Activity of essential oils and hydroalcoholic extracts from 12 plants against food spoilage yeasts: evaluation of their potential as new biopesticides

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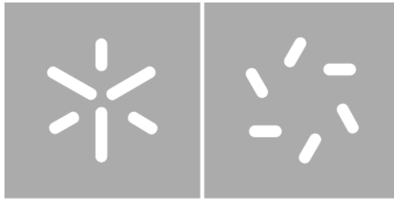

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Master Thesis

Master’s in Molecular Biology, Biotechnology and Bioentrepreneurship in Plants

Work conducted under the supervision of

Professor Maria João Sousa

january 2021

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STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

Universidade do Minho, 30 de janeiro de 2021

Bruna Daniela Fernandes Pereira

(Bruna Daniela Fernandes Pereira)

Atividade de óleos essenciais e extratos hidroalcoólicos de 12 plantas em leveduras de contaminação alimentar: avaliação do seu potencial como novos biopesticidas

RESUMO

O uso intensivo de pesticidas químicos nas últimas décadas tem trazido sérios problemas ao meio ambiente e à saúde humana. Consequentemente, o interesse pelos produtos naturais e respetiva aplicação tem sido alvo de interesse nos últimos anos. O principal objetivo deste estudo foi avaliar a atividade antimicrobiana de óleos essenciais e extratos hidroalcoólicos, visando a sua futura aplicação na agricultura e na conservação de frutas e vegetais frescos pós-colheita. Para tal, a atividade antimicrobiana de 12 extratos de plantas e óleos essenciais, conhecidos por possuírem atividade antimicrobiana contra um amplo número de microrganismos, foi testada contra as leveduras de contaminação alimentar, *Saccharomyces cerevisiae* e *Zygossacharomyces parabailii*. Os resultados mostraram que os óleos essenciais foram o material vegetal mais ativo. Todos os óleos essenciais exibiram atividade antimicrobiana nas espécies de leveduras estudadas; particularmente, o óleo essencial de *T. vulgaris* evidenciou o efeito mais potente, seguido por *C. flexuosus* e *C. citratus*. Devido à sua elevada atividade, foi avaliado o mecanismo de morte celular do óleo de *T. vulgaris*. Foi evidenciado que, embora o óleo de tomilho tenha induzido morte celular em concentrações reduzidas (100 µg/mL), apenas aumentou ligeiramente a permeabilidade da membrana e acumulação de espécies recativas de oxigénio. Adicionalmente, dado que os extratos hidroalcoólicos de *M. piperita* e *S. sclarea* não apresentaram atividade antimicrobiana e, pelo contrário, um ligeiro aumento de biomassa foi evidenciado nos ensaios de crescimento, um potencial efeito protetor do envelhecimento celular foi estudado na levedura *S. cerevisiae* por um período de 28 dias, sob restrição calórica extrema. Resultados promissores foram observados com o extrato de *S. sclarea*, cujas diferenças significativas na viabilidade celular entre células tratadas e não tratadas foram observadas ao longo do tempo do ensaio. Em conclusão, os resultados obtidos neste estudo confirmaram a possibilidade de utilização de óleos essenciais como agente antimicrobiano alternativo ou complementar na proteção contra leveduras de contaminação alimentar.

PALAVRAS-CHAVE

Óleo essencial, extrato hidroalcoólico, leveduras, atividade antimicrobiana, envelhecimento celular.

Activity of essential oils and hydroalcoholic extracts from 12 plants against food spoilage yeasts: evaluation of their potential as new biopesticides

ABSTRACT

The intensive use of chemical pesticides in recent decades has raised serious problems to the environment and human health. Consequently, the interest in natural products and their application has been of interest in recent years. The main objective of this study was to evaluate the antimicrobial activity of essential oils and hydroalcoholic extracts, envisaging their future application in agriculture and in the conservation of fresh fruits and vegetables postharvest. Hence, the antimicrobial activity of 12 plant extracts and essential oils, known to have antimicrobial activity against a large number of microorganisms, was tested against food spoilage yeasts, *Saccharomyces cerevisiae* and *Zygossacharomyces parabailii*. The results showed that essential oils were the most active plant material. All essential oils exhibited antimicrobial activity in the studied yeast species; particularly, the essential oil of *T. vulgaris* showed the most potent effect, followed by *C. flexuosus* and *C. citratus*. Due to its high activity, the mechanism of cell death of *T. vulgaris* oil was evaluated. It was evidenced that, although thyme oil induced cell death in low concentrations (100 μg/mL), it only slightly increased the membrane permeability and accumulation of reactive oxygen species. In addition, given that the hydroalcoholic extracts of *M. piperita* and *S. sclarea* did not show antimicrobial activity and, on the contrary, a slight increase in biomass was evidenced in the growth experiments, a potential protective effect of cell aging was studied in the yeast *S. cerevisiae* over a period of 28 days, under extreme caloric restriction. Promising results were observed with the extract of *S. sclarea*, whose significant differences in cell viability between treated and untreated cells were continuous over time. In conclusion, the results obtained in this study confirmed the possibility of using essential oils as an alternative or complementary antimicrobial agent to protect against food contamination yeasts.

KEYWORDS

Essential oil, hydroalcoholic extract, yeast, antimicrobial activity, cell aging.

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ABREVIATIONS AND ACRONYMS

CFU	Colony forming units
CLS	Chronological lifespan
EO	Essential oils
h	Hours
HAE	Hydroalcoholic extracts
MIC	Minimum inhibitory concentration
min	Minutes
OD	Optical density
PBS	Phosphate buffered saline
PI	Propidium iodide
ROS	Reactive oxygen species
SD	Standard deviation
TVEO	*Thymus vulgaris* essential oil
YEPD	Yeast extract peptone dextrose

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I. INTRODUCTION

Fruits and vegetables are fresh, living, or perishing parts of plants, rich in nutrients and fibre essential to the human nutrition [1], [2]. Their crops might be consumed fresh or after processing and are produced worldwide either on farms with conventional or organic agricultural production methods, or under environmentally controlled glasshouses [2]. However, they are among the most challenging food products to commercially produce and distribute because they remain metabolically and developmentally active and go under several physiological and compositional changes, being subjected to spoilage losses during production, transportation and storage [3], [4]. Furthermore, there is an imbalance between the growing food demand of the world population and global agricultural output, as well as discrepancies between supply and demand [5].

Every year, one third of world's food is lost or wasted, which amounts to about 1.3 billion tons per year [6]. In the European Union , around 89 million tonnes of food waste is generated and, in Portugal, it was estimated that food waste represents 17% of the annual food production, approximately 1 million tonnes [7], [8]. Food and Agricultural Organization (FAO) [9] report found that fruits and vegetables suffer higher loss and waste percentages than cereals and pulses, especially when cold storage or processing conditions are not adequate.

Food losses represent a waste of resources used in its production such as land, water, energy and inputs, having environmental impacts due to the CO_2 emissions during its production and economic effects because the product will be wasted or have inferior quality and value [10]. Food waste has a global carbon footprint of about 7% of all global greenhouse gas emissions caused by humans and its associated costs are estimated at 143 billion euros [8], [9]. Reduction of postharvest losses reduces cost of production, trade and distribution, lowers the price for the consumer and increases the farmer's income [11]. It is therefore needed to find ways to prevent such losses in the effort to help fight climate change, rise income, and improve food security. Additionally, with the increase in human population, which is expected to grow from 7.7 billion to 9.7 billion in 2050 [12], it is also needed to ensure that sufficient food is available to every inhabitant in our planet. It is estimated that for every one percent reduction in loss, it will be saved 5 million tons of fruit and vegetables per year [11].

Early intervention measures during crop development and harvesting through the use of good agricultural practices, such as use of fungicides, adequate sanitation, proper transportation and storage

conditions and careful handling, packing and transport will provide dramatic reductions in yield loss due to spoilage, enhancing the food safety and shelf life of fresh-cut produce [4], [13].

However, with the increase in the consumption of fresh produce and food trade, consumers have also become more aware about the safety of the foods they eat, demanding reduced or no use of pesticides [3], [14]. Consequently, concerns over the potential impact of disease management practices on the environment and/or on consumer health have requested alternative methods to combat diseases, such as the use of plant extracts and essential oils or their major compounds, which have shown a broad spectrum of activity against plant pathogens, particularly those responsible for postharvest diseases, and thus are a sustainable alternative to the use of synthetic pesticides [14]–[16].

I.1. Microorganisms in food spoilage

Most microorganisms that are initially observed on whole fruit or vegetable surfaces are soil inhabitants, members of a very large and diverse community of microbes that collectively are responsible for maintaining a dynamic ecological balance within most agricultural systems [4], [17]. It is estimated that between 27% and 42% crop losses produced is lost each year due to pests, including pathogens [5], [18]. Contamination can be introduced to the crop on the seed itself, during crop growth in the field, during harvesting and postharvest handling or during storage and distribution [4], [18].

Fresh fruits and vegetables present nearly ideal conditions for the survival and growth of many types of microorganisms due to their succulent nature [4], [11]. Some spoilage microbes are capable of colonizing and creating lesions on both healthy or damaged plant tissue, but they can also enter plant tissues during fruit development or through various specialized water and gas exchange structures of leafy matter [4], [17]. The presence of air, high humidity, and higher temperature during storage increases the chances of spoilage, being estimated that about 20-25% of the harvested fruits and vegetables decayed by pathogens is during postharvest handling [4], [19]. Due to worldwide trades, in which food products are often sold in different locations from their production sites, there is an urgent need to extend their shelf-life.

I.1.1. Yeasts

Yeasts are unicellular fungi widely known for their beneficial contributions in the fermentation of alcoholic beverages, bread, and other food products. Moreover, they are exploited for the production of metabolic products and whole cell mass, which might enhance the organoleptic characteristics of fruits and act as biocontrol agents [20], [21]. Several beneficial effects on human health and well-being have also been reported [21], [22], such as prebiotic and probiotic effects, bioavailability of nutrients, detoxification of mycotoxins, lowering of serum cholesterol, antioxidative properties and antimutagenic and antitumor activities. However, yeasts are also involved in spoilage of food products, making use of food components as growth substrates and transforming them into different metabolic end products, which alter the characteristics of the food [10].

Yeast spoilage is most evident on packaged products because carbon dioxide (gas) production will cause the containers of those products to swell, distort, and eventually explode due to the internal pressures that are developed [1], [10], [23]. Furthermore, yeasts grow as individual colonies, as a film of dull, dry, or slimy biomass that can cause discoloration, as a film of biomass that covers or floats on the surface of a product, or as haze, turbidity and sediments of cells within the product [10], [20], [23]. These visual signs of spoilage are usually accompanied by the development of yeasty, alcoholic, and other off-odours and off-flavours in the product [10].

I.1.1.1. Factors affecting yeast growth

A variety of factors influence yeasts and exert stress conditions that the cells must withstand, or otherwise die [24]. Stationary phase cells are, generally, more resistant to physical and chemical stresses than cells from the exponential growth phase. Cells exposed to sub-lethal stresses may initiate adaptive reactions to enhance their survival and growth [10].

The most important physical factor influencing the life of yeasts is temperature due to its ability to affect the limits and rate of growth and also influence other physiological and biochemical properties, such as tolerance to ethanol, growth in the presence of high concentrations of sugar and salt, resistance to preservatives, and production of spoilage metabolites [10]. Most yeasts exhibit optimal growth in the range 20-30 °C and very few species grow above 40-45 °C, but many grow at 2-10 °C, including spoilage species of *Saccharomyces* and *Zygosaccharomyces* [10], [24]. Under normal environmental conditions, yeast cells are quickly inactivated at 55-65 °C [10], [23].

With regard to ecological factors, water activity (a_w) is one of the most important factors affecting the growth of yeasts in foods [24]. Food spoilage yeasts have minimum a_w values of 0.90-0.95 for growth and some species are particularly osmo- or xerotolerant and grow at lower values, depending on the nature of the solute, temperature or other ecological factors, as well as by the physiological state of the cells [10], [24]. Such yeasts are most frequently associated with the spoilage of high sugar and high salt foods, and thus are of special importance to the food industry [10], [24]. The principal xerotolerant yeasts species belong to the genus *Zygosaccharomyces* [10], [24].

Yeasts prefer acid environments, having the best growth rate in the range pH 4.5-6.5 [1], [20]. Many species, however, grow at pH values as low as 1.3-1.7 [20], [23]. Acidity and pH synergistically interact with many other factors affecting yeast growth, such as temperature, water activity, sugar and salt concentration, which will influence product stability and shelf life [10].

Although yeasts are well known for their beneficial fermentations that produce bread and alcoholic drinks, they can grow with or without oxygen (facultative). In most natural habitats, and also in foods, normal atmospheric conditions prevail with high oxygen and low carbon dioxide concentrations and, thus, fruits and vegetables can be stored for extended periods under controlled or modified atmosphere, with decreased oxygen and increased carbon dioxide or nitrogen concentrations [24].

Additionally, a range of preservatives may be added to foods and beverages to control yeast growth. Many of these are weak organic acids such as sorbic, benzoic, acetic, and propionic acids and their effectiveness depends on their concentration, the pH of the food, and other food properties such as sugar and salt concentrations [10]. *Zygosaccharomyces* species, especially *Z. parabailii*, are notoriously resistant to these preservatives and can tolerate the maximum concentrations permitted [1], [20]. The two major end products of yeast growth and metabolic activity, ethanol and carbon dioxide, also affect yeast growth [10], [24]. Likewise, plant extracts can be inhibitory to yeasts and other microorganisms [25].

I.1.1.2. Fruits and vegetables spoilage yeasts

Yeasts can be found in a wide variety of environments, such as in plants, animal products, soil, water and insects owing to the variety of food substrates that yeasts can make use of, namely pectines and other carbohydrates, organic acids, proteins and lipids, and, consequently, are able to contaminate many foods, causing changes in odour, colour, taste and texture [1], [26]. Their association with the

phyllosphere of many crops ensures their presence on fruit and vegetables, and their entry into food processing plants [27].

Yeasts are present as part of the surface flora of healthy, undamaged fruits. However, damage to the structural integrity of fruits exposes their sugary, acidic tissues which are ideal for the growth of fermentative yeasts, such as yeast from the genera *Saccharomyces*, *Schizosaccharomyces* and *Zygosaccharomyces*, and eventual product spoilage [4], [28]. Processed fruit products, such as fruit juices, juice and pulp concentrates, canned fruits, dried fruits, glazed fruits, ready to-eat fruit slices, and fruit salads are also prone to fermentative spoilage by yeasts of the genera *Saccharomyces*, *Zygosaccharomyces*, *Hanseniaspora/Kloeckera*, *Candida*, and *Pichia* [4]. Products with high sugar concentrations are more likely to be spoiled by osmotolerant species such as *Zygosaccharomyces bailii, Zygosaccharomyces rouxii, Hanseniaspora valbyensis,* and *Schizosaccharomyces pombe* [10]. Species of *Cryptococcus*, *Rhodotorula*, *Sporobolomyces*, *Aureobasidium*, and *Metschnikowia* are also generally found in fruit products [28].

Yeasts are normal colonizers of vegetables and its activity becomes apparent when circumstances become favourable to them, for instance, during the fermentation of vegetables [1]. Many different yeast species have been identified in vegetables, including species of *Candida*, *Cryptococcus*, *Rhodotorula*, *Sporobolomyces*, *Trichosporon*, *Pichia* and *Torulaspora* [10], [29].

Furthermore, fresh-cut fruits and vegetables quality loss can be induced by *Rhodotorula mucilaginosa*, *Rhodotorula glutinis*, *Zygosaccharomyces bailii*, *Zygosaccharomyces bisporus*, and *Zygosaccharomyces rouxii* [4].

I.1.1.3. Control of spoilage outbreaks in the food industry

The control of food and beverage spoilage by yeasts generally depends on the maintenance of chemical and physical conditions that prevent yeast growth, such as low temperature, low pH/high acidity, high concentrations of sugar or salt, and addition of chemical or natural preservatives. However, due to their capacity of adaptation to changes in the external environment, yeast cells can adapt and acquire resistance to the continuous stress by shifting their metabolic and physiological behaviour to tolerate the new conditions [10]. Prevention and minimization of contamination are, therefore, key requirements in the management of yeast spoilage.

Spoilage management should begin in the field using an integrated strategy of good agricultural practices, which includes fungicide application in the field and postharvest and cross-contamination prevention measures in the packinghouse and storage facility [4], [13]. At time of harvest and throughout handling before storage and distribution, it is important to minimize wounds and bruising and to cull all damaged and diseased product [4]. Upon harvest, fresh fruits and vegetables benefit from immediate surface sanitation and rapid cooling to slow product metabolism and growth of spoilage microbes [4], [29]. Some additional commonly used methods include forced air refrigeration, vacuum cooling, and immersion in ice [4].

