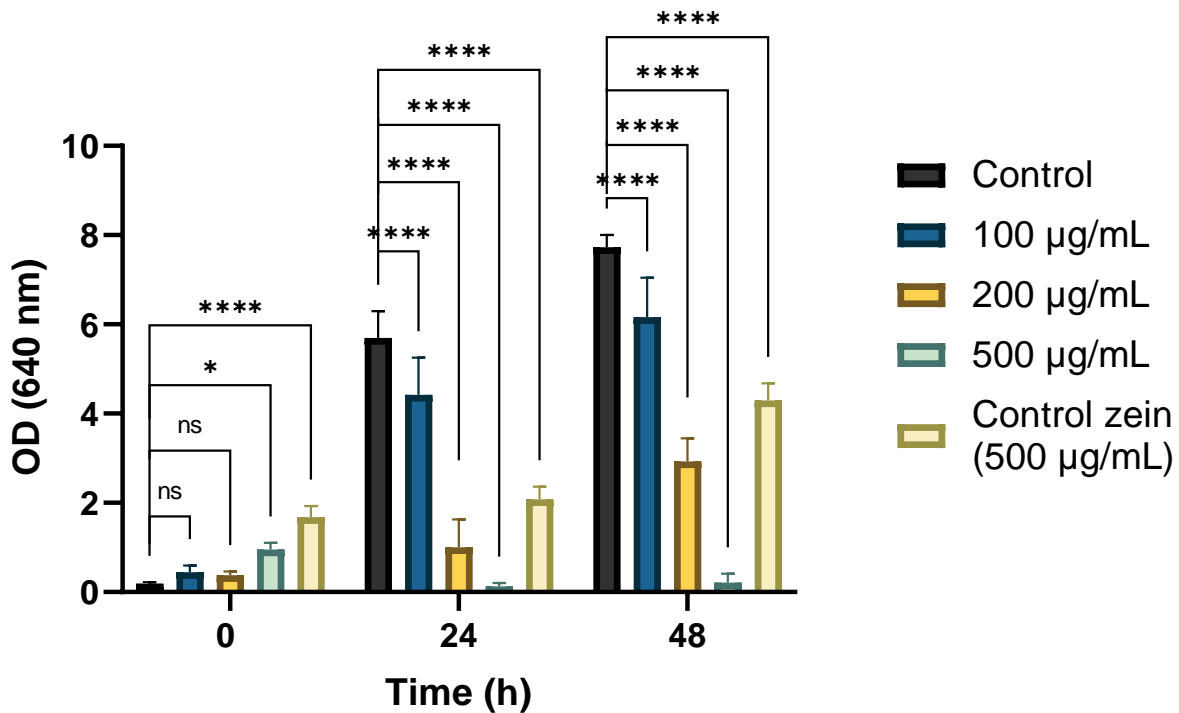
Figure 8.6 - Antimicrobial activity of zein particles *Thymus vulgaris* EO at concentration 2 mg/mL zein to 2 mg/mL against *Z. parabailii*. Control zein were at concentration 500 µg/mL. Optical density was measured at 640 nm. The reported values are means ± SD (n≥3). Statistical analysis was performed by the two-way ANOVA. *, **** and ns indicate p≤0.05, p≤0.0001 and not significant, respectively, compared with cells control.

At beginning of the assay a significant increase in absorbance is observed over the concentrations in all the zein-based formulations (Figure 8.3 to 8.6). This fact was due to the dispersed particles of zein that influences the absorbance, and at the time 24 and 48 hours, due to their precipitation. A similar effect was also described in literature, when the ethanol concentration decreases, the zein becomes insoluble and precipitates due to the less ordered structure and increasing exposure of hydrophobic groups (Li *et al.*, 2013).

This performance of the zein particles, suggests that can be due to high susceptibility to temperature, pH or ionic strength (Tinoco *et al.*, 2021), compromising the stability of zein particles and limiting their performance as active compound delivery. The use of copolymers, such as keratin (Tinoco *et al.*, 2021) and sodium caseinate (Li *et al.*, 2013), polysaccharides as chitosan derivatives or basic amino acids (i.e., lysine or arginine) (Martínez-López *et al.*, 2020), that are commonly added into the particle formulation, and in this case would improve dispersion stability

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observed, possibly increase the antimicrobial activity and avoid these limiting issues in future. Changes in the concentration of the zein solution, the electric field applied, the injection speed, or the needle-to-tip distance (Martínez-López *et al.*, 2020) are also described to alter the physicochemical parameters on the development of the zein-based particles that should be taken into consideration.

8.2.3.Keratin-based particles

To determine the antifungal activity of *Satureja montana* encapsulated in keratin-based particles 100, 200 and 500 μg/mL final concentrations of SMEO were used. Antifungal activity (Figure 8.7) was observed with this particles for all the concentrations compared to control, with significant differences ($p \leq 0.0001$). The MIC was reached at 48 hours with the concentration of 200 μg/mL.