In addition to these active control measures the application of standard procedures, new processing and preservation technologies, better material quality, good manufacturing practices and better packaging allows a reduction in the contamination to acceptable levels, assuring the quality and safety of all perishable foods and enhancing substantially the food safety and shelf life of fresh-cut produce [4], [17], [30]. Currently, the alternative approaches to prevent microbiological spoilage of foods include: thermal and nonthermal processing technologies; biopreservation or biocontrol; modified atmosphere packaging; antimicrobial packaging; and hurdle technology [31]. Another important technology to minimize microbial spoilage is adequate cold chain temperature management [4]. However, since most yeasts grow well at low temperatures, refrigeration does not prevent spoilage, only slows down its occurrence [10].

Antimicrobial packaging is designed to incorporate antimicrobial substances into the packaging system for delaying or preventing microbial growth and/or contaminations during postharvest transportation, storage, and retail display of the produce [32], [33]. These packages can incorporate active substances, like plant extracts and essential oils that can diminish the microbial activity [34], [35]. Combined with different preservation factors based on controlling temperature, water activity, acidity, redox potential and the use of preservatives and modified atmosphere, it is possible to achieve an enhanced level of product quality, safety, and stability [20], [29]. Moreover, the intensity of the individual preservation techniques can be kept comparatively low, minimizing the loss of quality, while the overall impact on microbial growth may remain high [29].

I. 1.1.4. Yeasts and food safety

Humans consume large populations of yeasts in their daily diet without adverse impact on their health. Unlike bacteria and viruses, yeasts are rarely associated with outbreaks of foodborne gastroenteritis,

intoxications or other infections [10], [36]. In recent years, nonetheless, there have been increasing reports of human infections with yeast species, including many that are commonly found in foods, for instance, *Saccharomyces cerevisiae, Candida krusei, Wickerhamomyces anomalus, Yarrowia lipolytica, Kluyveromyces marxianus, Rhodotorula* spp. [10], [23], [36]. Some species such as *Candida albicans* and *Cryptococcus neoformans* are opportunistic pathogens and cause a range of mucocutaneous, cutaneous, respiratory, central nervous system, organ or even systemic infections [36]. Likewise, *S. cerevisiae* has been associated with a wide variety of infections because of its extensive use in the food industry, which range from vaginitis in healthy patients and cutaneous infections, to systemic bloodstream infections and infections of essential organs in immunocompromised and critically ill patients [36], [37].

Usually, healthy individuals are not at risk, because infections are mainly restrained to people with weakened health and immune functions, albeit the growing use of these microorganisms in food products is raising concerns about the safety of yeasts in general [10], [36].

I.2. Biopesticides

The United Nations Organization for Food and Agricultural Organization (FAO) [38] has defined pesticides as: *any substance, or mixture of substances of chemical or biological ingredients which are meant for repelling, destroying or controlling any pest, or regulating plant growth*.

Currently, the management of plant pathogens depends heavily on synthetic pesticides, which have increased crop protection but with environmental and public health impacts [16], [18]. It has been estimated that about 2 million tonnes of pesticides are used on crops each year [39], [40]. Most pesticides reach a destination other than their target species, being present in air, water and soil [40], [41]. Their uninterrupted and indiscriminate use also lead to its presence in foods, fruits, vegetables and even in mother's milk and to the development of fungicide resistant populations of storage pathogens [16], [18], [40]. Further, the use of synthetic chemicals to control postharvest biodeterioration has been restricted due to their carcinogenicity, high and acute residual toxicity, hormonal imbalance, long degradation period and their adverse effects on food [15], [16], [41]. Therefore, there is an enduring demand for developing new disease management practices to enhance crop yields by reducing crop losses, minimizing the conventional pesticide usage, avoiding the accumulation of pesticide residues, increasing farmer's income and enhancing environmental health [40]. The increasing pressure placed by consumers to reduce or eliminate chemical fungicides in foods, the high cost of discovering, developing and registering

new synthetic pesticides and the rapid emergence of pest resistance have contributed to the growing interest in natural antimicrobial compounds [18].

Biopesticides, also known as green pesticides, are pesticides derived from organic sources which are considered environmentally friendly and reduce the negative impacts to human health. Plant extracts and essential oils are two of the most promising groups of natural compounds for the development of novel and safer antimicrobial compounds, agents promoting food preservation, and alternatives to treat infectious diseases [42]. Additionally, due to their large number of active components, the development of resistance to pathogens is not as common [40], [43]. Although natural pesticides can be very toxic for certain species, their environmental impact is reduced, as other species can digest them, and they do not accumulate in the environment or food chain and, consequently, can contribute to reduce the pest population and increase food production [40], [44].

I. 2.1. Plant extracts

Plants are fundamental to humans' daily lives as they produce secondary metabolites which are of potential use in medicine and other applications, such as antibiotics, analgesic, flavours, perfumes, insecticides, dyes, food additives, poisons, among others [45]. The growing use of plant extracts is primary because they are generally considered safe, easily accessible, affordable, and are widely acceptable and trusted by the majority of the population as a form of health care [46], [47].

Chemical analysis have shown that plant extracts consist of a complex mixture of multiple organic compounds, many of which are antimicrobial [47]–[49]. These include alkaloids, coumarins, essential oils, flavonoids, lectins, phenolics and polyphenols, polyacetylenes, polypeptides, quinones, tannins, saponins and steroids, which are deposited in specific parts of the plant such as leaves, flowers, bark, seeds, fruits, root, among others [16], [47], [50]. These compounds have a range of biologic, chemical, and physical activities. Plants such as allspice, clove, cinnamon, garlic, and thyme contain numerous antimicrobial compounds, which cause the disturbance of the cytoplasmic membrane; disruption of the proton motive force; electron flow; and the active transport and coagulation of cell contents [32], [47]. Amid these natural compounds, phenols constitute one of the major groups of herbal compounds acting as radical scavengers and antioxidants [51].

Fresh, frozen or dried plant materials can be used as a source for the extraction of secondary plant metabolites. They are usually air-dried to a constant weight before extraction [52]. For the extraction of

the desired active constituents, various solvents such as water, ethanol, chloroform, acetone, ethyl acetate and methanol are commonly used [15], [50]. The quality of a plant extract is determined by the plant parts, extraction solvents, extraction techniques and type of equipment used [52]. Hydroalcoholic solvent mixture (mixture of alcohol and water in varying proportions) is generally considered to give high extraction yields of plant extracts, which is owing to their expanded polarity range [50].

The use of botanicals and plant derivatives is still limited due to the need of high doses in order to obtain a satisfactory level of effect, no clear indications on their mammalian toxicity and standardization of plant extracts, which may differ according to the geographical area, season of plant harvest, condition of the harvest and the pre-harvest period and other factors [52]. Additionally, the commercial use of plant extracts faces several drawbacks, such as patent and intellectual rights, differences in extraction methods, complexity of the extracted mixture, difficulties in the purification steps and formulation and stability of the active ingredients [15], [52].

I.2.2. Essential Oils

Medicinal and aromatic plants produce essential oils as secondary metabolites in response to physiological stress, pathogen attack and ecological factors [53]. Essential oils are volatile natural complex secondary metabolites which can be isolated through hydrodistillation, steam distillation, or dry distillation of the plant material, including the whole plant or just the wood, bark, roots, leaves, flowers, fruits or seeds [41], [42], [54]–[56]. The plants can be fresh, partially dehydrated, or dried, but in the case of flowers, they must be fresh [57]. The oils are stored in secretory cells, cavities, canals, epidermal cells or glandular trichomes [41], [47], [57]. These volatile oils contained in herbs are characterized by strong aromatic components and responsible for different scents that plants emit [44], [47].

Essential oils are usually recognized as hydrophobic, but they are largely soluble in fats, alcohols, and most organic solvents, including ethanol and diethyl ether [56], [57]. The relative density of essential oils is commonly lower than that of water, with the exception of a few cases (cinnamon, sassafras, and vetiver) [55], [56]. At room temperature, these oils are typically liquid and usually range from colourless to slightly yellowish in colour, being easily transformed from a liquid to a gaseous state at room or slightly higher temperature without undergoing decomposition [44], [55], [57]. They have a high refractive index and optimal rotation, as the result of many asymmetrical compounds, which could be of interest for their identification and quality control [53], [56], [57].

There are 17,500 aromatic plant species among higher plants and approximately 3,000 essential oils are known out of which only about 300 are commercially important for pharmaceuticals, cosmetics and perfume industries apart from pesticidal potential [43], [54], [58]. Genera capable of elaborating the compounds that constitute essential oils are distributed in a limited number of families, such as Myrtaceae, Lauraceae, Rutaceae, Lamiaceae, Asteraceae, Apiaceae, Cupressaceae, Poaceae, Zingiberaceae and Piperaceae [41], [54], [59].

I.2.2.1. Chemical structure

The chemical structure of essential oils components is a crucial characteristic since it affects the precise mode of action. The composition pattern, concentration of individual components, and yield of the essential oil can vary with the plant genetics, geographical origin, type of plant part, stage of development or seasonal sampling period, physiology of the whole plant, environmental factors, cultivation conditions, postharvest techniques and the species and age of a targeted organism [56], [59]. That is, chemical structure can differ in plants that are botanically identical.

Being natural mixtures of very complex nature, essential oils may consist of about 20–60 active substances at quite different concentrations [41], [42], [60]. Generally, the major components determine the biological properties of the essential oils. Essential oils consists predominantly of terpenes (mostly monoterpenes and sesquiterpenes), terpenoids (oxygenated compounds such as phenols, alcohols, aldehydes, ketones or ethers) and aromatic and aliphatic compounds, all characterized by low molecular weight [42], [61], [62].

Terpenes are made from combinations of several isoprene units (C5). Occurring in the cytoplasm of plant cells, biosynthesis of terpenes proceeds via the mevalonate and mevalonate-independent pathways and consists of synthesis of the isopentenyl diphosphate precursor [42], [55], [61]. The main terpenes are the monoterpenes (C10) and sesquiterpenes (C15), but longer chains also exist [42], [60], [61]. Turpentine, damask rose, tea tree oil, coriander, peppermint, lemon, caraway, wormwood, eucalyptus, and chenopodium are examples of monoterpene-rich oils; and sandalwood, German chamomile and ginger constitute examples of sesquiterpenes [57].

A terpene containing oxygen is called a terpenoid, which can be subdivided into alcohols, phenols, esters, aldehydes, ethers, ketones, and epoxides [42], [61], [63]. Terpenoids tend to be both volatile and

thermolabile and may be easily oxidized or hydrolysed depending on their structure [43]. Terpenoids include thymol, carvacrol, linalool, linalyl acetate, citronellal, piperitone, menthol and geraniol [63].

The phenylpropanoids are a family of organic compounds with an aromatic ring and a three-carbon propene tail and are synthesized by plants from the amino acids phenylalanine and tyrosine via the shikimate pathway [55], [56]. Aromatic compounds are derived from phenylpropenes, which constitute a subfamily of phenylpropanoids and are characteristic of certain species, and include safrole, eugenol, isoeugenol, anethol, vanillin, aldehyde anasique, cinnamaldehyde, trans-anethole, methyl chavicol and myristicin [42], [60], [64]. These compounds occur less frequently than the terpenes. The principal plant sources for aromatic compounds are anise, cinnamon, clove, fennel, nutmeg, parsley, sassafras, star anise, tarragon, and some botanical families (Apiaceae, Lamiaceae, Myrtaceae, Rutaceae) [57], [61]. Phenylpropenes are not only essential for physiological function in plants, but also make important contributions to the fragrances and flavours of many plant species, playing an important role in attracting both pollinators and seed dispersers, repelling predators, protecting themselves from heat or cold, inhibiting seed germination, communicating with other plants and utilizing chemical constituents in the oil as defence materials [44], [53], [55], [65].

I.2.2.2. Mode of action

The main mode of action of essential oils in eukaryotic cells is thought to be through disruption of the cell membrane. Terpenoids and terpenes act as function of their lipophilic properties, allowing them to easily pass through and accumulate in cell membranes, disturbing the cytoplasmic and mitochondrial membranes and causing an increase of permeability. Hence, the central effects are related with inhibition of electron transport, protein translocation and enzyme-dependent reactions [42], [61], [63], [66]. Leakage of intracellular constituents and impairment of microbial enzyme systems can occur, and extensive loss of the cell contents will induce the cell death by apoptosis and necrosis [28], [34], [43], [67].

Most monoterpenes, constituting 90% of the essential oils, serve as chemical messengers, making them especially useful for synomones and alarm pheromones, and have the ability to cause a drastic reduction in the number of intact mitochondria and Golgi bodies, impairing respiration and photosynthesis and decreasing cell membrane permeability [54], [61], [63]. As the membrane integrity is being damaged, it further affects pH homeostasis, equilibrium of inorganic ions, nucleic acid synthesis, cytokine

interactions, reactive oxygen species (ROS) accumulation and balance of ATP pools [33], [61], [68]. Thus, the mitochondria and the endoplasmic reticulum appear to be important sites in their mechanisms of action [69].

In yeasts, the content of ergosterol, a major sterol in the membrane responsible for cell integrity, has been shown to be reduced by the action of different essential oils [61], [69]–[73]. Various studies also show an inhibition of wall formation, spore germination, mycelial elongation, growth, sporulation and viability of spores [69], [72], [74]–[77].

I.2.2.3. Synergistic effect

Each compound has its own structure and biological activity and in the presence of other active molecules can decrease or increase its activity [41]. A synergistic effect is observed when the combination of substances is greater than the sum of the individual effects, resulting in higher bioactivity. Synergistic effects can be produced if the constituents of an extract affect different targets or interact with one another, improving the solubility and, consequently, enhancing the bioavailability of one or several substances of an extract [42]. Constituents found in low amounts enhance the effectiveness of the major constituents while also reducing the dose of polluting substances and the risk of developing resistance [41]–[43], [54]. Thus, mutual synergistic action may play a very important role in terms of efficacy, being advantageous in pre- and postharvest protection [41], [42].

Negative synergism, antagonism, can also occur between the essential oil or their components and the other ingredients present in the total formulation through a decrease in the activity of two compounds in combination as compared with their individual activity. Moreover, an additive effect can be observed when the combined effect of two or more agents is equal to the sum of their individual activity [33], [47].

However, synergism, antagonism and additive effects between different phytochemicals or other chemical compounds has to be further investigated [33].

I.2.2.4. Biological activity

In nature, essential oils play an important role in the protection of the plants, having antimicrobial, antioxidant, anti-inflammatory, antiviral and insecticidal activities and also protecting against herbivores

by reducing their appetite for such plants [42], [54], [61]. Furthermore, they may attract some insects to favour the dispersion of pollens and seeds, or repel undesirable others [57], [61], [78].

Antimicrobial activity

A wide variety of essential oils are known to possess antimicrobial properties, which might be due to the presence of active constituents, mainly to isoprenes, such as monoterpenes, sesquiterpenes and related alcohols, along with other hydrocarbons and phenols [56]. Among the different constituents, phenolic compounds exhibit the highest antimicrobial activity, of which thymol and carvacrol from oregano and thyme, and eugenol from clove are the most active against a broad spectrum of microorganisms [33], [41], [42], [55]. p-cymene, limonene, menthol, eugenol, anethole, estragole, geraniol, thymol, γ-terpinene, and cinnamyl alcohol comprise other constituents of essential oils with antimicrobial activity [42], [63].

Furthermore, essential oils have shown an ability to protect foods against pathogenic and spoilage microorganisms. It seems that monoterpenes, specifically α-pinene, β-pinene, α-terpinene, play, beside phenolics, a considerable role in disturbing the membrane function in yeasts [28]. Numerous essential oils containing non-phenolic main compounds have shown high toxicity against yeasts, including juniper, lemon, marjoram, clary sage, basil, ginger or lemon balm [25], [79], [80]. Antifungal activity of orange oil, tea tree oil , turpentine oil, rosemary oil, peppermint oil, thyme oil, oregano oil and clove oil against *S. cerevisiae* have also been documented in a previous study [66].

Antioxidant Activity

Oxidation damages various biological substances and subsequently causes many chronic and degenerative diseases, including cancer, liver disease, Alzheimer's disease, aging, arthritis, inflammation, diabetes, Parkinson's disease, atherosclerosis [15], [55], [58].

Phenolics and secondary metabolites with conjugated double bonds usually show substantial antioxidative properties due to their redox properties, which have an important role in neutralizing free radicals and also in peroxide decomposition [35], [51], [55]. Thymol and carvacrol are the most active compounds and their activity is related to their phenolic structure, which permit them to act as hydrogen donors, reducing agents, singlet oxygen quenchers and metal chelators [35], [55], [81]. The antioxidant

activity of essential oils is also due to certain alcohols, ethers, ketones, aldehydes, and monoterpenes, including linalool, 1,8-Cineole, eugenol, geranial/neral, citronellal, isomenthone, menthone, terpinenes and α-Terpinolene [55], [64], [82], [83].