On the other hand, the empty keratin-based particles not only did not inhibit its growth, but a significant ($p \leq 0.0001$) increase in optical density was obtained compared to the control. This is in agreement with the literature, since it has been described that components of particles such as keratin did not cause cellular toxicity, and materials based on keratin, such as hydrogels, scaffolds, and films, were demonstrated to have a proliferative stimulating effect (Gonçalves, 2017; Rouse & Van Dyke, 2010; Sierpinski *et al.*, 2008; Tinoco *et al.*, 2018).
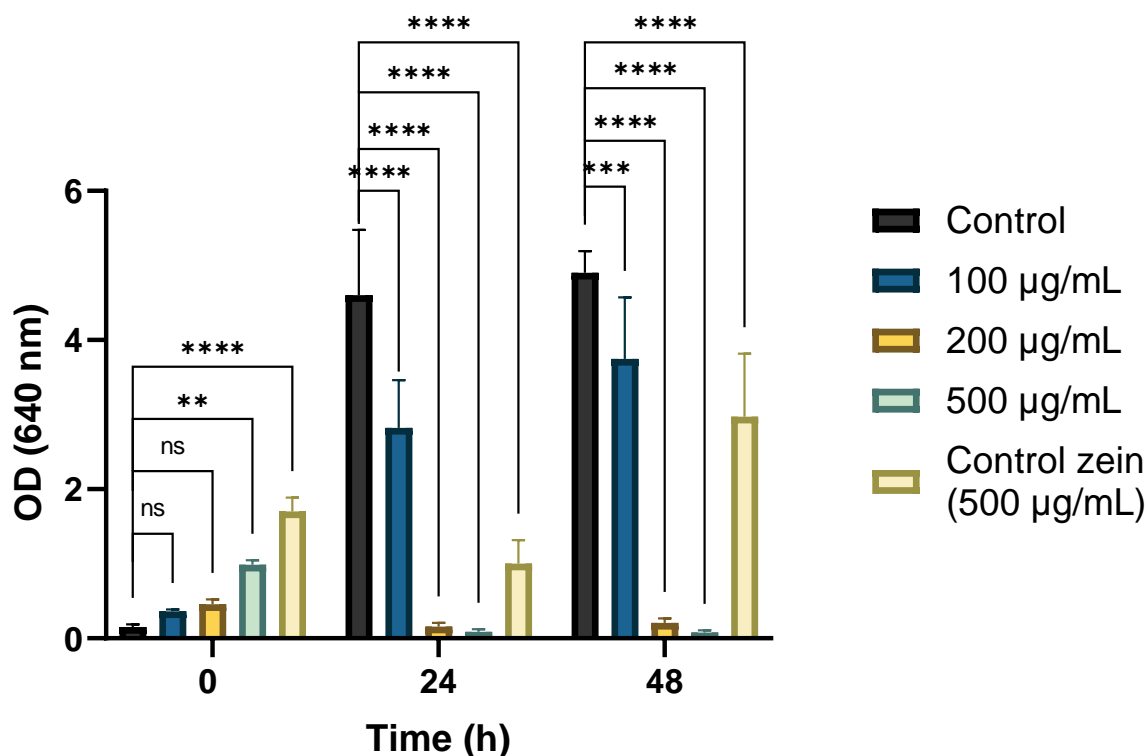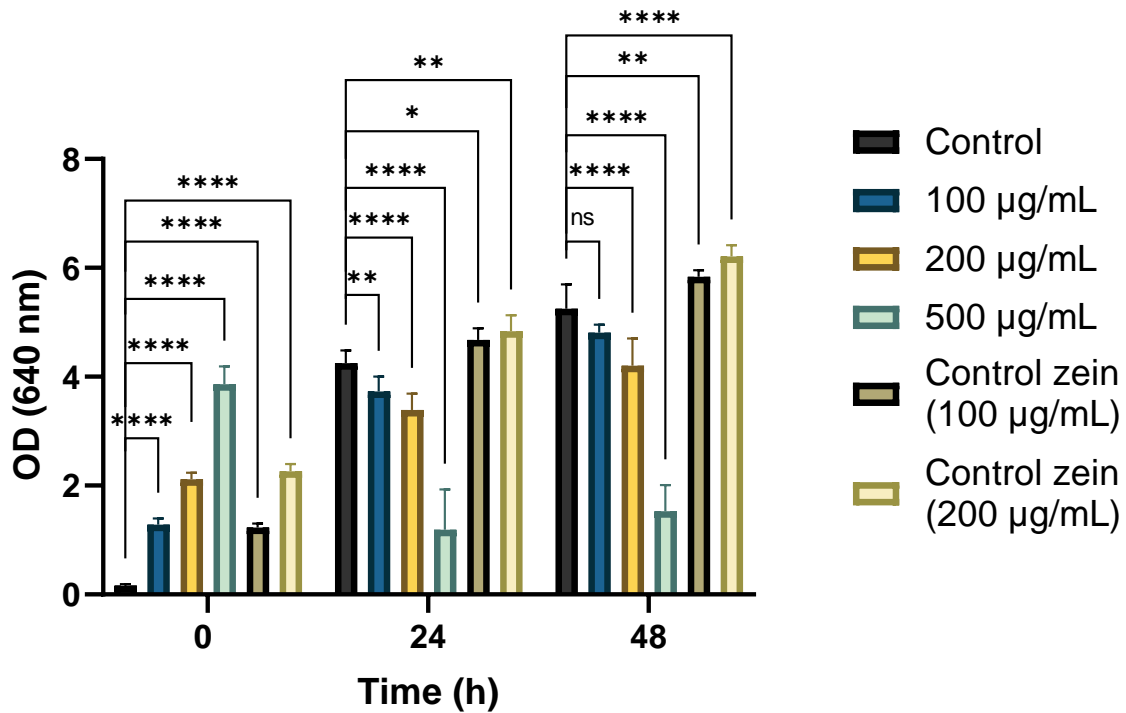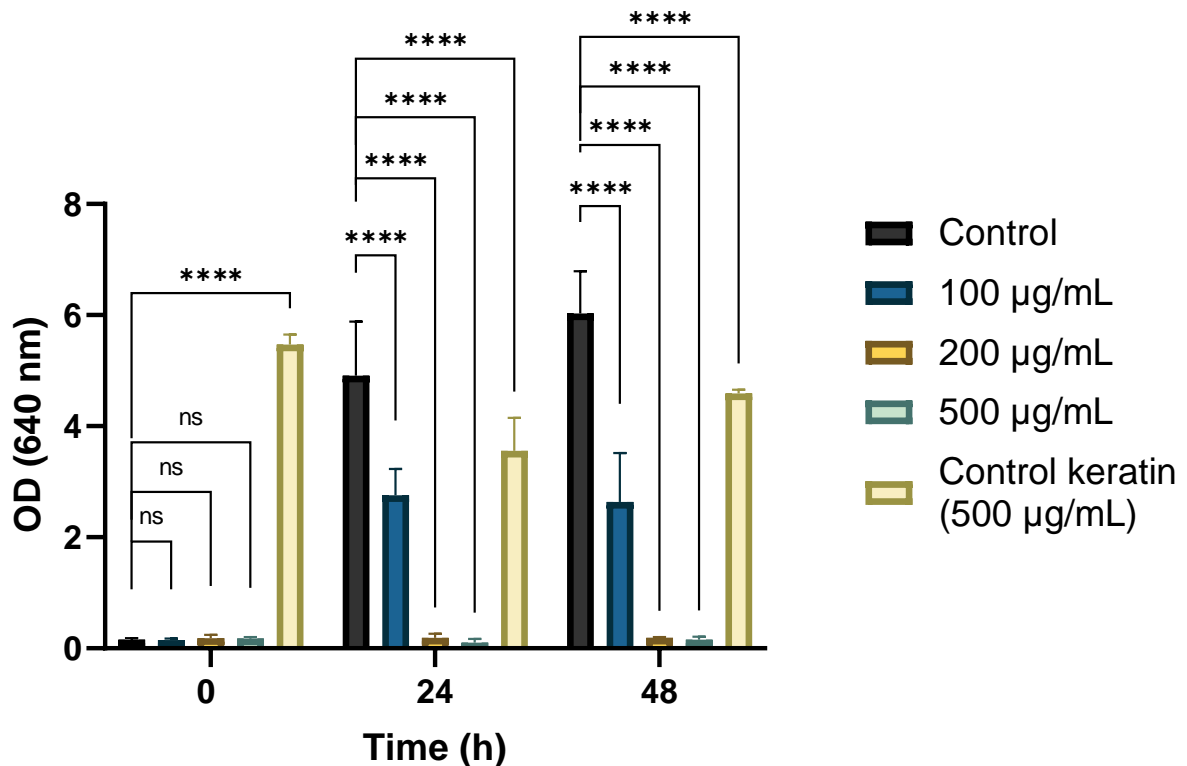
Figure 8.7. - Antimicrobial activity of keratin particles with *Satureja montana* EO against *Z. parabailii*. Control keratin were at concentration 500 µg/mL. Optical density was measured at 640 nm. The reported values are means ± SD (n≥3). The control keratin values at 24 and 48 hours were rectified by subtracting the value for time 0 for better interpretation of the results. Statistical analysis was performed by the two-way ANOVA. **** and ns indicate p≤0.0001 and not significant respectively, compared with cells control.

In order to investigate the antifungal activity *T. vulgaris* EO encapsulated in keratin-based particles was tested 100, 200 and 500 µg/mL final concentrations of TVEO. Antifungal activity from TVEO keratin-based particles (Figure 8.8) was observed for all the concentrations compared to control until 24 hours of assay (p≤0.0001), after that the growth of *Z. parabailii* recovered for 100 and 200 µg/mL. The MIC was reached with the concentration of 500 µg/mL.

Again, empty keratin-based particles did not inhibit *Z. parabailii* growth, but actually a significant increase in optical density was observed when compared to the control, suggesting that keratin derivatives have proliferative-stimulating activity in agreement with the literature (Gonçalves, 2017; Rouse & Van Dyke, 2010; Sierpinski *et al.*, 2008; Tinoco *et al.*, 2018).
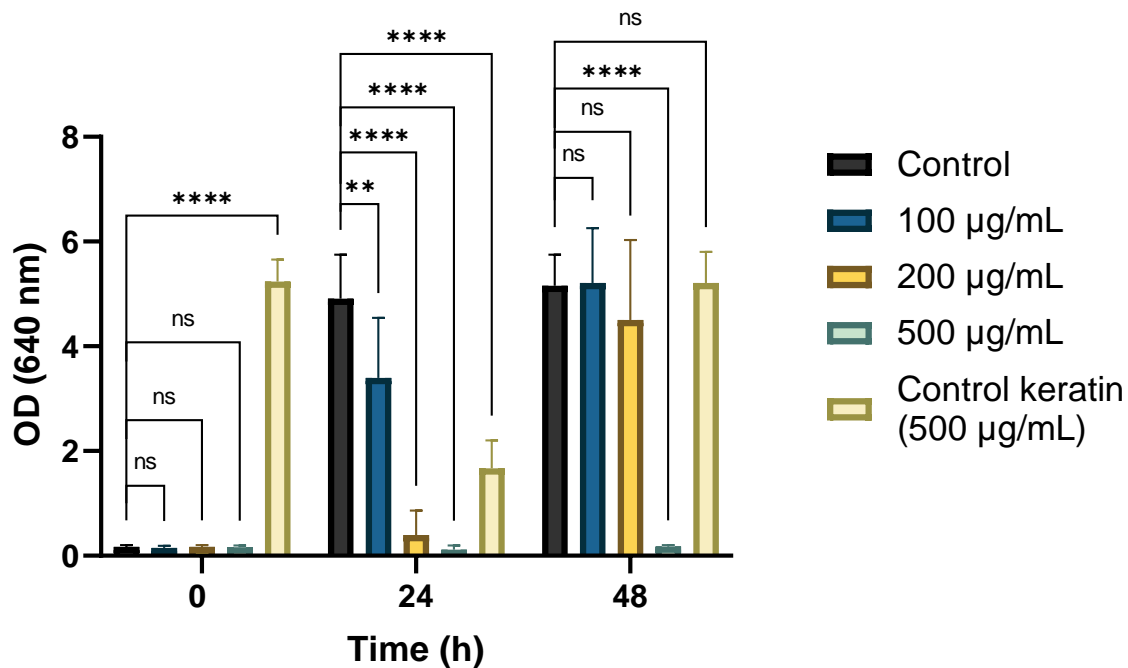
Figure 8.8 - Antimicrobial activity of keratin particles *Thymus vulgaris* EO against *Z. parabailii.* Control keratin were at concentration 500 µg/mL. Optical density was measured at 640 nm. The reported values are means ± SD (n≥3). The control keratin values at 24 and 48 hours were rectified by subtracting the value for time 0 for better interpretation of the results. Statistical analysis was performed by the two-way ANOVA. **, **** and ns indicate p≤0.01, p≤0.0001 and not significant, respectively, compared with cells control.

8.2.4. Liposome particles

For assessing the antifungal activity of essential oil from *Satureja montana* encapsulated in liposome particles to test 100, 200 and 500 µg/mL final concentrations of SMEO were used. *Z. parabailii* growth was reduced from 100 µg/mL when treated with SMEO liposome-based particles (Figure 8.9). However, the MIC cannot be primarily determined by analyzing absorbance. The minimum lethal concentration (MLC) of this formulation was determined by combining antimicrobial activity data with CFUs (Figure 8.11), and was reached at the concentration 500 µg/mL.

The low electrical charge values of the particles used in this study (from - 3.150 to - 0.636 mV), might provide crucial information on the particle morphological instability and on the antifungal performance since the interaction of particles with their environment is determined by their surface charge. In fact, the high absorbance values for the different concentrations, may be

due to poor colloidal stability in the suspension, unable to withstand aggregation of the particle, and have minimal electrostatic repulsion.
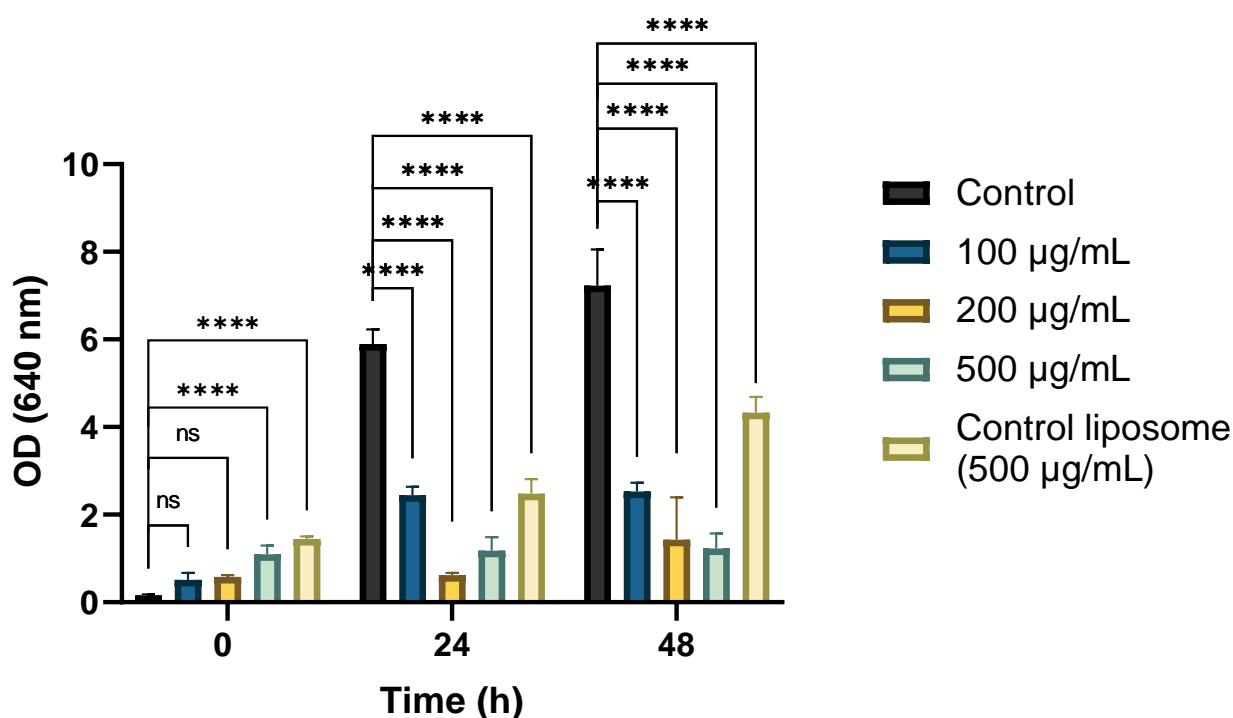
Figure 8.9. - Antimicrobial activity of liposome particles with *Satureja montana* EO particles against *Z. parabailii*. Control liposome were at concentration 500 µg/mL. Optical density was measured at 640 nm. The reported values are means ± SD (n≥3). Statistical analysis was performed by the two-way ANOVA. **** and ns indicate p≤0.0001 and not significant respectively, compared with cells control.

For assessing the antifungal activity of essential oil from *Thymus vulgaris* encapsulated in liposome particles were used to test 100, 200 and 500 µg/mL final concentrations of TVEO. As to antimicrobial activity of liposome particles of *T. vulgaris* EO, *Z. parabailii* growth was slightly reduced for 100 µg/mL and 200 µg/mL EO but without significance (Figure 8.10). For the 500 µg/mL concentration a significant decrease was obtained for 24 h of growth but not for 48 h. So, the MIC could not be determined only by measuring absorbance. The MLC of this formulation was established using a combination of optical density data and CFUs determination (Figure 8.12), which revealed that was reached at a concentration of 500 µg/mL.

The difficulty to determine antifungal efficacy using absorbance shows that the particle experiences some morphological change, as previously noted with the same encapsulation type in

S. montana EO (Figure 8.9). The particles used presented low electrical charges (-2.13 to -0.818 mV) that can create instability in interactions with their surroundings and cause instability in its absorption by the cell.
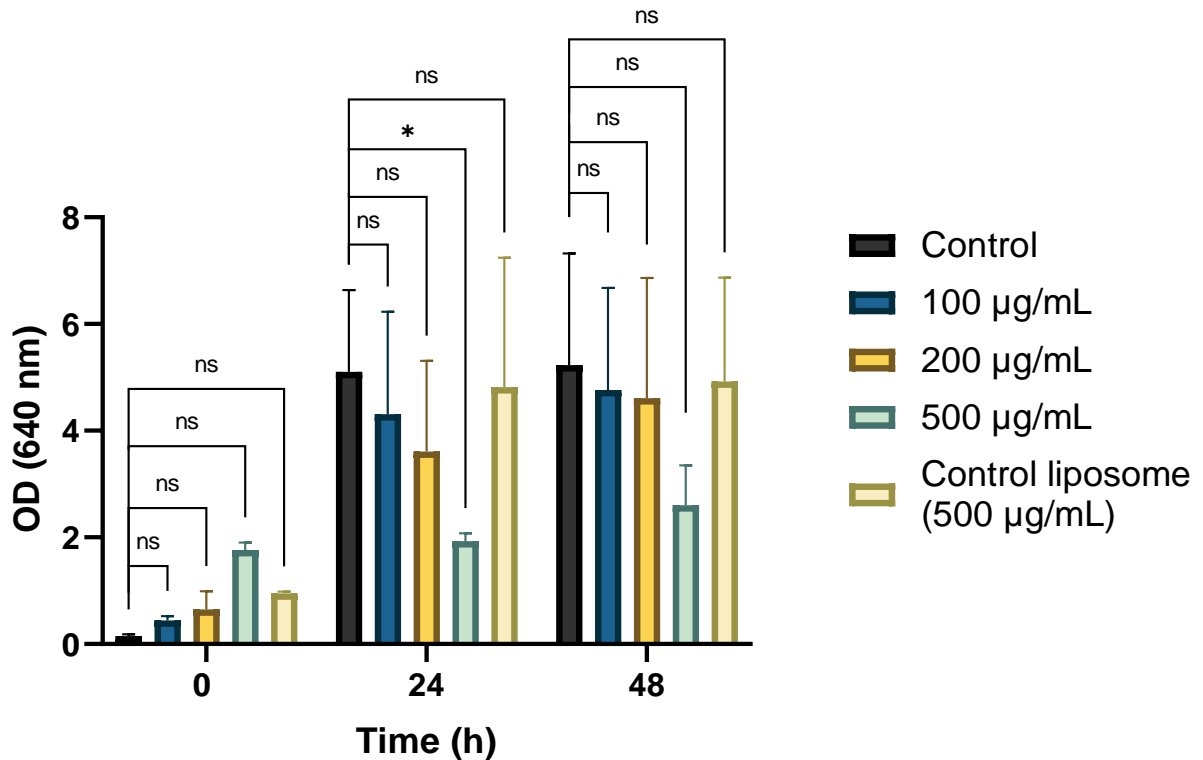
Figure 8.10. - Antimicrobial activity of liposome particles of *Thymus vulgaris* EO against *Z. parabailii.* Control liposome were at concentration 500 µg/mL. Optical density was measured at 640 nm. The reported values are means ± SD (n≥3). Statistical analysis was performed by the two-way ANOVA. * and ns indicate p≤0.05 and not significant, respectively, compared with cells control.