Owing to their natural antioxidant capacity, essential oils can replace synthetic antioxidants and prevent various degenerative diseases and lipid oxidation in food systems [35], [55].

Anti-Inflammatory Activity

Inflammation is a biological and protective response of body tissue to combat invaders and to remove dead or damaged host cells. Inflammation has been known to be associated with certain diseases including hypertension, cancer, stroke, rheumatism, allergies, or arthritis [55], [58].

The anti-inflammatory activity of essential oils may be attributed not only to their antioxidant activities but also to their interactions with signalling cascades involving cytokines and regulatory transcription factors, and on the expression of pro-inflammatory genes [55]. Linalool, linalyl acetate and 1,8- cineole, present in many plant essential oils, have been shown to have anti-inflammatory activity [84], [85]. Essential oils, therefore, represent a novel option in the treatment of inflammatory diseases.

Repellent and Insecticidal Activity

Essential oils have a broad range of compounds with a variety of insecticidal and repellent mechanisms. Commonly, essential oils can be inhaled, ingested, or skin-absorbed by insects, interfering with basic physiological, behavioural, metabolic, and biochemical functions [55], [59]. Compounds from essential oils can exert their activities on insects through neurotoxic effects involving several mechanisms, notably through GABA, octopamine synapses, and the inhibition of acetylcholinesterase [41], [43], [59], [78]. The insecticidal constituents of many plant extracts and essential oils are monoterpenoids [86], [87]. On the other hand, most insect repellents are terpenoids [88]. In some cases, the same terpenoid can repel certain undesirable insects while attracting more beneficial insects to favour the dispersion of pollens and seeds [59]. Well known essential oils with bioactivity, either as an insecticide or repellent, are clove, thyme, mint, cinnamon and oregano [86], [89]–[94].

Cancer preventive properties

Essential oil constituents have cytotoxic and antitumor activities. The volatile oils have the capacity to act as antioxidants and interfere with mitochondrial functions of mammalian cells. As a result, they diminish metabolic events, such as increased cellular metabolism, mitochondrial overproduction and permanent oxidative stress, which are characteristic of malignant tumour development [95].

Various types of malignancies like, glioma, colon cancer, gastric cancer, human liver tumour, pulmonary tumours, breast cancer, and leukaemia are reported to be lowered after treatment with plant essential oils [61], [95]. In fact, geraniol from palmarosa oil, citral present in lemongrass oil, terpinen-4-ol from tea tree oil and nutmeg, garlic and lemon balm oil have shown promising activities against different malignant tumour development [96]–[100]. In general, terpenoids as well as polyphenol constituents of plant oils prevent tumour cell proliferation through necrosis or induction of apoptosis [61], [64]. Hence, those molecules are supposed to have potential anticancer activities and, therefore, of extreme importance in prevention and therapeutics strategies, as for instance chemotherapy [55].

I.2.2.5. Commercial Applications

Essential oils have proven industrial applications in the manufacture of perfumes, cosmetics, soap, shampoos, or cleaning gels [57]. Moreover, they enhance the shelf life and quality of the products [14]. These volatile oils are also used in a wide variety of consumer goods such as confectionery food products, beverages and for flavouring foods [57]. Another commercial application of these oils is as therapeutic agents in aromatherapy or as active principles or excipients of medicine and pharmaceutics [57].

Due to their reported antibacterial, antifungal, antiviral, nematicidal, insecticidal and antioxidant properties, they are commonly used in the nutritional and agricultural fields [57]. Essential oils can be incorporated into packaging containing the antimicrobial compound or as plant and crop protectants, such as pesticides [35], [57]. Pesticides based on plant essential oils or their active substances have demonstrated efficacy against a range of plant pathogens responsible for postharvest diseases [101]–[104]. They may be applied as fumigants, granular formulations or direct sprays with a range of effects including lethal toxicity, growth inhibition, repellence and/or oviposition deterrence [44].

I.2.2.6. Safety and Challenges

The increasing use of essential oils worldwide has raised concerns in what regards eventual adverse health and environmental impacts. In general, the common use of plant essential oils in drugs and foods does not show significant toxicity to humans, other mammals, birds, and fish, being rapidly absorbed and metabolized in the liver and excreted by the kidneys if accepted mean daily oral doses are respected [14], [44], [59], [78]. Albeit, there is evidence showing that when essential oils are inappropriately used, they can give rise to adverse effects in humans, such as skin irritation, headache and nausea [14], [40].

Owing to their volatility, environmental impacts of essential oil-based pesticides can be expected to be minimal [105]. However, essential oil products are less potent than most other pesticides, so greater quantities are required, and thus the concentrations that are effective against the respective disease can be phytotoxic to certain sensitive plants, causing further chemical deterioration [14], [105]. Essential oils also requisite greater application rates and may need frequent reapplication when used outdoors [15], [44].

Higher concentrations may also be a concern regarding flavour, since exceeding the acceptable flavour and/or odour thresholds would imply a stronger herbal aroma, that may become unacceptable to consumers [63]. Besides flavour, essential oils may interact with food components and result in unwanted appearance characteristics [33].

Despite their promising properties, problems related to their volatility, poor water solubility and tendency to oxidise have to be addressed [54]. These reported characteristics could diminish their efficacy in cosmetics, food, and pharmaceutical industries.

In fact, several factors appear to limit the success of volatile oils, most notably scarcity of the natural resource, product standardization and quality control, refinement of pesticide products, protection of technology (patents) and regulatory approval [54], [59], [105].

The authorization processes are complex and costly, and its manufacture is usually at a low scale because production is restricted by the limited availability of active substances and, therefore, expensive or disadvantageous [43]. Greater production of certain oils would require substantial cost, which smaller companies may not be willing to invest [42], [44]. Furthermore, the chemical profile of plant species can vary naturally depending on genetic, geographic, climatic, annual or seasonal factors [44], [59]. Therefore, obtaining a standardized product, which is important for regulatory approval, is a challenge. However, due to safety of some essential oils and their active substances, they can be used without

toxicological and ecotoxicological studies which are essential for registering commercial products [44], [59], [78].

Although the contact effect of essential oils is good, in the presence of air, light, moisture, and high temperatures rapid evaporation into the environment and gradual biodegradation of active substances occur, leading to low persistence of the effect [43]. Therefore, formulation of essential oil-based products is also an important area of concern. Nanoformulations based on natural or synthetic polymeric materials that encapsulate the active ingredient are a very active field of research, as controlled released formulations allow smaller quantities of pesticides to be used and are highly effective over a given period of time [44], [78]. They can increase water solubility, dissolution rate, dispersion uniformity, prevent the degradation of active ingredients and improve their bioavailability for long periods [41], [44]. The development of new formulations, however, needs to be accessed for its safety to humans and the environment. Nevertheless, with the growing awareness of consumers, essential oils and phytochemicals are expected to be continuously exploited.

I.3. Plant species

I.3.1. *Mentha aquatica* L.

Mentha spp. are part of the family of Lamiaceae and includes about 30 species grown in temperate climate zones around the world [64]. *Mentha aquatica* L., commonly known as water mint, is a perennial herb growing on the banks of flowing waters, ponds and lakes from West Asia to tropical Africa and Europe [106].

Water mint extract contains flavones, limonene, trans-βocimene, rosmarinic acid, quercetin, naringenin, caffeic acid, chlorogenic acid and other compounds such as rutin and ferulic acid [106], [107]. The major constituents of the essential oil are menthofuran, linalyl acetate, 1,8-cineole, menthone, pulegone, menthol, piperitenone oxide, β-caryophyllene, limonene, terpinen-4-ol, germacrene D, caryophyllene oxide, elemol and viridiflorol [108]–[111]. However, its composition varies among different studies, which may be due to different environmental conditions as well as differences on harvesting time [106].

The plant is known for its allergenic, analgesic, antipyretic, antiseptic, antispasmodic, carminative, decongestant, deodorant, diaphoretic, digestive, diuretic, antiemetic, insecticides, sedative, stimulatory and vermifuge actions [112].

I.3.2. *Mentha spicata* L.

Mentha spicata L., commonly known as spearmint, is a cross breeding species resulted from *M. longifolia* and *M. rotundifolia*, and native of Africa, temperate Asia, and Europe [113], [114]. It is considered one of the most important spices in the world and has been used as a medicinal and aromatic plant.

Extracts of spearmint leaves are characterised mainly by a high content of phenolic compounds such as rosmarinic acid, luteolin, and apigenin derivatives, some of which have been shown to have antioxidant properties [115], [116]. Spearmint oil is a complex mixture of terpenic hydrocarbons and aromatic compounds obtained through hydrodistillation of the leaves of the plant, of which carvone, menthone and limonene are its major constituents [113], [114], [117]. Carvone is responsible for the characteristic odour of spearmint oil, being widely used in chewing gums, beverages, cosmetic products, perfumery, sweets, toothpastes and mouth washes [112]–[114], [117].

Spearmint has been used as a valuable source of antioxidants for the nutraceuticals, food and cosmetic industries [113], [118]. It also provides strong antispasmodic, antiseptic, diuretic, stimulant, insecticide, mutagenic, fungicidal, bactericidal, antiviral, nematicidal properties [112]–[114]. Furthermore, it is used to reduce fever, provide relief from depression and asthma and treat headaches, indigestion and nausea [113], [118].

I.3.3. *Mentha piperita* L.

Peppermint (*Mentha piperita* L.), a natural hybrid of the two species *Mentha spicata* L. and *Mentha aquatica* L., is a perennial, with a fresh, slightly sweet, peppery and strong menthol notes [81], [119]. A light yellow or greenish volatile oil with an intense mint aroma is obtained after steam distillation of the aerial parts [15], [119]–[121].

The plant extracts are rich in a wide variety of secondary metabolites such as tannins, phenols (caffeic acid, rosmarinic acid, eugenol and α-tocopherol), steroids, carotenes, betaine, choline, flavonoids and

volatile oils, which were found to have antimicrobial properties [119], [122]. The essential oil is mostly made up of menthol and menthone, and their ratio is the major determinant of the flavouring quality of this essential oil [42], [58], [64]. Peppermint oil also contains neomenthol, isomenthone, menthofurane, additionally including among others limonene, pulegone, alpha- and beta-pinene,trans-sabinene hydrate [112], [121].

The extracts of various alcoholic solvents were found to have different levels of antioxidant activity due to the presence of an enormous amount of flavonoid and phenolic compounds [15], [122]. Moreover, research showed that the flavonoids, phenolic acids and essential oils of *M. piperita* exhibit antifungal, antiviral, cholegogic, carminative, spasmolytic, anti-allergic, and other types of pharmacological activity [119], [123]. The volatile oil is mostly used due to its pleasant mint flavour and aroma and the cooling anaesthetic effect, being widely used in toothpowder, toothpaste, chewing tobacco, mouth fresheners, perfumes, analgesic balms, confectionary, cough drops, chewing gums, candies and the tobacco industry [15], [119]–[121].

I.3.4. *Mentha pulegium* L.

Mentha pulegium L., also called pennyroyal mint, is a perennial aromatic herb, whose leaves have a strong mint-like odour and are used in tea for the treatment of fevers, headaches, minor respiratory infections, digestive disorders, menstrual complaints and various minor diseases [112], [124].

M. pulegium plants are sources of alkaloids, glycosides, saponins, tannins, flavonoids and phenolic compounds, which are responsible for the antioxidant activity [124]. Its essential oils are mainly composed of pulegone and menthone, which are known to have strong pesticidal and antioxidant properties, as well as menthol, isomenthone and piperitone [91], [125]–[127]. Pennyroyal dry parts and essential oil are used in medicine, gastronomy as culinary herb, aromatherapy and cosmetics [126], [128]. The herb is antiseptic, antispasmodic, carminative, diaphoretic, emmenagogue, anti-inflammatory, antimicrobial, sedative and stimulant [124], [125].

M. pulegium volatile oil has shown to be a natural way to help in antibacterial, antifungal and insecticidal issues and, thus, can be used as an antibiotic, a bio-insecticide and an organic food preservative [91], [126].

I.3.5. *Salvia sclarea* L.

The genus *Salvia* is probably the largest genus in the Lamiaceae family, consisting of almost 1000 species spread throughout the world.

Salvia sclarea L., commonly known as clary sage, is an aromatic plant native to Southern Europe and cultivated worldwide, mainly in the Mediterranean region and Central Europe [129], [130]. Clary sage oil is obtained from inflorescences, which possesses a very strong aromatic scent, and thus has an economic value for the flavour and fragrance industries, where it is used as flavouring agent to give aroma to tobacco, mixed herbs, cocktails and liqueurs [131]–[133].

Clary sage has bioactive compounds such as salvinolone, salvipisone, acetylsalvipisone, sclerapinone and sclareol [129]. The extract also contains relevant quantity of polyphenols which probably mostly contribute to its pharmacological effects [134], [135]. The most important components in the oils are alcohols (linalool, terpineol), esters (linalyl acetate, α-terpinyl acetate, geranyl acetate) and germacrene D [130], [132], [136]. Sclareol, a diterpene alcohol, is also widely reported [130], [132], [136], [137].

Among the essential oil compounds, linalool is known for its antibacterial and acaricidal properties and its attractiveness to a broad spectrum of pollinators, herbivores and parasitoids, and germacrene D for its pheromone activity [131], [132]. Furthermore, the volatile oil and extracts of the aerial parts of clary sage have demonstrated analgesic, anti-inflammatory, antimicrobial, antioxidant, antiviral and genotoxic properties and is used as an antidepressant, antiseptic, antispasmodic and carminative [129], [130], [133].

I.3.6. *Salvia officinalis* L.

Sage (*Salvia officinalis* L.) is a common herbal plant native to the Mediterranean region, however, it is widely cultivated in various parts around the world [138]–[140]. Sage essential oil is extracted by distillation of partly dried raw material flowering plants or only sage leaves [121].

Phytochemical investigation of sage revealed a great number of bioactive compounds, among which the most important are essential oil and a wide range of phenolic compounds, flavonoids and phenolics [138], [141]. The essential oil is mostly composed of α-thujone, 1,8-cineole (eucalyptol), α-humulene, β-thujone, β-caryophyllene, camphor, borneol, isobutyl acetate, camphene, linalool, α and β-pinene, viridiflorol [112], [138]–[140].

The plant is known to have a wide range of biological activities, such as antibacterial, fungistatic, virustatic, insecticidal, antioxidant, anti-inflammatory, antispasmodic, astringent, antiseptic, eupeptic, antihidrotic and stimulant properties [112], [139], [142], [143]. Furthermore, the extracts and essential oils of *S. officinalis* are used as spices and for healing of a large range of diseases such as those of the nervous system, heart and blood circulation, and respiratory system [140], [142], [144].

I.3.7. *Thymus vulgaris* L.

Thymus spp. belongs to the Lamiaceae family and comprises over 400 species. Common thyme, *Thymus vulgaris* L., is an evergreen herb used for its flavour and indigenous to the Mediterranean region and neighbouring countries, Northern Africa and parts of Asia. The medicinal parts are the oil extracted from the fresh, flowering herb: the dried leaves; the striped and dried leaves; and the fresh aerial part of the flowering plant [112]. The highest level of essential oil production usually occurs during the flowering period of the plant [121].

The main components of thyme essential oil are thymol, γ-terpinene, carvacrol, p-cymene, borneol and linalool [62], [112], [121]. Thymol is characterized by strong bactericidal, fungal and anti-parasitic properties, with relatively low toxicity to humans and animals [121]. Oils containing predominantly thymol are generally considered of superior quality. Other compounds found in the oil includes monoterpenes, such as α-tujen, α-pinene, α-terpinen, camphor myrcen, geraniol, sabinene hydrate (thuyanol), 1,8-cineol (eucalyptol), terpin-4-ol and camphor [56], [121]. Sesquiterpene hydrocarbons have also been identified, which includes α-humulene, α-kopene, kubeben, α-gurjunen, germacren D, α- and γ-muurolen, and γ and δ-cadinene [121].

Thyme essential oil has also been used as a flavouring agent for a variety of food products, sauces, meats, canned foods, and as an antiseptic agent for its antimicrobial properties [145].

I.3.8. *Cymbopogon flexuosus* Steud.

Cymbopogon spp., also referred to as lemongrass, belongs to one of the most important essential oil yielding of the Poaceae (Gramineae) family. Most of the *Cymbopogon* species biosynthesize and accumulate essential oils predominantly in the young and rapidly expanding leaves and floral

tops/inflorescence [146]. The quality of the lemongrass essential oil is measured by its citral content, the major constituent of lemongrass oil [64], [147].