8.2.5. Minimum Inhibitory Concentration (MIC) and Minimum Lethal Concentration (MLC)

Aiming to analyze if the EOs under study when encapsulated, besides inducing growth arrest, could also induce cell death in the culture, next, the CFU values of SMEO encapsulated, in the most significant inhibitory concentrations evaluated previously (200 and 500 µg/mL), with the different materials were compared to those of the free essential oil for the time point of 48 h of incubation.

In the case of *Satureja montana* EO, for the concentration of 200 µg/mL, it was clear that liposome-based particles had a considerably decreased antifungal activity (p≤0.0001) against *Z. parabailii*, while the zein particles maintained and keratin increased their effect compared to free EO (Figure 8.11). However, for 500 µg/mL, the maximum concentration applied in the present study, no CFU formation was observed, this being a lethal concentration for all the different formulations and for the free EO.
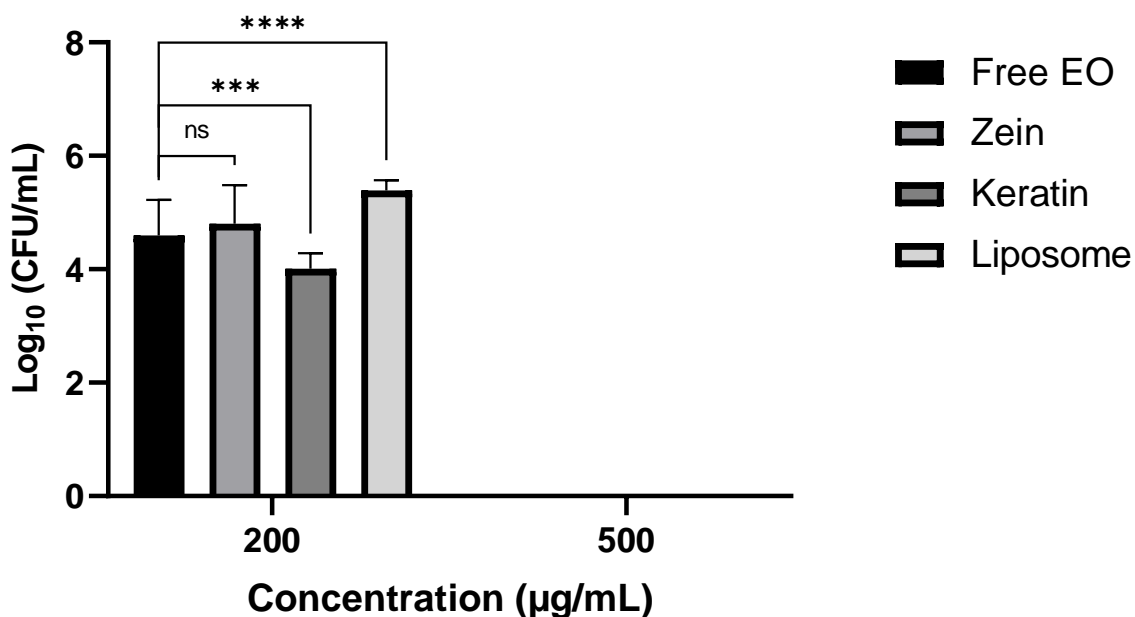
Figure 8.11. – CFU of cell suspensions of *Z. parabailii* treated with the *Satureja montana* EO at 200 and 500 µg/mL, free or encapsulated in different formulations, at time point of 48 hours of treatment. The reported values are means ± SD (n≥3). Statistical analysis was performed by the two-way ANOVA. ***, **** and ns indicate p≤0.001, p≤0.0001 and not significant respectively, compared to 200 µg/mL of free essential oil, that was considered control.

The CFU values of *Z. parabailii* cultures treated with encapsulated TVEO, at the more inhibitory concentrations evaluated previously (200 and 500 µg/mL), were assessed to analyze if the EOs under study when encapsulated, besides inducing growth arrest, could also induce cell death in the culture. In addition, the effects of the particles formulations were compared to the free essential oil (Figure 8.12).

The CFU values of *Z. parabailii* cultures treated with encapsulated TVEO (Figure 8.12) for 48 h, showed that zein-based particles had a considerably decreased antifungal activity (p≤0.0001 when compared to the free essential oil. The results allow to conclude that there is still significant

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growth (since the number of CFUs is considerable higher than that of free EO) in this treatment conditions and so that the minimum inhibitory concentration for this formulation is 500 μg/mL, which is also the lethal concentration.

Furthermore, the TVEO encapsulated in the keratin-based particles and in the liposomes maintained its activity compared to control, without significant differences. It was also possible to observe that no colonies were formed at the maximum concentration of 500 μg/mL in all the formulations, demonstrating the cytotoxic lethality already established in the free essential oil (TVEO), the control.
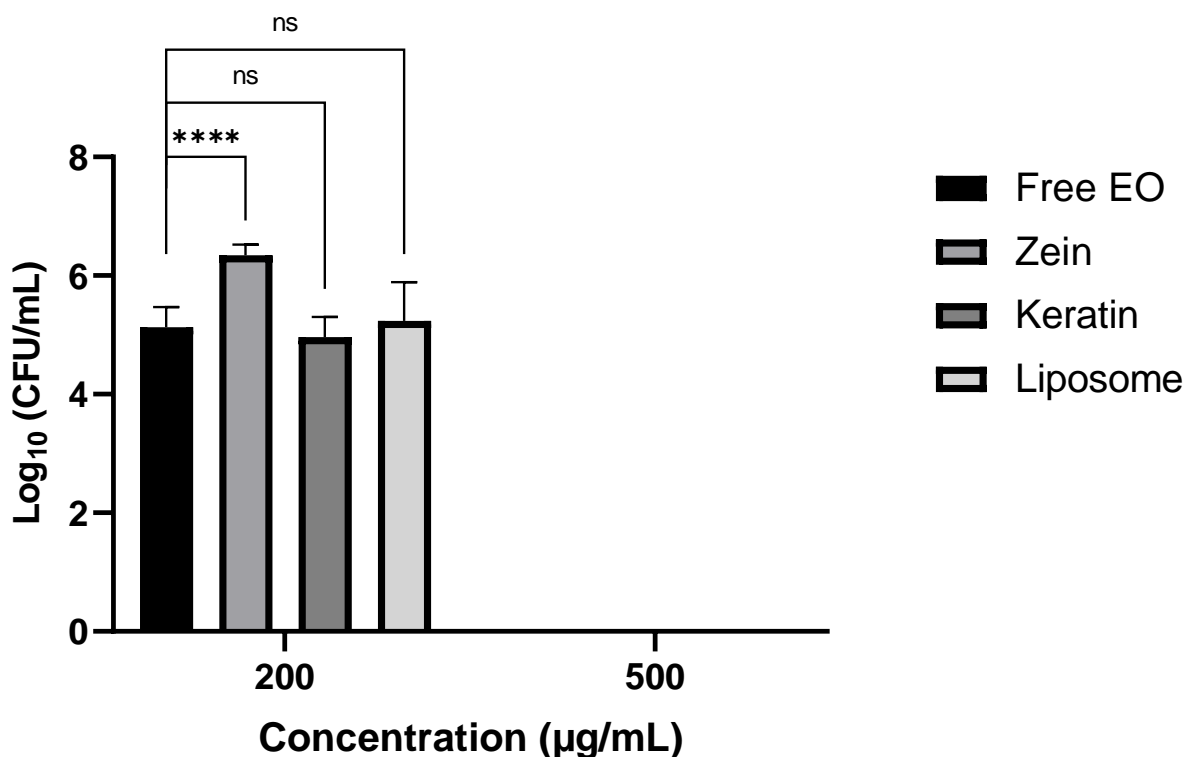
Figure 8.12. - CFU of cell suspensions of *Z. parabailii* treated with the *Thymus vulgaris* EO at 200 and 500 μg/mL, free or encapsulated in different formulations, at time point of 48 hours of treatment. The reported values are means ± SD (n≥3). Statistical analysis was performed by the two-way ANOVA. **** and ns indicate $p \leq 0.0001$ and not significant respectively, compared to 200 μg/mL of free essential oil, that was considered control.

In summary (Table 8.4), with *S. montana* OE the MIC of 200 μg/mL was obtained in three of the five formulations analyzed, the free EO, zein particles at 2 mg/mL and keratin-based particles. Regarding *Thymus vulgaris* EO, the lowest MIC obtained was 200 μg/mL, and it was

reached only with free essential oil. These findings raised a concern since the encapsulation of the essential oil *Thymus vulgaris* could be restricting the action of the primary active components. It was also demonstrated that between free EOs the two were similar active when compared to *S. montana* essential oil obtaining in both a MIC of 200 µg/mL.

Nonetheless, according to Kalli *et al.* (2022) the polygodial sesquiterpene, derived from *Polygonum hydropiper*, is the most promising antifungal and common pesticide against Z. *parabailii*, with a low minimum fungicidal concentration (MFC) of 50 µg/mL (213 M), which is better than the results reported in this study.

Despite the fact that the EOs of thyme and savory are mostly made of thymol and carvacrol, both of which are highly efficient in antimicrobial activity and permeabilization of cell membrane (Maccelli *et al.*, 2020; Ni *et al.*, 2021; Pateiro *et al.*, 2021; Santoro *et al.*, 2018), and their composition are similar, the antibacterial action after encapsulation is quite different. Savory revealed to be superior in growth inhibition of spoilage yeast Z.*parabailii* due to positive performance in more than 50% of encapsulating formulations tested. The MIC values for plant derivatives, such as essential oil, define strong inhibition of antifungal activity at concentrations up to 500 µg/mL (Aligiannis *et al.*, 2001).

Table 8.4. - Summary table of the minimum inhibitory concentrations (MIC) and minimum lethal concentrations (MLC) of the free and encapsulated essential oils of *Satureja montana* and *Thymus vulgaris*, obtained at the antimicrobial activity assays. NE means not evaluated, respectively

Yeast *Z. parabailii*		Concentration (µg/mL)					
			Particles				
		EO	Zein			Keratin	Liposome
			1 mg/mL	2 mg/mL	10 mg/mL		
Satureja montana	MIC	200	500	200	>500	200	500
	MLC	500	500	500	500	500	500
Thymus vulgaris	MIC	200	NE	500	NE	500	500
	MLC	500	NE	500	NE	500	500

8.3. Effect of *Satureja montana* and *Thymus vulgaris* EOs on plasma membrane integrity

Since it was observed that the EOs under study, both free and encapsulated, beside inhibiting growth could also induce cell death, next we questioned if loss of plasma membrane integrity could underlie the cell death inducing effect. To clarify this issue, the particles with the best performance, the keratin particles, were used. This analyzes could also help to discriminate between necrotic and apoptotic-like cell death. For assessing plasma membrane integrity, the *Zygosaccharomyces parabailii* cells were stained with propidium iodide (PI). Propidium iodide is the most popular technique for monitoring cell membrane integrity in yeast (PI). It is a fluorescent nucleic acid intercalator that can only enter cells with a damaged cell membrane and can be used in low and high throughput applications (Carmona-Gutierrez *et al.*, 2018).

Z. parabailii cells were incubated for a period of 48 hours with 200 and 500 µg/mL of both EOs in the keratin-based formulations and cells samples were collected at predetermined time points (0, 1, 5, 24 and 48 hours of treatment), Samples were then stained with PI and analyzed flow cytometry and fluorescence microscopy (Figure 8.13 to 8.18 and Appendix table A.1).

Maximum percentage of PI positive cell (94%) was obtained at the concentration of 500 µg/mL of *Satureja montana* EO at time point 48 hours (Figure 8.13(A) and 8.14). Meanwhile, there no loss of membrane integrity was observed for the concentration of 200 µg/mL SMEO. For the particles with *Thymus vulgaris* EO a maximum of 8.11% of PI positive cell was obtained at 24 hours of treatment with 500 µg/mL, that reveled to be inefficient in terms of damaging the integrity of the *Z. parabalii* membrane. The results obtained for PI assay were consistent with the optical density data obtained for antifungal activity against *Z. parabalii* (Figure 8.13 (A)).

According to Donsì & Ferrari (2016), EOs function at the level of microorganism plasma membranes; in this situation, where keratin-based particles of *S. montana* EO were evaluated, the results are consistent with this conclusion. Also, thymol and carvacrol, main compounds of both essential oils used in this study, have been found to act on microbial cells, producing structural and functional cell membrane damage, increased permeability, and eventually cell death (Ni *et al.*, 2021).

To complement data collected using PI, it is imperative to incorporate it with methodologies that allow for experimentally assessing cell viability, which reflects a cell's ability to proliferate (Carmona-Gutierrez *et al.*, 2018). Our previous results (Section 8.2.5) showed that there were no viable cell at 48 h of treatment with 500 μg/mL of both *S. montana* and *T. vulgaris* EOs, either free or encapsulated. Together, analysis of CFU and membrane integrity results shows that loss of CFU occurs concomitantly with loss of membrane integrity for the SMEO keratin particles, which is consistent with the potential occurrence of necrosis for these particles.

On the other hand, for TVEO keratin particles, there is no significant increase in loss of plasma membrane integrity which indicates that loss of cell viability may not be due to a necrotic process. Further assays, however, are necessary since PI results are only from one experiment.

(A)