East Indian lemongrass (*Cymbopogon flexuous* Steud.) is a tall perennial aromatic grass [64], [148]. The essential oil it produces is mainly composed of cyclic and acyclic monoterpenes, particularly citral, geraniol, citronellol, citronellal, linalool, elemol, 1,8-cineole, limonene, â-carophyllene, methylheptenone, geranyl acetate and geranyl formate [146]. The geraniol, geranyl acetate, α-bisabolol and isointermedeol compounds have been individually reported for their cancer cell cytotoxicity and limonene and borneol are known for their immunostimulatory activity, analgesic and anaesthetic activities, respectively [148]. The citral compound is used as antifungal, bactericidal and insecticidal agents and is responsible for powerful inhibitory effects on growth of a number of yeasts and filamentous fungi [149], [150].

Furthermore, *C. flexuosus* oil is thought to help with stress-related disorders and has been shown to have antioxidant, allelopathic, anthelmintic, anti-inflammatory, insect and mosquito repellent activities [146], [148], [151].

I.3.9. *Cymbopogon citratus* Stapf.

Cymbopogon citratus Stapf., known as West Indian lemongrass, is an aromatic perennial plant widely used in tropical countries, especially in Southeast Asia [152], [153].

C. citratus contains various phytoconstituents such as volatile oils, tannins, saponins, steroids, flavonoids, alkaloids, phenols, and anthraquinones, which may be responsible for the different biological activities, such as antiamoebic, antibacterial, antidiarrheal, antifungal, antimalarial, antimutagenicity, antiseptic, anti-inflammatory, antioxidant, hypoglycemic, cardioprotective and neurobehavioral properties [105], [152], [154]. Citral, myrcene, geraniol, limonene, burneol, citronellyl acetate, citronellal and geranyl acetate are the major compounds found in the essential oil [112], [152], [154], [155]. Its primary bioactive constituents are obtained from its leaves, stem, and roots [15], [152].

West Indian lemon grass has been used as a food ingredient, in cosmetics and as folk medicines in several regions of the world [154]. Popularly, aqueous infusion of this plant is said to have bioactive efficacy against nervous and gastrointestinal disturbances when administered orally, due to the phenol compounds found in wild lemongrass leaves, which can act as antioxidants, and so, be used both in the field of health and food [105], [153], [154], [156].

I.3.10. *Coriandrum sativum* L.

Coriander (*Coriandrum sativum* L.) is a culinary and medicinal plant from the Apiaceae family, native to the Mediterranean region [157]–[159]. The plant is grown widely all over the world and mainly cultivated for its aromatic leaves and seeds [77], [158], [160]. The seeds are ground and used as a spice, particularly in Eastern Europe, and contain an essential oil responsible for its pleasant flavour [158].

Coriander essential oil is obtained by steam distillation and the monoterpenoid linalool is the main component [158]–[160]. Typical compositional analysis of coriander oil also includes geraniol, terpinen-4-ol, a-terpineol, g-terpinene, a-pinene, p-cymene, camphor and geranyl acetate, limonene, β-phellandrene, eucalyptol, borneol, β-caryophyllene, citronellol, thymol, linalyl acetate, caryophyllene oxide, elemol and methyl heptenol [112], [158], [159], [161].

The seeds are mainly responsible for the medical use of coriander and have been used as a drug for indigestion, against worms, rheumatism, diarrhea, sub-acid gastritis, pain in the joints, dyspeptic complaints, loss of appetite, convulsion, insomnia and anxiety [157], [158], [160]. The essential oils and various extracts from coriander have been shown to possess antibacterial, antifungal, antioxidant, antidiabetic, anticancerous, stomachic, spasmolytic and carminative and antimutagenic activities [157], [158], [161].

I.3.11. *Apium graveolens* L.

Apium graveolens L., commonly known as celery, is a commercially important seed spice belonging to the family Apiaceae. Celery plant is indigenous to Southern Europe, Asia, and Africa and some parts of South and North America [162], [163].

Celery herb (leaves and stalks), seeds, volatile oil, and oleoresins are extensively used as flavouring ingredients in many food products [163]–[165]. The volatile oil or essential oil contained in the seed is isolated by steam distillation and has a characteristic odour due to a series of phthalide derivatives, in particular 3-n-butyl phthalide, 3-n-butyl-4,5-dihydrophthalide (sedanenolide), and sedanolide [163], [164], [166], [167]. Its oil contains limonene as a major constituent, several mono- and sesquiterpene hydrocarbons, a number of alcohols and some carbonyl compounds including the phtalides, of which sedanenolide, 3-n-butylphthalide, cnidilide, neocnidilide, ligustilide, and 3-isobutylidene3a,4-dihydrophthalide have been reported [165], [166]. The phthalides from celery are the most significant

bioactive compounds exhibiting many health benefits like protection against cancer, high-blood pressure, and cholesterol [163].

Traditionally, seeds of *A. graveolens* have been used against arthritis, rheumatic pain, jaundice, female obstruction and genitourinary treatments and as a treatment to relieve teeth and ear aches, skin diseases and headaches [162], [164], [167]–[169]. The dried leaves are also used for lowering blood pressure and relief of asthma and bronchitis [162], [163]. Celery volatile oil has been shown to have antifungal activity, and it is active against bacteria [165], [168]. Additionally, celery has aphrodisiac, anthelmintic, antispasmodic, carminative, diuretic, emmenagogue, laxative, sedative, stomachic, stimulant, and toxic properties [163], [167], [169].

I.3.12. *Anethum gravelons* L.

Dill (*Anethum graveolens* L.) is an annual and sometimes biennial medicinal plant from the family Apiaceae, and native to Mediterranean region, Southeastern Europe and Central Southern Asia [170], [171]. Essential oil is present in all parts of the plant, but its content is highest in the seeds, whose major components are carvone, limonene and α-phellandrene [172]–[174].

Dill seed and herb are used as flavouring agents in food industry for salads, sauces, soups, seafoods, potatoes and especially in pickled vegetables. However, essential oil is mostly used due to its characteristic aroma and flavour, which is also used in perfumery to aromatize cosmetics, detergents, soaps, as carminative and for control of flatulence, colic, and hiccups in infants and children [170], [171], [175], [176]. Furthermore, it has been reported that dill has antioxidant, insecticidal, anti-inflammatory, antihyperlipidemic, diuretic, antisecretory, antidiabetic, hypotensive, antispasmodic, antiemetic, anti-hypercholesterolaemic, laxative effect and anticancer activity [170], [171], [173], [177]. Dill oil has been shown to have antifungal activity and to inhibit several species of bacteria [173], [174], [176].

I.4. Scope of this thesis

Despite the high number of studies on the antimicrobial effects of plant extracts and essential oils, most studies have focused on pathogenic bacteria. Moreover, mechanism of action of essential oils has not been extensively investigated. Due to their reported biological activities and promising advantages, there is still urgent need to continue to investigate them *in vivo* and *in vitro*. Therefore, the objectives of

the present study were: (i) to investigate the inhibitory capacity of hydroalcoholic extracts and essential oils from the genera *Thymus*, *Coriandrum*, *Apium*, *Salvia*, *Mentha*, and *Cymbopogon* on the growth of food spoilage yeast, *Saccharomyces cerevisiae* and *Zygosaccharomyces parabailii*; (ii) to study the mechanism underlying cell death induced by the most active essential oil; and (ii) to analyse the protective effect of hydroalcoholic extracts on *S. cerevisiae* chronological lifespan.

Overall, the purpose of this study is to assess the response of yeast cells to the tested plant material in order to evaluate the potential applicability of the plant extracts and essential oils on agriculture as a mean of reduction of crop losses and, additionally, provide an overview about the antimicrobial mode of action of essential oils.

II. MATERIALS AND METHODS

II.1. Plant material

Leaves, stems and inflorescences of *Mentha piperita* L., *Mentha Pulegium* L., *Mentha aquatica* L., *Salvia sclarea* L. and leaves and stems of *Cymbopogon citratus* Stapf. were commercially obtained from Ervital (Portugal). Essential oils (EO) of *Mentha aquatica* L. (flowers and stems) and *Mentha pulegium* L. (leaves and some stems) were also purchased from Ervital.

The following essential oils were supplied from Florihana (France): *Mentha piperita* L. (flowering tops from France), *Mentha spicata* L. (flowering plant from Marocos), *Salvia sclarea* L. (flowering tops from France), *Salvia officinalis* L. (flowering tops from Spain, France and Albania), *Cymbopogon flexuous* Steud. (plant from India and Nepal), Cymbopogon citratus Stapf. (leaves from Portugal), *Thymus vulgaris* L. (flowering tops from Spain), *Coriandrum sativum* L. (seeds from France and Hungary), *Anethum graveolens* L. (seeds from France and Hungary) *and Apium graveolens* L. (seeds from India).

II.2. Preparation of the hydroalcoholic extracts

The extracts were individually prepared by macerating the plant material into smaller particles with a mill (Moulinex). The dried powder (25 g) was mixed with 500 mL of an ethanol-ultrapure H_2O (70:30) solution on a magnetic stirrer, at 400 rpm. After 2 hours, 10 g of charcoal (Merk) was added and stirred for 2-3 min. The solutions were filtered twice: first with a Whatman paper filter n° 114, buchner funnel, 1 L filter and Millipore vacuum pump, and secondly with Whatman paper filter n° 1. Then, the ethanol was evaporated in a rotatory evaporator (Heidolph) at 35 °C, under vacuum at 65-30 mBar (Vacuum pump V-700, Buchi) and rotation at 100 rpm. Condensation was carried out by a refrigeration system (Frigiterm, P Selecta) at 5 °C and the remaining crude extract was distributed in centrifuge tubes, containing volumes of 25 to 30 mL of extract in each one to be frozen (-20 °C). Lastly, the extracts were lyophilized for 4 to 5 days.

For preparing hydroalcoholic extract (HAE), the powdered plant samples were reconstituted in deionized water to obtain a stock solution with the desired concentration. Ethanol was not used to dilute the samples due to similar dilution efficacy observed. Lastly, the extracts were strained through a Whatman filter no. 4 (pore size 20-25 mm) and the cleared filtrates were used immediately or stored in the freezer at 4°C until it could be assayed.

II.3. Strains and culture conditions

Antimicrobial activity was evaluated against two yeast strains: *Saccharomyces cerevisiae* BY4741 and *Zygosaccharomyces parabailii* ISA1307. *S. cerevisiae* BY4741, a model organism, was further tested on the following experiments. Yeast cells were grown at 30 °C and maintained at 4 °C on yeast extract peptone dextrose (YEPD) medium agar plates with the following composition: 2% (w/v) glucose, 2% (w/v) bactopeptone, 1% (w/v) yeast extract and 2% (w/v) agar, being replicated periodically.

II.4. Antimicrobial activity and Minimum Inhibitory Concentration (MIC)

To evaluate the antimicrobial activity of the plant material, the microorganisms were grown in liquid media (YEPD) to early exponential phase (OD_{640nm} = 0.5-0.6) [25]. Then the cells were centrifuged at 5000 rpm for 5 min, resuspended in sterilized water and centrifuged again. The suspension was adjusted with fresh medium to an optical density of 0.1 in a final volume of 3 mL or 5 mL per glass tube for the HAE or EO assay, respectively. A requisite amount of HAE or EO was added to each tube to achieve the following concentrations: 250, 500, 1000, 2000, 4000 and 8000 µg/mL or 100, 200, 500, 1000, 2000 µg/mL, respectively. Concentrations of essential oils were calculated assuming a density of 1. Moreover, oils were measured and not weighed to ease the procedure; however, concentration were represented in µg to facilitate comparison with the extracts. Samples without any extract or oil treatment were considered as negative controls. All cultures were incubated in duplicate at 26 °C, 160 rpm. Growth was inferred by optical density after being cultured for 24 h and 48 h.

The minimum inhibitory concentration (MIC) was defined as the lowest concentration of HAE or EO at which the microorganism does not demonstrate significant growth.

II.5. Cell death-inducing activity

II.5.1. Viability assays

To investigate the antifungal effects of the most active essential oil, cell death induction was analysed by evaluating the number of colony forming units (CFU) at varying time points after exposure to EO. Cells of *S. cerevisiae* BY4741 were incubated in YEPD medium at 30 °C to early exponential phase (OD_{640nm} = 0.5–0.6). Then, cells were centrifuged, washed in sterile water, and centrifuged again. The suspension was adjusted with fresh medium to OD_{640nm} = 0.3 in a final volume of 5 mL per glass tube and 50 and 100

µg/mL of EO was added. All cultures (including a control without EO) were incubated at 26 °C, 160 rpm. Cells without EO were used as negative control.

At predetermined time points (0, 30, 60, 120, 180, 240 and 300 min following the addition of oil), 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 plate could be counted.

Colony counts were determined after incubation of the plates at 30 °C for 24-48 h. Colony count data for each experiment were converted into percentages relative to the colony count at time zero to normalize data and correct for slight variations in starting inocula concentrations between experiments. Time-kill curves were then constructed by plotting % of CFU against the exposure time (min).

II.5.2. Cell membrane integrity

In order to detect the membrane integrity of *S. cerevisiae* cells treated with essential oil, yeast suspensions were stained with propidium iodide (PI). PI is a fluorescent probe that only penetrates cells with severe membrane lesions, resulting in increased red fluorescence [178].

To this end, *S. cerevisiae* suspensions were grown overnight in YEPD medium at 30 °C, in a shaker at 200 rpm, until an OD_{640nm} of 0.5-0.6 was reached. The suspensions were centrifuged, washed and the cellular concentration adjusted with fresh medium to OD_{640nm} = 0.3. Different concentrations of EO (50 and 100 µg/mL) were then added. Cells without EO were used as negative controls.

At predetermined time points (0, 60, 180 and 300 min after oil addition), 300 µL of culture was collected and centrifuged. For a staining positive control, one sample without EO was submitted to a heat treatment (100 °C) for 10 min to lyse cells before further treatment, and then centrifuged and processed as the other samples. The pellets were resuspended in 100 µL of PBS and incubated with 5 µg/ml of PI (Molecular Probes, Eugene, U.S.A.) for 10 min at room temperature in the dark [179]. After being centrifuged and concentrated in 30 µL of PBS, a sample (3.5 µL) was transferred to a glass slide, and a coverslip was added to be observed under a Leica DM500 + CTR500 + ebq100 Microscope with red fluorescence filter. The percentage of PI-positive cells, which represents dead cells, was quantified. At least 300 cells of three independent experiments were evaluated.

II.5.3. Reactive oxygen species (ROS) production

Mitochondria dysfunction monitored by ROS production was assessed using the MitoTracker Red CM-H2Xros (Molecular Probes, Eugene, U.S.A.) staining, which specifically detects the ROS generation in mitochondria by passively diffusing across the plasma membrane and accumulate in active mitochondria [180].

S. cerevisiae suspensions were grown overnight in YEPD medium at 30 °C in a shaker at 200 rpm until an OD_{640nm} of 0.5-0.6 was reached. The suspensions were centrifuged, washed and the cellular concentration adjusted with fresh medium in the absence or presence of 150 mM acetic acid, pH 3, to OD_{640nm} = 0.3. Different concentrations of EO (50 and 100 µg/mL) were then added to the tubes without acetic acid, which was used as a positive control. Cells without EO were used as negative controls.

At predetermined time points (0, 60, 180 and 300 min after oil addition), 300 µL of culture was collected, centrifuged, and resuspended in 100 µL of PBS. The samples were incubated with 0.4 µg/mL MitoTracker Red CM-H2XRos at 30 °C for 15 min and protected from the light [179]. For the positive control, acetic acid treated cells were analysed after two hours of treatment.

The samples (3.5 µL) were transferred to a glass slide, and a coverslip was added to be observed under a Leica DM500 + CTR500 + ebq100 Microscope with red fluorescence filter. Cells with red fluorescence were considered to contain mitochondrial ROS. At least 300 cells of three independent experiments were evaluated.

II.6. Chronological Lifespan

To investigate the potential protective effect of HAE on yeast cells, an aging experiment with *M. piperita* and *S. sclarea* was performed, as described by Santos *et al.* [181]. Cells of *S. cerevisiae* BY4741 were cultured at 26 °C, 160 rpm, in YEPD medium until stationary phase was reached. At the third day, cells were centrifuged and resuspended in sterile water adjusting cell concentration to an OD_{640nm} of 1.0. Incubation in water, which is also referred to as "extreme calorie restriction", prevents any regrowth of yeast cells and causes entry into a longer-lived but low metabolism stationary phase [182], [183]. Two conditions were tested: supplementation with 2000 µg/mL of HAE during the time of yeast cultivation but not on aging, and during caloric restriction.

Three-day old cultures are considered to be the 100% survival point and, thus, day 0 of the aging experiment. The experiments were carried out in plastic tubes with loose plastic caps and incubated at 26 °C, 160 rpm. At day 0, 1, 8, 15, 21 and 28, evaluation of the number of surviving cells was performed by serially diluting a 50 μL aliquot of culture in sterile water and spreading five aliquots of 40 μL onto the surface of YEPD medium agar plates. Cell viability was assessed by CFU of the culture aliquots incubated for 24 h at 30 °C. The dilution factor was chosen to ensure 50-250 colonies per plate could be counted.