(B)
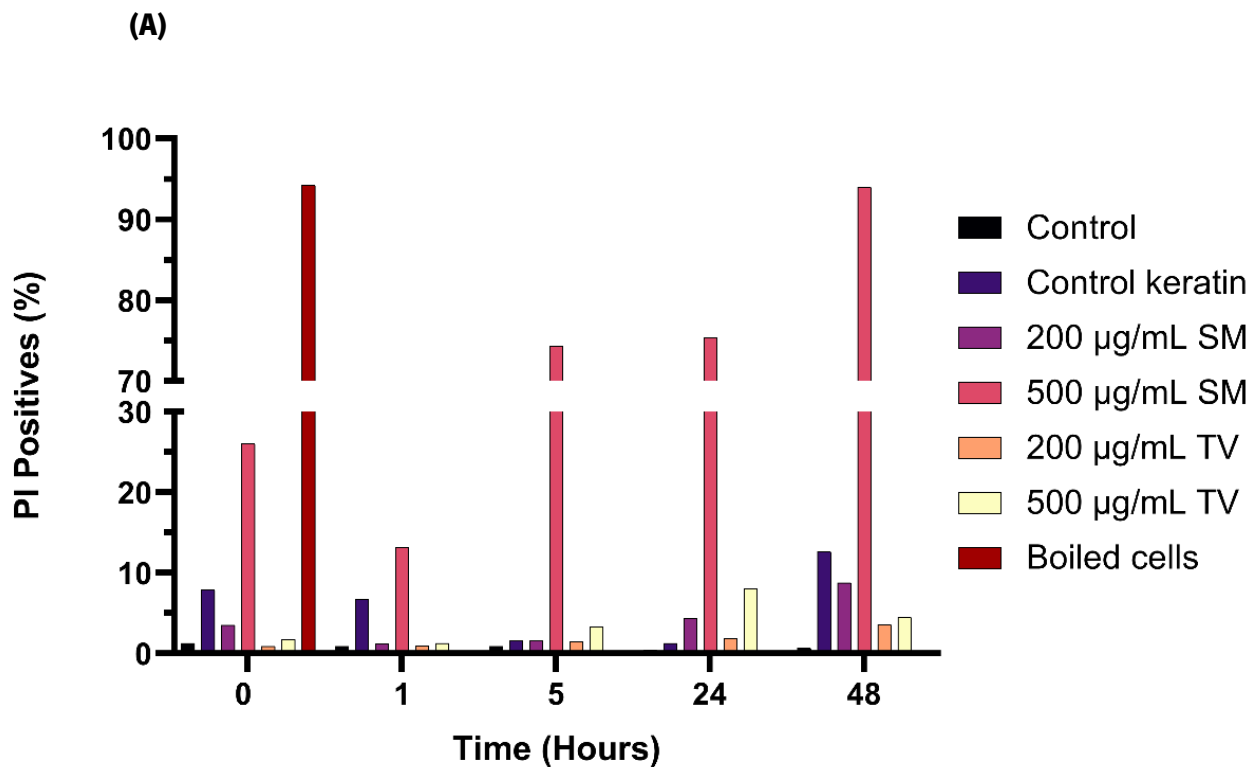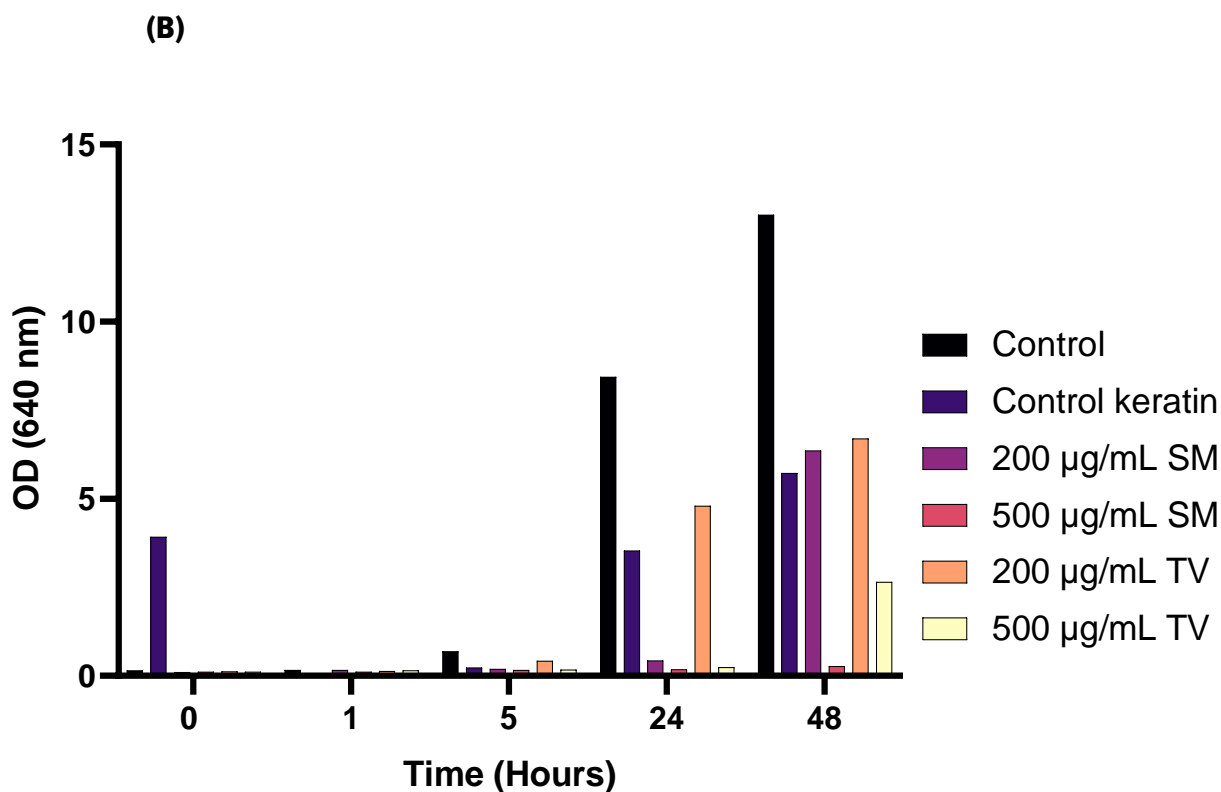
Figure 8.13. – **(A)** Analysis of cell membrane integrity of *Z. parabailii* cells treated with keratin particles of *Satureja montana* (SM) and *Thymus vulgaris* (TV) EOs and stained with propidium iodide (PI). Boiled cells are used as a positive control for PI staining. For flow cytometry analysis the cells were collected at different time points 0, 1, 5, 24 and 48 hours of treatment and then stained with 2 μg/mL of PI. Control keratin were at concentration 500 μg/mL. Results are expressed as percentage of PI positive population. **(B)** Antimicrobial activity of keratin particles against *Z. parabailii*. Optical density was measured at 640 nm. Control keratin were at concentration 500 μg/mL. For the control keratin condition, values of time 1, 5, 24 and 48 hours were rectified by subtracting the value for time 0 for better interpretation of the results. SM and TV means *Satureja montana* and *Thymus vulgaris*, respectively. All the reported values are result of one experiment (n=1).

The MIC previously established with *S. montana* for keratin particles was 200 μg/ml, and in the case of the results obtained in flow cytometry, it is also possible to see that at a concentration of 200 μg/ml, *Z. parabailii* growth is inhibited only for the 24 hours of assay (Figure 10.1(B)). Based on the results obtained from optical density the cause for the recovery of growth in the final 24 hours of the test remains uncertain; it could be related with the volume of the suspension or even oxygen availability in the solution as the experiment was performed in an higher volume (Blackburn, 2006). It could also be due to the stability of the encapsulation of the EO in the particle, as some weeks passed since their preparation, opposite to the first experiments of the section.

Despite Gonçalves (2017) and Tinoco *et al.* (2018) mention that the particles stability is unaffected after three months of storage, in this study suggests that EO loses their encapsulation stability. On the other hand, some yeast retains the ability to repair cell membrane damage even after staining positive for PI in response to certain stress shocks (Carmona-Gutierrez *et al.*, 2018)..

The efficiency of SMEO encapsulation at 500 μg/mL had exceptionally positive performance (Figure 8.14), with percentages above 70% after 5 hours of testing and reaching a maximum of 94.01% after 48 hours. The antifungal efficacy and viability of the keratin-based particles with SMEO were also validated, confirming that the concentration of 500 μg/mL keratin-based particles is the minimum lethal concentration.
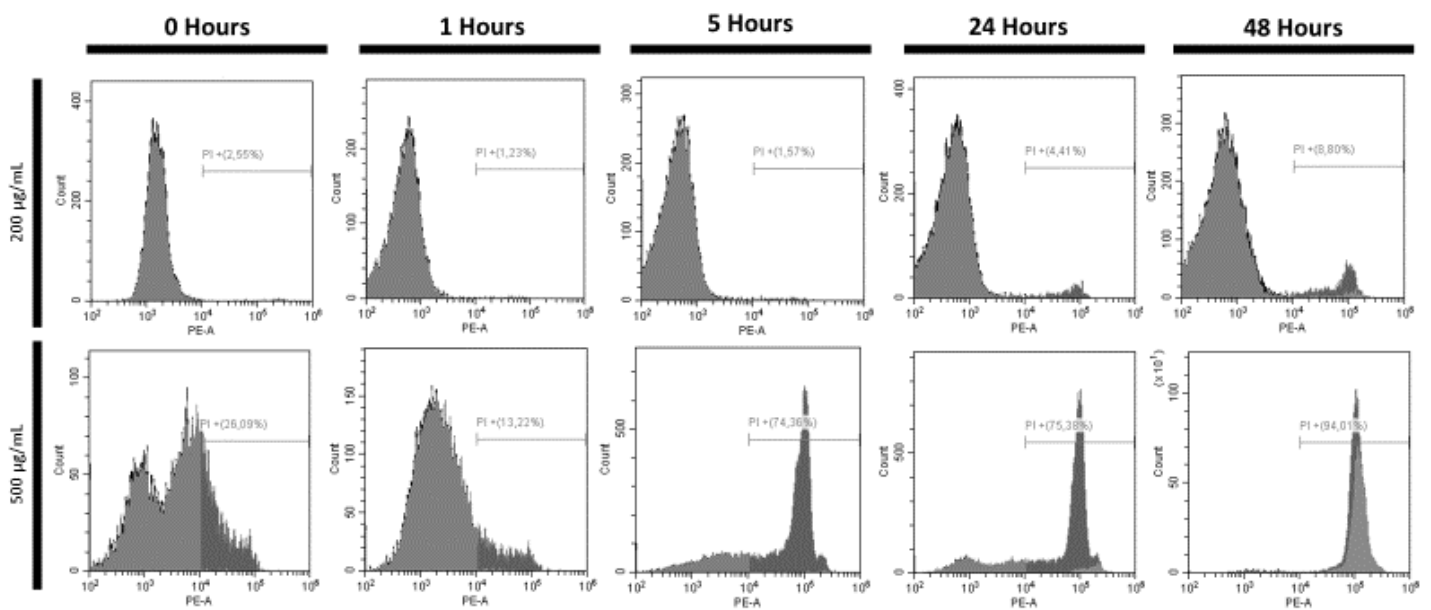
Figure 8.14 - Flow cytometry analysis of cell membrane integrity with keratin particles of *Satureja montana* (SMEO) with concentrations 200 and 500 μg/mL against *Z. parabailii* stained with propidium iodide (PI). For flow cytometry analysis the cells were collected at different time points 0, 1, 5, 24 and 48 hours of treatment and then stained with 2 μg/mL of PI. Results are expressed as percentage of PI positive population. The reported values are result of one experiment (n=1).

The incorporation of the keratin particles inside the cell and its direct association with the percentage of PI positive cells were also investigated during the research using flow cytometry. This was possible, as keratin particles were stained with green Fluorescein Isothiocyanate (FITC).

At a concentration of 500 μg/mL of the SMEO keratin-based particles, a population rise trend is detected in the right quadrants, which is consistent with an increase in the percentage of

PI positive cells. In addition, there is an upward tendency from the lower to upper quadrants, which corresponds to a rise in the percentage of green fluorescence (Figure 8.15). This can also be seen in fluorescence microscopy at time points of 24 and 48 hours incubation, where a correlation between FITC and PI positive can be seen (Figure 8.16). These data suggests that membrane permeability is directly related to SMEO keratin-based particles incorporation.
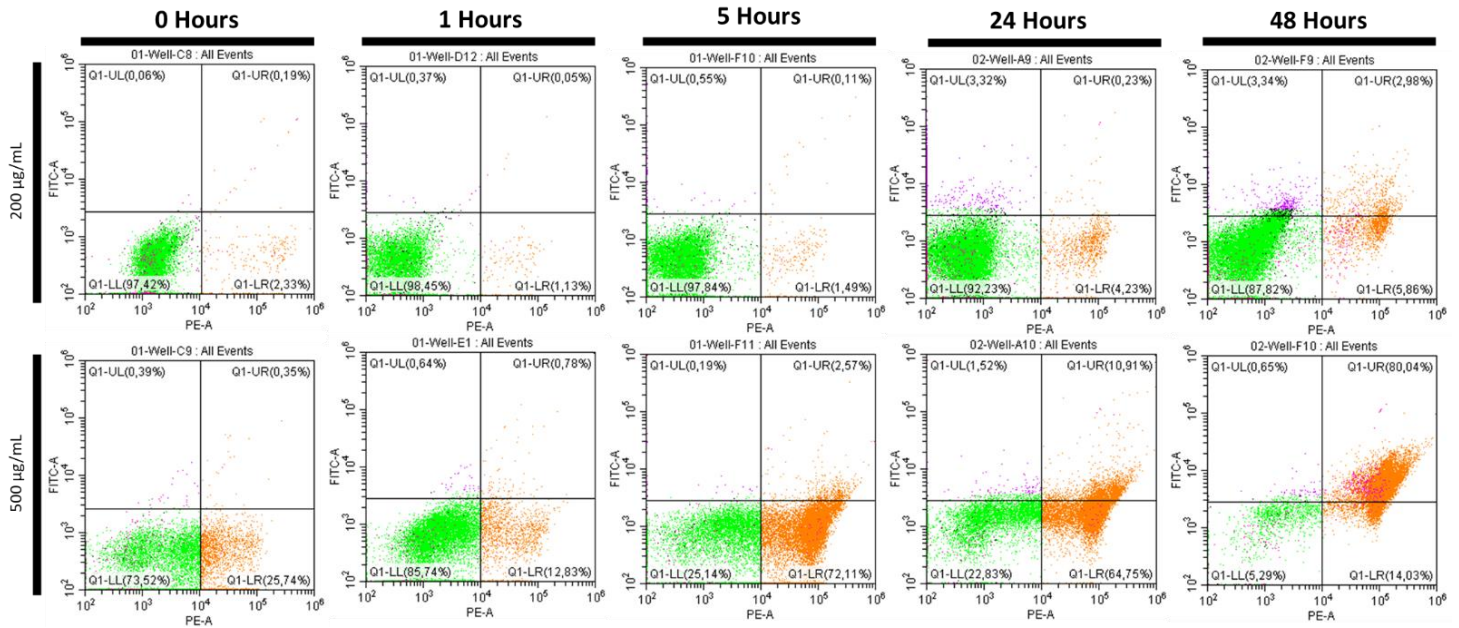
Figure 8.15 - Flow cytometry analysis of *Z. parabailii* cell membrane integrity after treatment with keratin FITC marked particles of *Satureja montana* (SMEO) at concentrations of 200 and 500 μg/mL. For flow cytometry analysis the cells were collected at different time points 0, 1, 5, 24 and 48 hours of treatment and then stained with 2 μg/mL of PI. Results are expressed as percentage of PI positive population. The reported values are result of one experiment (n=1).

However, it is unclear where the particles are contained inside specific cellular structures (Figure 8.16); primarily, visual alterations are detected at the yeast volume level, with a more rounded form rather than a narrow silhouette. The use of FITC was also crucial in understanding that even after the membrane is permeable, particles is detected inside and is not released into the solution, suggesting that it is not only reserved, but also present in various cell structures (Donsì & Ferrari, 2016; Sousa, 2016; Zhu *et al.*, 2021).
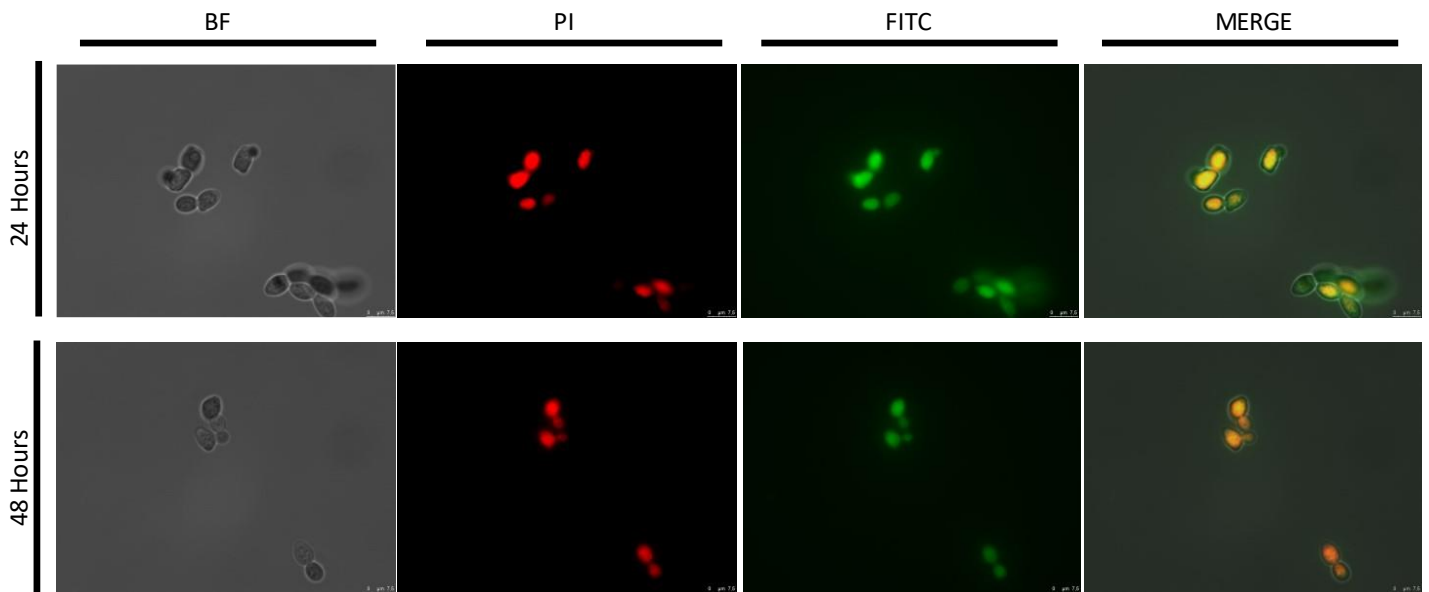
Figure 8.16 - Fluorescence microscope analysis of *Z. parabailii* cells treated with keratin particles of *Satureja montana* (SM) at concentration of 500 µg/mL and stained with propidium iodide (PI). For fluorescence microscopy analysis the cells were collected at different time points of 24 and 48 hours of treatment and then stained with 2 µg/mL of PI. Results were exhibit in bright field (BF), PI (Propidium Iodide), FITC (Fluorescein isothiocyanate) and merge of all layers. The reported values are result of one experiment (n=1).