Colony count data for each experiment were converted into percentages relative to the colony count at time zero to normalize data and correct for slight variations in starting inocula concentrations between experiments. Cell viability curves were then constructed by plotting % of CFU against time (days).

II.7. Reproducibility and statistical analysis of the results

Antimicrobial activity was repeated at least twice (n=2) with 2 replicates each and cell death assays and aging experiments were carried out in triplicate (n=3) and duplicate (n=2), respectively. The results obtained are represented by mean and standard deviation (± SD) values and analysed using two-way ANOVA analysis of variance. Statistical analyses were carried out using GraphPad Prism Software v6.00 (GraphPad Software, California, USA). P-values lower than 0.05 were assumed to represent a significant difference.

III. RESULTS AND DISCUSSION

III.1 Activity of plant material against yeast

Among promising alternative methods to control food spoilage much attention is being paid to the use of natural products, such as plant extracts and essential oils, and their activity. The *in vitro* antimicrobial activity of plant material has been extensively studied and demonstrated against a number of microorganisms, usually using direct-contact antimicrobial assays, such as different types of diffusion or dilution methods, as reviewed by different authors [25], [49], [184]–[189].

In this study, the antimicrobial activity of plant extracts and essential oils of different species were assessed by evaluating yeast growth, as inferred by optical density, after being cultured with crescent concentrations of hydroalcoholic extract (HAE) or essential oil (EO), over a period of 48 h.

III.1.1 Antimicrobial activity of hydroalcoholic extracts

The effects of HAE from *Mentha piperita and Salvia sclarea* at concentration from 500 to 8000 μg/mL were tested on growth *Saccharomyces cerevisiae* BY4741 and *Zygosaccharomyces parabailii* ISA 1307 (Figure 1 and 2). In addition, the HAE from *Mentha pulegium*, *Mentha aquatica* and *Cymbopogon citratus* were investigated against *S. cerevisiae* (Figures 3-5).

It was observed that *M. piperita* was the most active HAE, being able to significantly reduce *Z. parabailii* growth, as shown on Figure 1, and thus suggesting an antimicrobial potential. Bupesh *et al.* [190] obtained a MIC of 10000 μg/mL with aqueous extract of *M. piperita* against *Bacillus subtillis* and *Pseudomonas aerogenosa*, which suggests that, in spite of the differences in microorganisms and extract solvent used, higher concentrations might have promising results. However, *M. piperita* activity was not significantly evidenced on *S. cerevisiae*, which despite inhibiting yeast growth with the highest concentration after 24 h, resumed its growth after 48 h. *M. pulegium* and *M. aquatica* did not show significant differences against *S. cerevisiae* as well and even evidenced a slight increase in yeast growth with some of the concentrations tested, as illustrated in Figures 3 and 4. Moreover, *M. aquatica* was the least inhibitory *Mentha* HAE. In agreement with our results, Gulluce *et al.* [191] and Scherer *et al.* [192] reported no significant antimicrobial activities of *Mentha* spp. extracts against different microorganisms.

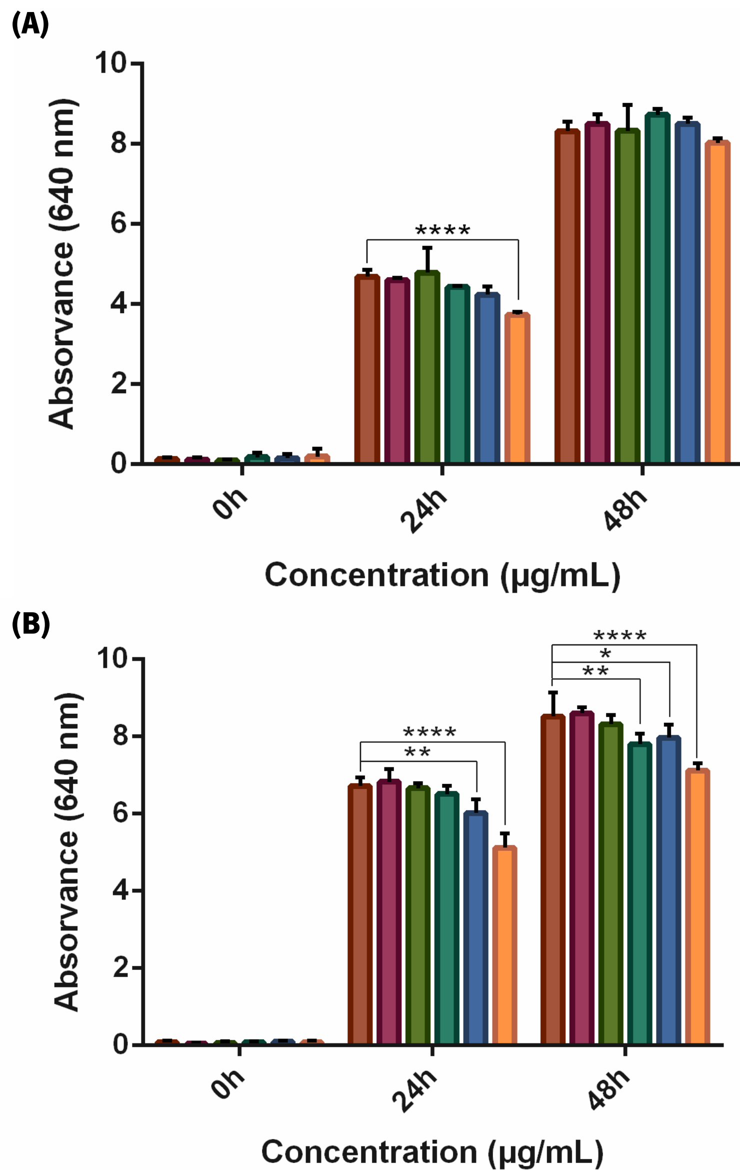
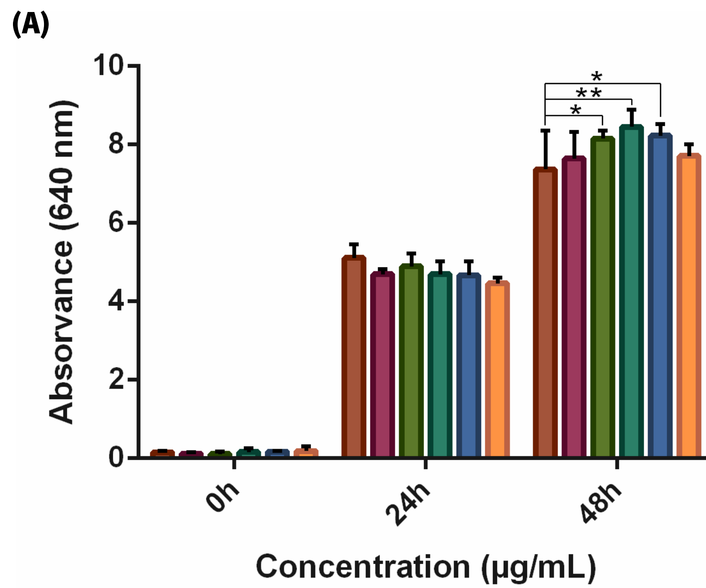
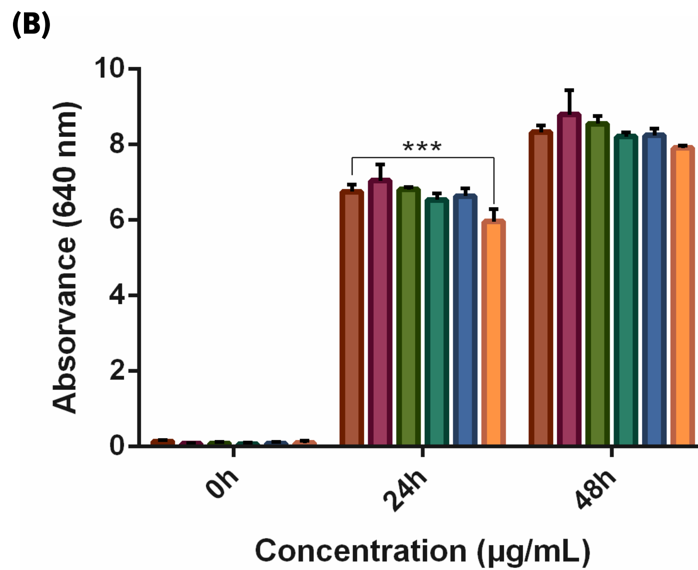


Figure 1 - Antimicrobial activity of *Mentha piperita* L. hydroalcoholic extract (HAE). Absorbance, measured at 640 nm, for the different HAE concentrations added in media with (A) *Saccharomyces cerevisiae* BY4741 and (B) *Zygosaccharomyces parabailii* ISA1307 after 24 and 48 h incubation. The data represent mean values with standard deviations of at least two independent experiments (n=2) with 2 replicates each. *, ** and **** indicate $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.0001$, respectively, compared with control. Every condition tested reported differences of $p \leq 0.0001$ between each time point.





Control 500 μg/mL 1000 μg/mL 2000 μg/mL 4000 μg/mL 8000 μg/mL

Figure 2- Antimicrobial activity of *Salvia sclarea* L. hydroalcoholic extract (HAE). Absorbance, measured at 640 nm, for the different HAE concentrations added in media with (A) *Saccharomyces cerevisiae BY4741* and (B) *Zygosaccharomyces parabailii* ISA1307 after 24 and 48 h incubation. The data represent mean values with standard deviations of at least two independent experiments (n=2) with 2 replicates each. *, ** and *** indicate $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively, compared with control. Every condition tested reported differences of $p \leq 0.0001$ between each time point.

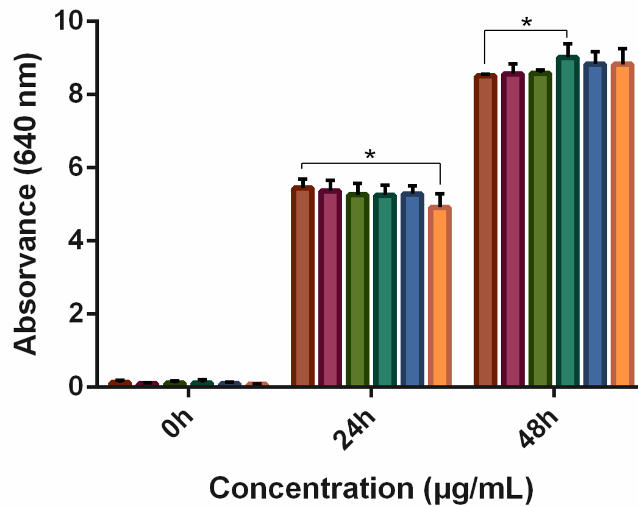


Figure 3- Antimicrobial activity of *Mentha pulegium* L. hydroalcoholic extract (HAE). Absorbance, measured at 640 nm, for the different HAE concentrations added in media with *Saccharomyces cerevisiae* BY4741 after 24 and 48 h incubation. The data represent mean values with standard deviations of at least two independent experiments (n=2) with 2 replicates each. * indicates p≤0.05 compared with control. Every condition tested reported differences of p≤0.0001 between each time point.

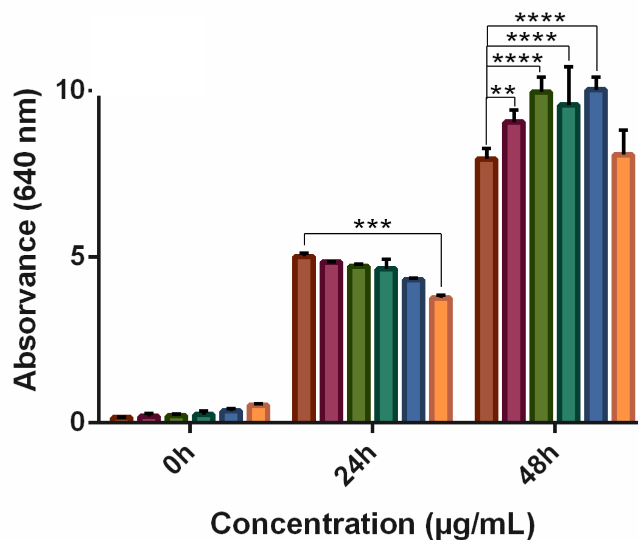


Figure 4- Antimicrobial activity of *Mentha aquatica* L. hydroalcoholic extract (HAE). Absorbance, measured at 640 nm, for the different HAE concentrations added in media with *Saccharomyces cerevisiae* BY4741 after 24 and 48 h incubation. The data represent mean values with standard deviations of at least two independent experiments (n=2) with 2 replicates each. **, *** and **** indicate p≤0.01, p≤0.001 and p≤0.0001, respectively, compared with control. Every condition tested reported differences of p≤0.0001 between each time point.

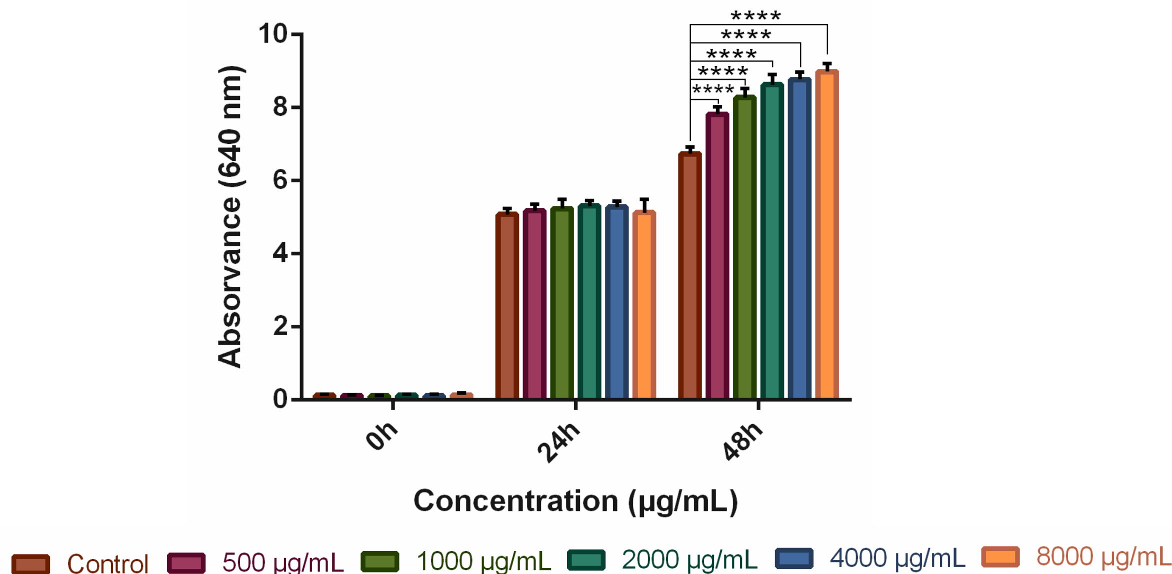


Figure 5 - Antimicrobial activity of *Cymbopogon citratus* Stapf. hydroalcoholic extract (HAE). Absorbance, measured at 640 nm, for the different HAE concentrations added in media with *Saccharomyces cerevisiae* BY4741 after 24 and 48 h incubation. The data represent mean values with standard deviations of at least two independent experiments (n=2) with 2 replicates each. **** indicates $p \leq 0.0001$ compared with control. Every condition tested reported differences of $p \leq 0.0001$ between each time point.

Stimulation of *S. cerevisiae* growth was also observed with *S. sclarea* (Figure 2A) and *C. citratus* (Figure 5). This growth might be an adaptative response of yeast to the HAE or a stimulation by some of its constituents. However, with the exception of *C. citratus* HAE, such increase was not maintained at the highest concentration, which could be due to an hormetic kind of stress response of cells in which "*lower concentrations of the plant material increase the survival of the cells by stimulating biological processes that allow to maintain cellular stress at a level which is below a threshold of toxicity; while higher concentrations of this chemical compound decrease the survival of the cells by creating stress which exceeds such threshold*" [193], [194]. *C. sage* HAE did not display a similar response against *Z. parabailii,* evidencing higher sensitivity to HAEs.

The lowest toxic activity was observed with *C. citratus*, whose *S. cerevisiae* growth was stimulated after 48 h with all concentrations, as shown on Figure 5. Higher concentrations would have to be tested to confirm if such response was further increased. Almeida *et al.* [195] reported MICs ranging from 31250 to 62500 µg/mL and no inhibition for concentrations lower than 15620 µg/mL against five *Candida* species, after 24 h treatment with HAE, which are quite superior to the concentrations tested in this study

and, once more, suggests that concentrations tested were not sufficient. Schuck *et al.* [196] did not find inhibitory activity of the HAE against *C. albicans*, however a fungistatic effect was reported using *C. citratus* volatile oil.

Minimum inhibitory concentrations (MIC) could not be determined because HAE did not, or only slightly, reduced yeast growth and, as stated, an increase in growth was even observed for some plant species. Due to the reduced activity of the HAEs, a lower number of plant species was tested. Higher concentrations might have to be tested, albeit, if promising results were obtained at such concentrations, they would not be easily implementable *in vivo*, and so, would be of limited interest.