Regarding the use of keratin-based particles with *T. vulgaris* (Figure 8.17), the results obtained were not consistent with those previously obtained, in the case of microbial activity and cell viability. The MIC previously determined was 500 µg/mL, but in the flow cytometry experiment growth was still observed for this concentration. As for the SMEO, this fact may be due to some experimental error as this experiment was only performed once, or to factors such as the different volume of the suspension, even oxygen availability in the solution or stability of the EO encapsulation in the particles (Blackburn, 2006).
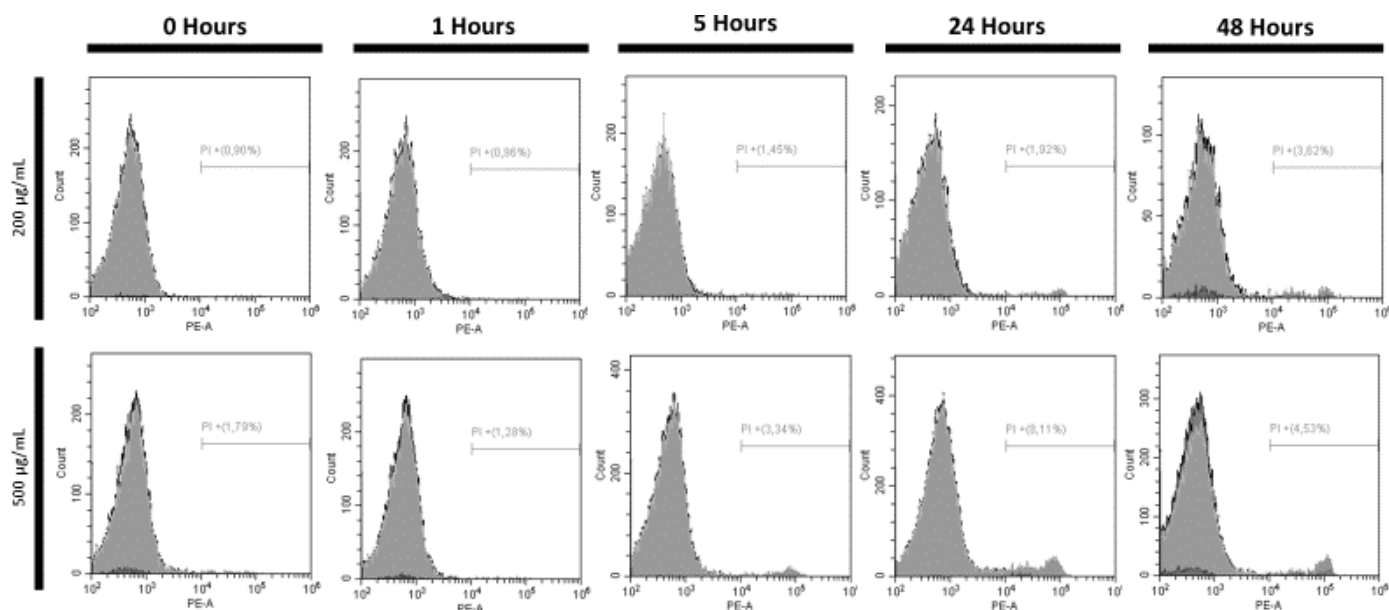
Figure 8.17 - Flow cytometry analysis of *Z. parabailii* cells treated with keratin particles of *Thymus vulgaris* (TVEO) with concentrations 200 and 500 µg/mL and stained with propidium iodide (PI). For flow cytometry analysis the cells were collected at different time points 0, 1, 5, 24 and 48 hours of treatment and then stained with 2 µg/mL of PI. Results are expressed as percentage of PI positive population. The reported values are result of one experiment (n=1).

9. CONCLUSION AND FUTURE PERSPECTIVES

In recent decades, the potential application of essential oils as antifungal agent has been extensively researched. However, the volatile and powerful flavor characteristics, as well as the quantities and prices connected with obtaining it, are some of the main disadvantages. The encapsulation of oils can address this deficit by improving and enhancing its application efficiency in fields such as agriculture and/or the food industry while maintaining a green and environmentally sustainable approach.

Three types of particles were used in the experiments: zein, keratin, and liposomes, all of which appeared to have similar size, zeta potential, and PDI to those described in the literature. Except in the case of liposomes, which had low electrical charge values (between -3,150 and -0.636 mV), indicating low colloidal stability, according to the literature.

All antimicrobial activity experiments resulted in a decrease in *Z. parabailii* growth. Starting at 100 µg/mL, *Satureja montana* essential oil was demonstrated to maintain performance while encapsulated, which was not the case with *Thymus vulgaris* essential oil. The MIC of *S. montana* EO was 200 µg/mL in three of the five formulations studied: free EO, zein particles, and keratin-

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based particles. Meanwhile, with *T. vulgaris* EO, the lowest MIC was 200 μg/mL, which was achieved only with free essential oil. These findings raised concerns since the essential oil *T. vulgaris* encapsulation may be limiting the activity of the principal active components. It's critical to understand the limitation of the encapsulation processes because they'll be crucial in future research.

Regarding the result of flow cytometry, the concentration of 500 μg/mL of *S. montana* EO keratin-based particles, reached a maximum of 94% of PI positive cells after 48 hours. These findings show that the cell membrane loses its integrity, which is consistent with necrosis as the cell death mechanism. Meanwhile, for keratin particles with *T. vulgaris* EO, a maximum of 8.11% was obtained after 24 hours of assay. However, it was unclear whether the established MIC is effectively confirmed with data on antimicrobial activity and cell viability due to the lack of replicates of the assay.

In fluorescence microscopy experiments, it was evidenced at 24 and 48 hour time points, that there is a link between FITC and PI positive results for 500 μg/mL of *S. montana EO* keratin-based particles. In fact, the evidence suggests that SMEO keratin-based particles effect is directly related to membrane permeability. However, the location of the particles within the various cellular structures could not be assessed.

To conclude, in order to effectively boost the application of the essential oil *T. vulgaris*, it will be necessary to explore additional particle formulations for the essential oil, such as keratin:zein (Tinoco *et al.*, 2021), as well as to elucidate the mechanisms of inhibition of the EOs in the different formulations described in this study. It will be important also to evaluate the performance of keratin particles of *S. montana* essential oil as a biopesticide under various pH, temperature, and acidity circumstances, as *Z. parabailli* is exceptionally resistant to stress.

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A. APPENDIX

Table A.1. - Analysis of cell membrane integrity with keratin particles of *Satureja montana* (SM) and *Thymus vulgaris* (TV) against *Z. parabailii* stained with propidium iodide (PI). Boiled cells are used as a positive control for PI staining. For flow cytometry analysis the cells were collected at different time points 0, 1, 5, 24 and 48 hours of treatment and then stained with 2 µg/mL of PI. Results are expressed as percentage of PI positive population. The reported values are result of one experiment (n=1)

PI positive (%)		Control	Control keratin	Keratin *Satureja montana*		Keratin *Thymus Vulgaris*	
				200	500	200	500
	0	AF	2.56	1.43	1.06	2.00	0.41	1.01
	0	PI	1.28	7.89	2.55	26.09	0.90	1.79
	1	AF	0.31	1.77	0.97	2.37	0.21	0.33
	1	PI	0.89	6.80	1.23	13.22	0.96	1.28
Time (Hours)	5	AF	0.84	1.07	0.61	1.85	0.40	0.20
	5	PI	0.92	1.6	1.57	74.36	1.45	3.34
	24	AF	1.54	0.32	0.14	0.27	0.04	0.12
	24	PI	0.49	1.3	4.41	75.38	1.92	8.11
	48	AF	1.39	5.24	1.89	42.46	0.41	1.16
	48	PI	0.72	12.65	8.80	94.01	3.62	4.53