Even though weak effects were observed, *Z. parabailli* exhibits higher sensibility towards HAE treatment, which is evidenced by a significant yeast growth reduction with lower concentrations of *M. piperita* against *Z. parabailii* compared with *S. cerevisiae* (Figure 1) and significant growth stimulation on a range of concentrations of *S. sclarea* against *S. cerevisiae* but not *Z. parabailii* (Figure 2) . However, it would be necessary to test more extracts against *Z. parabailii* to confirm its higher sensitivity with this plant material. The susceptibility differences between these two yeast strains may be related to various modes of action of the plant material or cell wall structural differences and/or metabolism, which enables them to overcome or adapt to a potential antimetabolic effect of a given HAE compared to the other yeast [197]–[199].

Although the results obtained for the HAE suggest unsatisfactory antimicrobial activities in the control of the pathogenic yeasts tested, many studies [45], [195], [200]–[202] present impressive results with regard to microorganism control by those plant species using different types of solvents, confirming the capacity of these plants to act as natural antimicrobials. These differences might be due to the fact that the efficiency of extraction is influenced by several parameters such as nature of phytochemicals, the method used for extraction, particle size, the solvent used, as well as the effect of nosy substances [203]. The polarity of solvent greatly influences antimicrobial activity of medicinal plants according to Padalia *et al.* [204] and Srikacha and Ratananikom [205]. Therefore, the low antimicrobial activity of hydroalcoholic extracts could be due to this solvent polarity.

III.1.2 Antimicrobial activity of essential oils

The effects of EO from *Thymus vulgaris*, *Cymbopogon flexuosus, Cymbopogon citratus*, *Mentha piperita* , *Mentha spicata*, *Mentha aquatica, Mentha pulegium, Coriandrum sativum*, *Anethum graveolens*, *Salvia officinalis*, *Salvia sclarea, Apium graveolens* at concentration from 100 to 2000 µg/mL were tested on growth of *Saccharomyces cerevisiae* BY4741 and *Zygosaccharomyces parabailii* ISA 1307 (Figures 6-17).

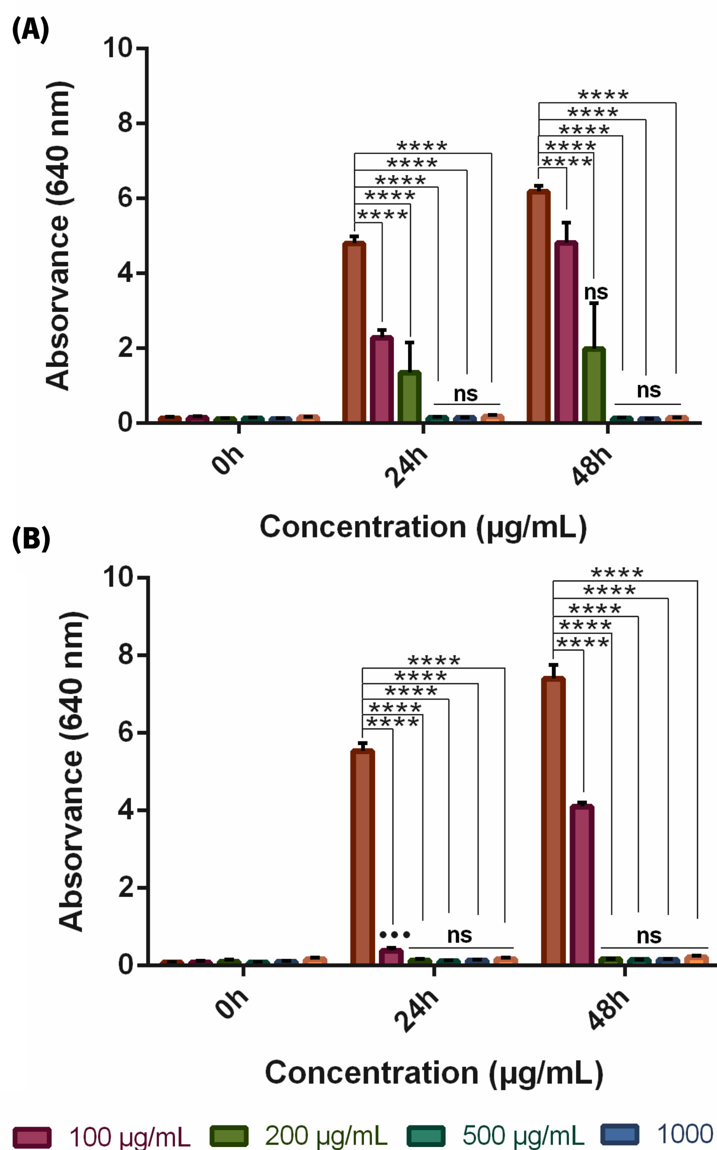


Figure 6- Antimicrobial activity of *Thymus vulgaris* L. essential oil (EO). Absorbance, measured at 640 nm, for the different EO concentrations added in media with (A) *Saccharomyces cerevisiae* BY4741 and (B) *Zygosaccharomyces parabailii* ISA1307 after 24 and 48 h incubation. The data represent mean values with standard deviations of at least two independent experiments (n=2) with 2 replicates each. **** indicates p≤0.0001 compared with control. Every condition tested reported differences of p≤0.0001 between each time point, unless represented with ns or ●●●, which indicate differences not significant or p≤0.001, respectively.

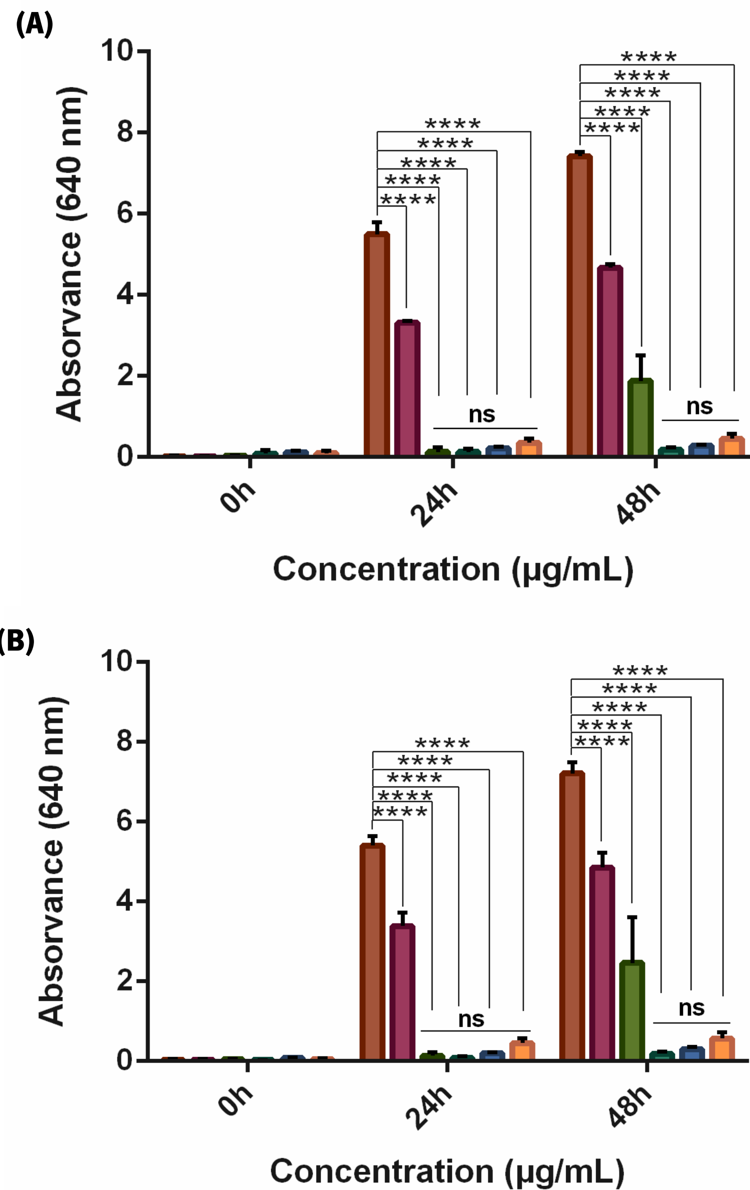
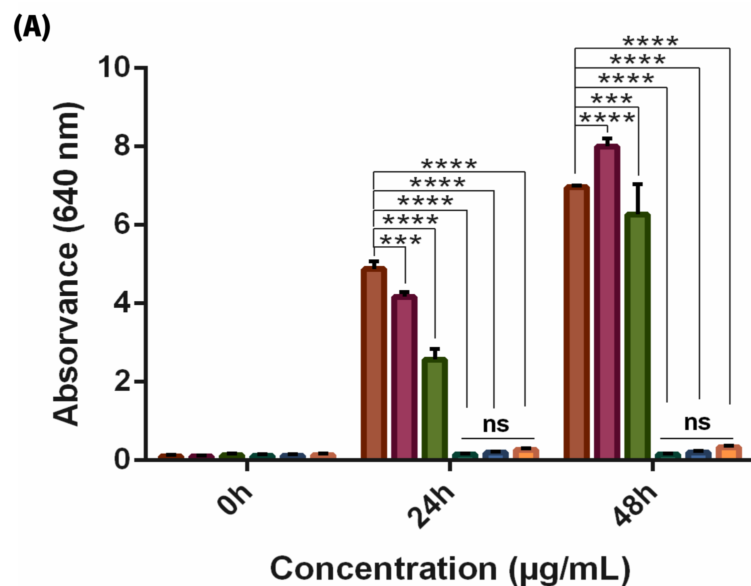


Figure 7 - Antimicrobial activity of *Cymbopogon flexuosus* Steud. essential oil (EO). Absorbance, measured at 640 nm, for the different EO concentrations added in media with (A) *Saccharomyces cerevisiae* BY4741 and (B) *Zygosaccharomyces parabailii* ISA1307 after 24 and 48 h incubation. The data represent mean values with standard deviations of at least two independent experiments (n=2) with 2 replicates each. **** indicates p≤0.0001 compared with control. Every condition tested reported differences of p≤0.0001 between each time point, unless represented with ns (not significant).



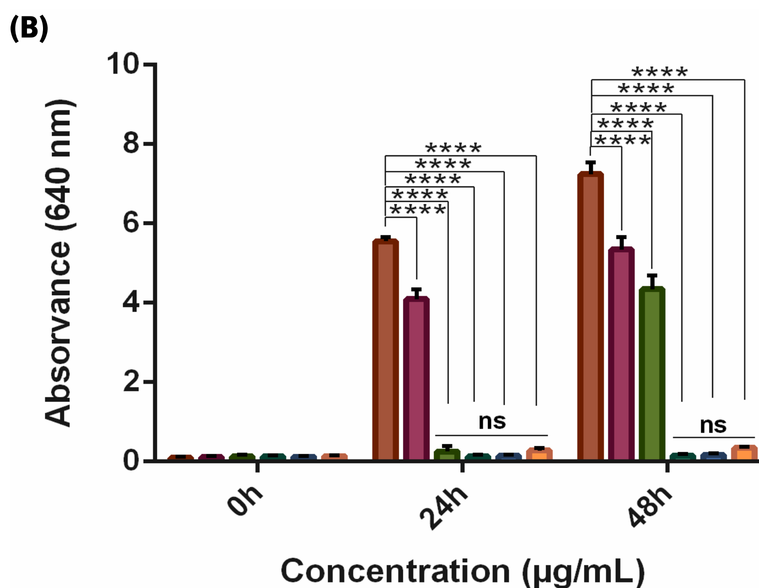
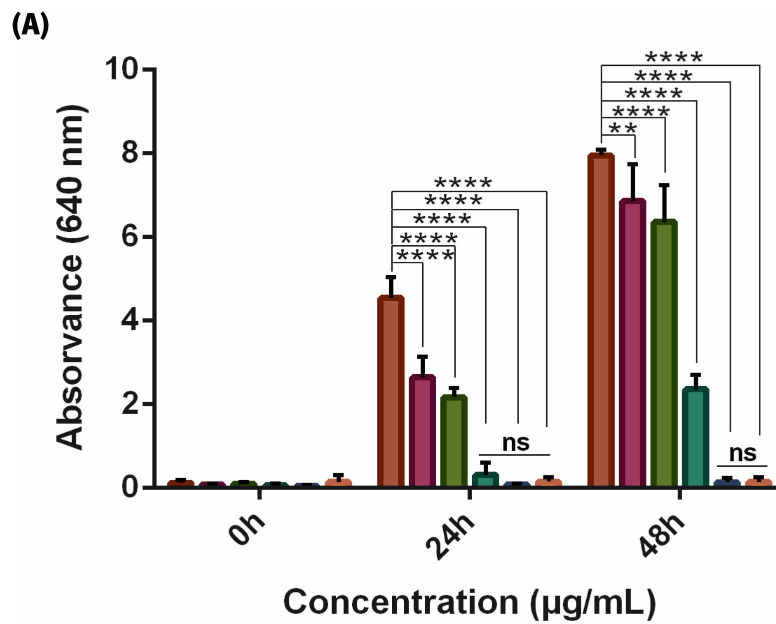


Figure 8 - Antimicrobial activity of *Cymbopogon citratus* Stapf. essential oil (EO). Absorbance, measured at 640 nm, for the different EO concentrations added in media with (A) *Saccharomyces cerevisiae* BY4741 and (B) *Zygosaccharomyces parabailii* ISA1307 after 24 and 48 h incubation. The data represent mean values with standard deviations of at least two independent experiments (n=2) with 2 replicates each. *** and **** indicate $p \leq 0.001$ and $p \leq 0.0001$ compared with control. Every condition tested reported differences of $p \leq 0.0001$ between each time point, unless represented with ns (not significant).



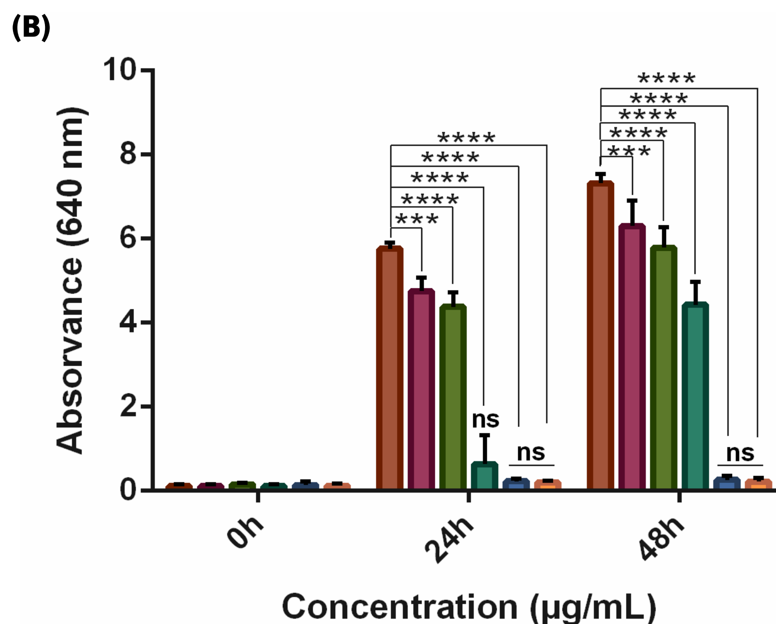
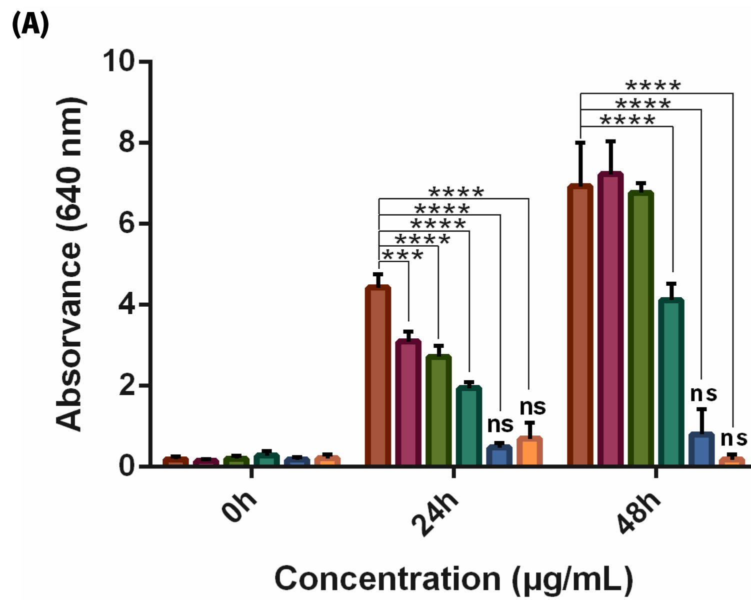
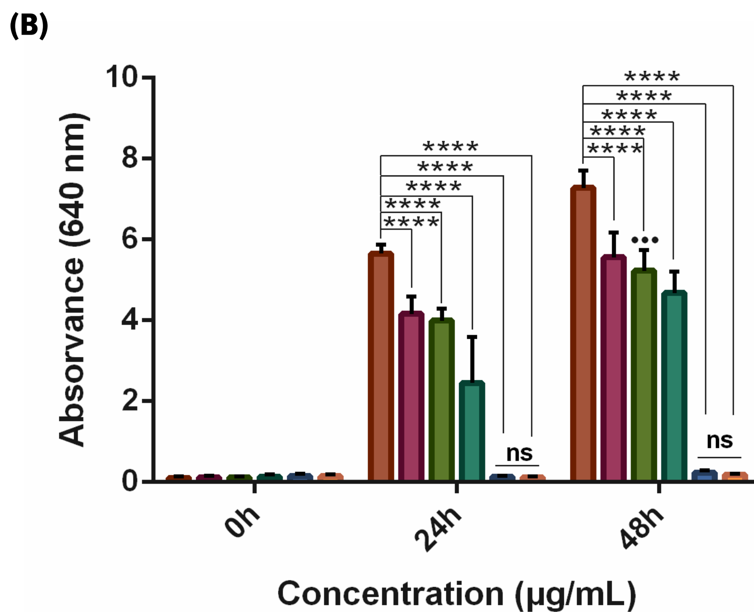


Figure 9 - Antimicrobial activity of *Mentha piperita* L. essential oil (EO). Absorbance, measured at 640 nm, for the different EO concentrations added in media with (A) *Saccharomyces cerevisiae* BY4741 and (B) *Zygosaccharomyces parabailii* ISA1307 after 24 and 48 h incubation. The data represent mean values with standard deviations of at least two independent experiments (n=2) with 2 replicates each. **, *** and **** indicate p≤0.01, p≤0.001 and p≤0.0001, respectively, compared with control. Every condition tested reported differences of p≤0.0001 between each time point, unless represented with ns (not significant).





Control 100 µg/mL 200 µg/mL 500 µg/mL 1000 µg/mL 2000 µg/mL

Figure 10 - Antimicrobial activity of *Mentha spicata* L. essential oil (EO). Absorbance, measured at 640 nm, for the different EO concentrations added in media with (A) *Saccharomyces cerevisiae* BY4741 and (B) *Zygosaccharomyces parabailii* ISA1307 after 24 and 48 h incubation. The data represent mean values with standard deviations of at least two independent experiments (n=2) with 2 replicates each. *** and **** indicate $p \leq 0.001$ and $p \leq 0.0001$, respectively, compared with control. Every condition tested reported differences of $p \leq 0.0001$ between each time point, unless represented with ns or ●●●, which indicate differences not significant or $p \leq 0.001$, respectively.

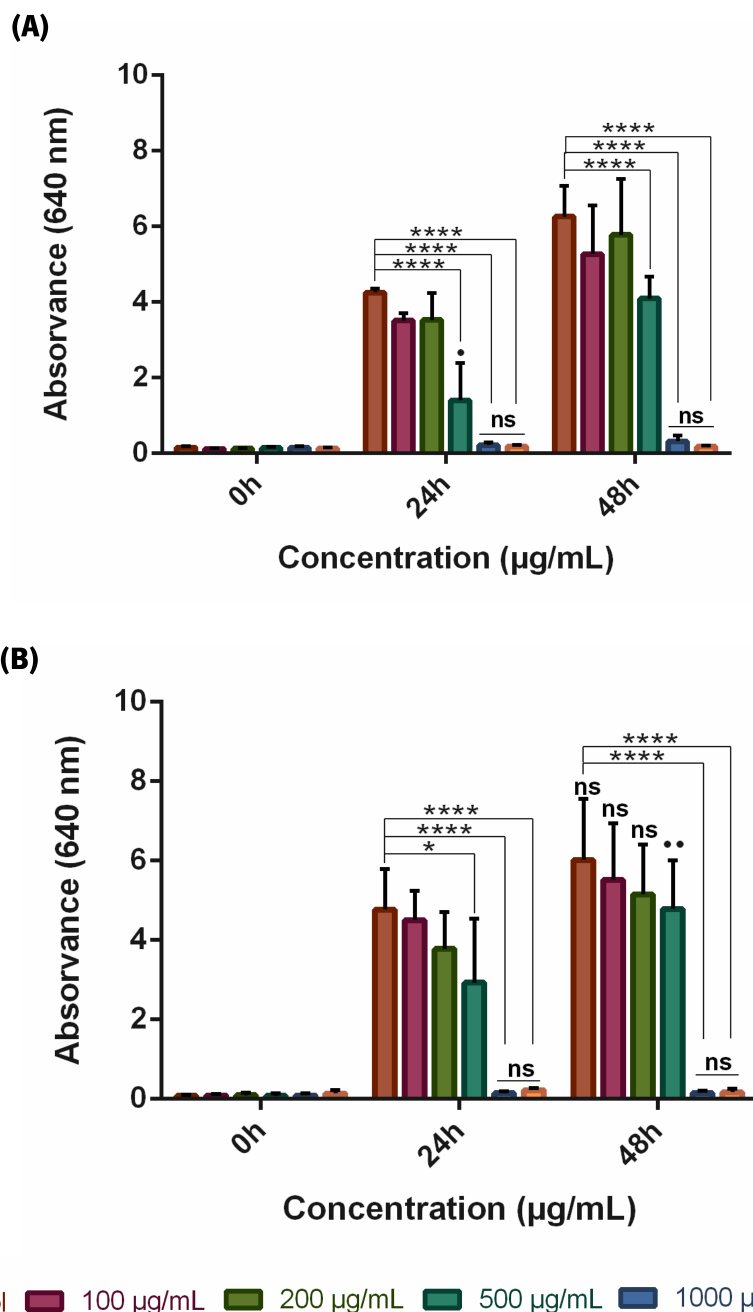


Figure 11 - Antimicrobial activity of *Mentha aquatica* L. essential oil (EO). Absorbance, measured at 640 nm, for the different EO concentrations added in media with (A) *Saccharomyces cerevisiae* BY4741 and (B) *Zygosaccharomyces parabailii* ISA1307 after 24 and 48 h incubation. The data represent mean values with standard deviations of at least two independent experiments (n=2) with 2 replicates each. * and **** indicate $p \leq 0.05$ and $p \leq 0.0001$, respectively, compared with control. Every condition tested reported differences of $p \leq 0.0001$ between each time point, unless represented with ns, ● or ●●, which indicate differences not significant, $p \leq 0.05$ or $p \leq 0.01$, respectively.

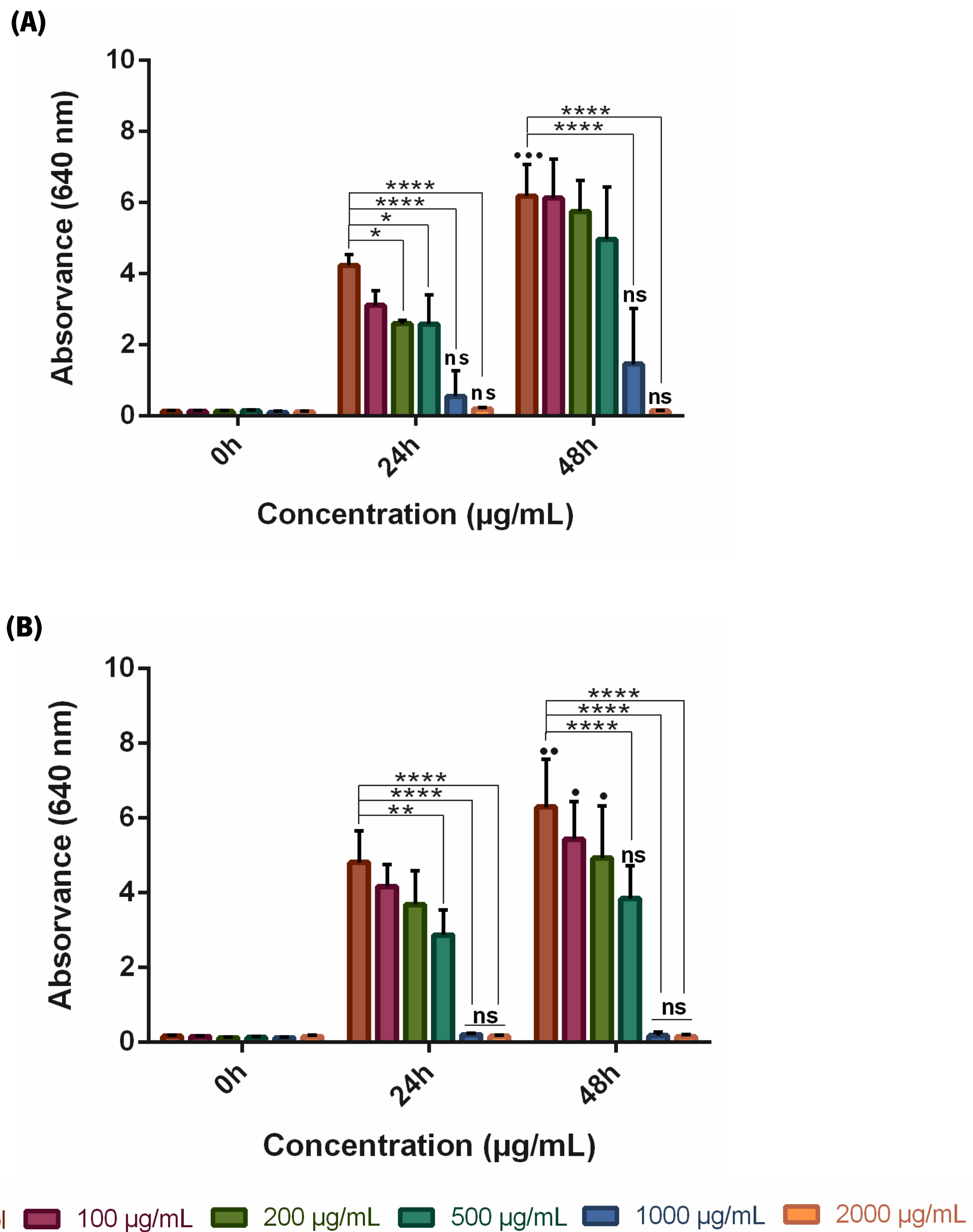
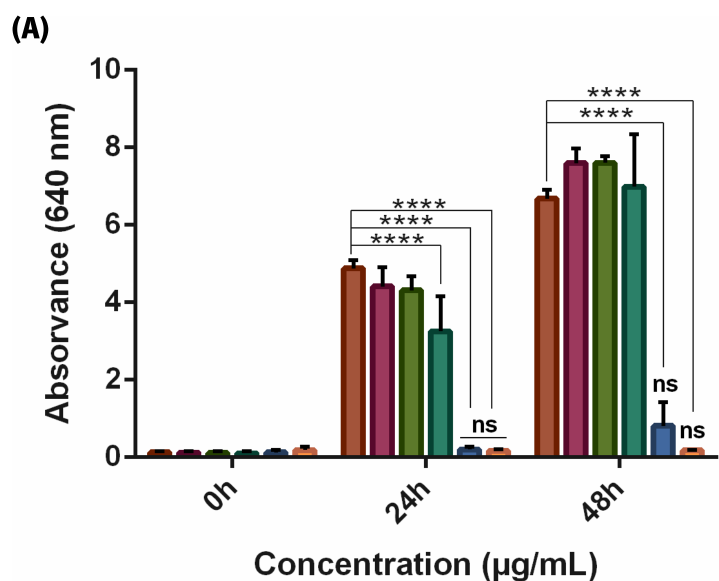
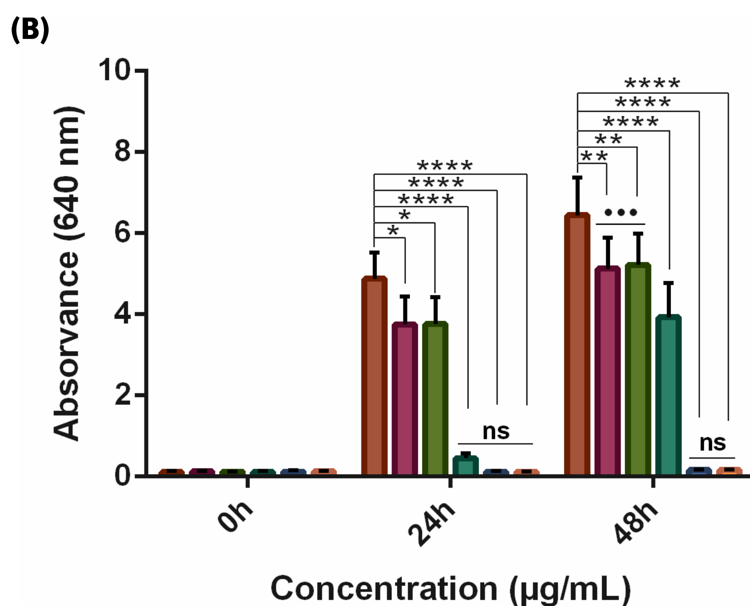


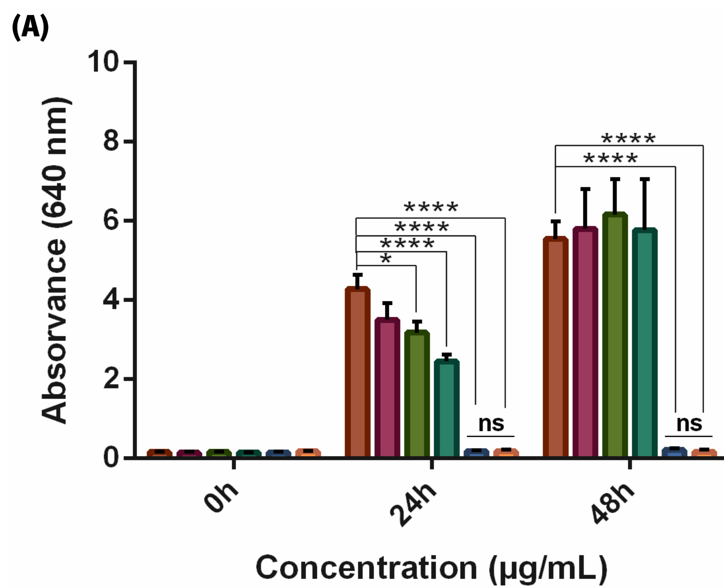
Figure 12 - Antimicrobial activity of *Mentha pulegium* L. essential oil (EO Absorbance, measured at 640 nm, for the different EO concentrations added in media with (A) *Saccharomyces cerevisiae* BY4741 and (B) *Zygosaccharomyces parabailii* ISA1307 after 24 and 48 h incubation. The data represent mean values with standard deviations of at least two independent experiments (n=2) with 2 replicates each. *, ** and **** indicate p≤0.05, p≤0.01 and p≤0.0001, respectively, compared with control. Every condition tested reported differences of p≤0.0001 between each time point, unless represented with ns, ● or ●●, which indicate differences not significant, p≤0.05 or p≤0.01, respectively.





Control 100 µg/mL 200 µg/mL 500 µg/mL 1000 µg/mL 2000 µg/mL

Figure 13 - Antimicrobial activity of *Anethum graveolens* L. essential oil (EO). Absorbance, measured at 640 nm, for the different EO concentrations added in media with (A) *Saccharomyces cerevisiae* BY4741 and (B) *Zygosaccharomyces parabailii* ISA1307 after 24 and 48 h incubation. The data represent mean values with standard deviations of at least two independent experiments (n=2) with 2 replicates each. *, ** and **** indicate $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.0001$ compared with control. Every condition tested reported differences of $p \leq 0.0001$ between each time point, unless represented with ns or ●●●, which indicate differences not significant or $p \leq 0.001$, respectively.



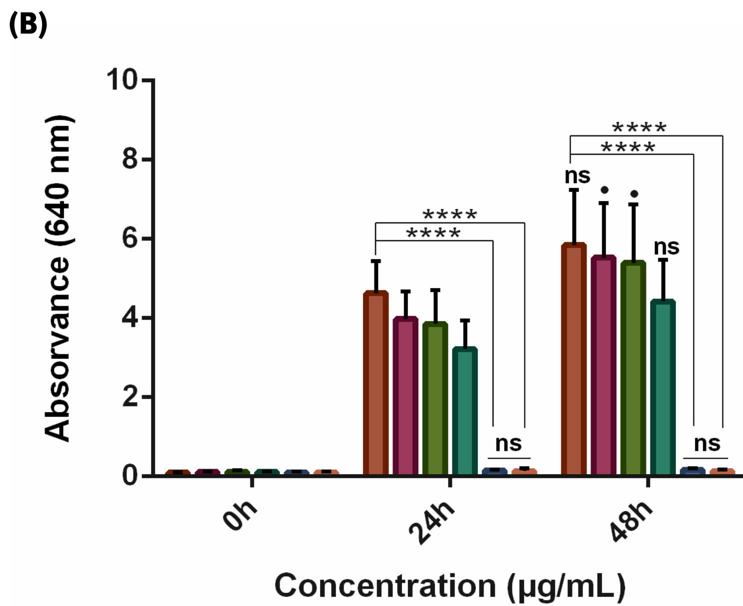
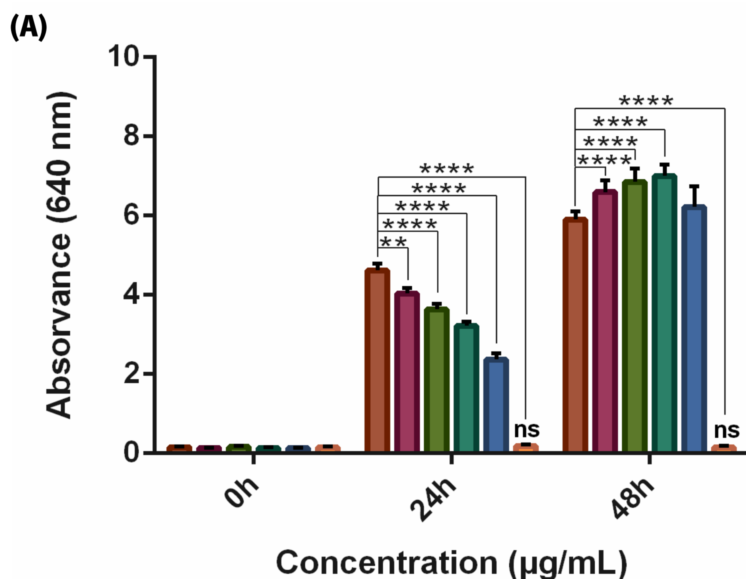
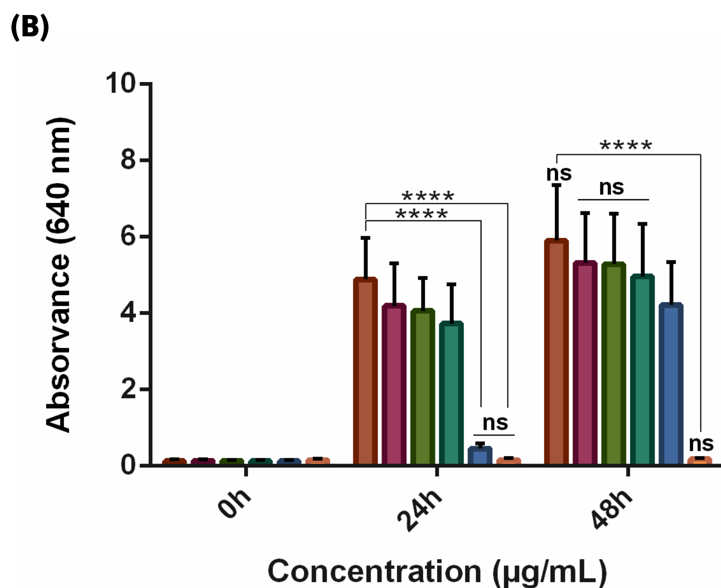


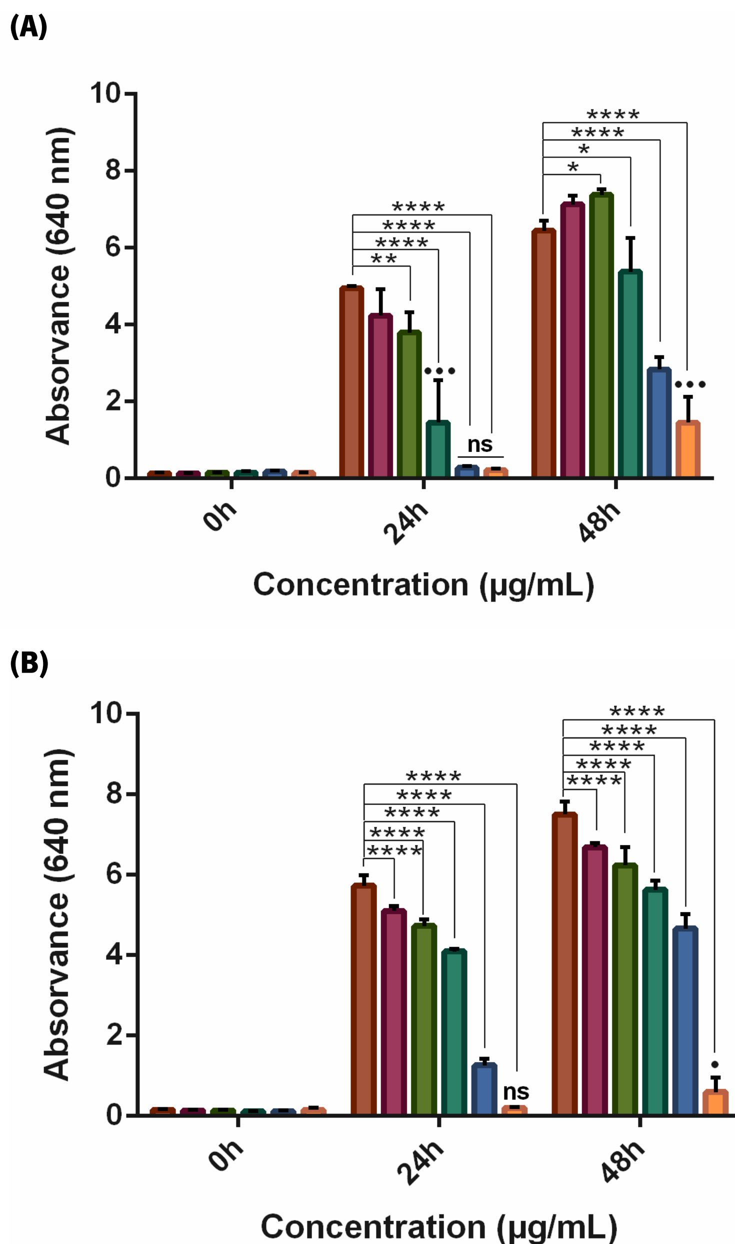
Figure 14 - Antimicrobial activity of *Coriandrum sativum* L. essential oil (EO). Absorbance, measured at 640 nm, for the different EO concentrations added in media with (A) *Saccharomyces cerevisiae* BY4741 and (B) *Zygosaccharomyces parabailii* ISA1307 after 24 and 48 h incubation. The data represent mean values with standard deviations of at least two independent experiments (n=2) with 2 replicates each. * and **** indicate $p \leq 0.05$ and $p \leq 0.0001$ compared with control. Every condition tested reported differences of $p \leq 0.0001$ between each time point, unless represented with ns or ●, which indicate differences not significant or $p \leq 0.05$, respectively.





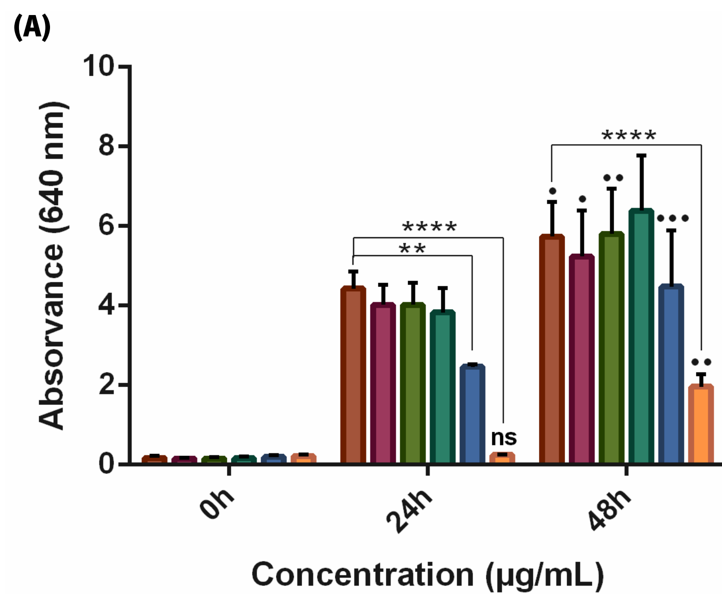
Control 100 µg/mL 200 µg/mL 500 µg/mL 1000 µg/mL 2000 µg/mL

Figure 15 - Antimicrobial activity of *Salvia officinalis* L. essential oil (EO). Absorbance, measured at 640 nm, for the different EO concentrations added in media with (A) *Saccharomyces cerevisiae* BY4741 and (B) *Zygosaccharomyces parabailii* ISA1307 after 24 and 48 h incubation. The data represent mean values with standard deviations of at least two independent experiments (n=2) with 2 replicates each. ** and **** indicate p≤0.01 and p≤0.0001, respectively, compared with control. Every condition tested reported differences of p≤0.0001 between each time point, unless represented with ns (not significant).



Control 100 µg/mL 200 µg/mL 500 µg/mL 1000 µg/mL 2000 µg/mL

Figure 16 - Antimicrobial activity of *Salvia sclarea* L. essential oil (EO). Absorbance, measured at 640 nm, for the different EO concentrations added in media with (A) *Saccharomyces cerevisiae* BY4741 and (B) *Zygosaccharomyces parabailii* ISA1307 after 24 and 48 h incubation. The data represent mean values with standard deviations of at least two independent experiments (n=2) with 2 replicates each. *, ** and **** indicate $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.0001$, respectively, compared with control. Every condition tested reported differences of $p \leq 0.0001$ between each time point, unless represented with ns, ● or ●●●, which indicate differences not significant, $p \leq 0.05$ or $p \leq 0.001$, respectively.



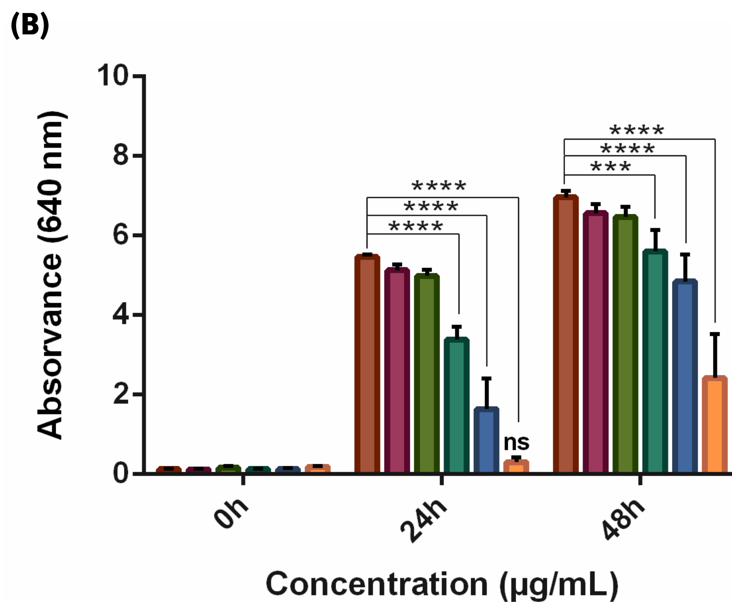


Figure 17- Antimicrobial activity of *Apium graveolens* L. essential oil (EO). Absorbance, measured at 640 nm, for the different EO concentrations added in media with (A) *Saccharomyces cerevisiae* BY4741 and (B) *Zygosaccharomyces parabailii* ISA1307 after 24 and 48 h incubation. The data represent mean values with standard deviations of at least two independent experiments (n=2) with 2 replicates each. **, *** and **** indicate $p \leq 0.01$, $p \leq 0.001$ and $p \leq 0.0001$ compared with control. Every condition tested reported differences of $p \leq 0.0001$ between each time point, unless represented with ns, ●, ●●● or ●●●●, which indicate differences not significant, $p \leq 0.05$, $p \leq 0.001$ or $p \leq 0.0001$, respectively.

Minimum inhibitory concentration (MIC) results revealed that most tested plant EO evidence antimicrobial activity against *S. cerevisiae* and *Z. parabailii* with MIC values ranging from 200 to 2000 µg/mL, as shown in Table 1. Aligianis *et al.* [206] proposed a classification of the antimicrobial activity for plant materials related to the MIC results as follows*:* strong inhibition – MIC up to 500 µg/mL; moderate inhibition – MIC between 600 and 1000 µg/mL; and weak inhibition – MIC above 1000 µg/mL.

Table 1 - Anti-yeast activity of the tested essential oils. Minimum inhibitory concentration (MIC), expressed as µg/mL, of essential oils against *Saccharomyces cerevisiae* BY4741 and *Zygosaccharomyces parabailii* ISA1307 after 24 and 48 hours. ND means not detected.

Essential oil	***S. cerevisiae***		***Z. parabailii***	
	24 hours	48 hours	24 hours	48 hours
Thymus vulgaris L.	500	500	100	200
Cymbopogon flexuosus Steud.	200	500	200	500
Cymbopogon citratus Stapf.	500	500	200	500
Mentha piperita L.	500	1000	500	1000
Mentha spicata L.	1000	1000	1000	1000
Mentha aquatica L.	1000	1000	1000	1000
Mentha pulegium L.	1000	1000	1000	1000
Coriandrum sativum L.	1000	1000	1000	1000
Anethum graveolens L.	1000	1000	500	1000
Salvia officinalis L.	2000	2000	1000	2000
Salvia sclarea L.	1000	ND	2000	ND
Apium graveolens L.	2000	ND	2000	ND

According to this classification, *T. vulgaris* (thyme) EO presents a strong activity, being highly effective at inhibiting *S. cerevisiae and Z. parabailii* with 100-500 µg/mL of EO (Figure 6). Thyme oil starts to significantly reduce microorganism growth with 100 µg/mL after 24 h of EO treatment, exhibiting the most promising results at lower concentrations. Its high activity has been related to phenolic compounds, such as thymol and carvacrol, which have been found to have substantial antimicrobial activity [80],

[184], [206]. In our study, the major compounds of the commercial oil used were thymol (45.85%), O-cymene (17.32%), γ-terpinene (11.7%), linalool (4.46%) and carvacrol (3.22%), as presented in Appendix A. In accordance with these results, *T. vulgaris* EO has been described by other authors to be composed predominantly by thymol (20-55%), followed by p-cymene (14-45%), carvacrol (1-10%), γ-terpinene (5-10%) and linalool (up to 8%) [112], [121]. Kunicka-Styczyńska [207], whose *T. vulgaris* sample was similar to ours, reported thyme oil as having the highest fungistatic activity among the essential oils tested (MIC of 500 µg/mL against *S. cerevisiae*, after 3 days). Moreover, Combrinck *et al.* [208] verified that the efficacy of the sample tested in their study, characterized by 63.1% thymol and high levels of linalool (21.3%), was echoed by the activity of thymol, as the inhibition of pathogens by the pure compound was similar or superior to that of the oil. Similarly, antimicrobial activity in our thyme EO sample can be attributed mainly to thymol, which accounts for approximately half of its content. Synergism between thymol and O-cymene, found as two main components of the tested thyme oil, can also explain its high activity [209], [210].

Apart from *T. vulgaris* EO, characterization of the composition of the tested oils is still being carried out within the scope of the project in which this work integrates and, thus, antimicrobial activity cannot be individually correlated to specific compounds present in those oils. Nevertheless, *Cymbopogon* spp. exhibits similar activity to *T. vulgaris*; however, opposed to the latter EO, MIC on *Z. parabailii* is higher after 48 h (500 µg/mL), which could be explained by higher antifungal activity shown by thymol in comparison to citral, geraniol and limonene, some of the main components reported for *Cymbopogon* oils responsible for their antimicrobial properties [82], [146], [149], [211].

Despite similar MIC, *C. flexuosus* appears to have better antimicrobial activity than *C. citratus*, as a higher inhibition of tested yeasts was observed (Figure 7). As a matter of fact, a significant growth at 200 µg/mL *C. citratus* EO treatment against *S. cerevisiae* was reported after 48 h, as shown in Figure 8A. Difference between oils of the same genus was reported by Silva *et al.* [198] on gram negative and gram positive bacteria, who speculated that the higher citral content or synergistic action between major and minor components in the essential oil mix might account for it.

Essential oils of *Mentha* spp. exhibit strong to moderate growth inhibition. *M. piperita* was the most effective, reporting extremely significant differences with all concentrations tested in comparison to control, as illustrated on Figure 9, and a lower MIC after 24 h of EO treatment. *M. spicata* exhibits similar results to *M. piperita* (Figure 10), howbeit a higher concentration was needed to significantly inhibit

growth. *M. pulegium.* and *M. aquatica* evidenced identical activities; however, *M. aquatica* presents better activity against *S. cerevisiae*, whereas *M. pulegium* was more effective against *Z. parabailii* (Figure 11A and 12B, respectively). *M. aquatica* higher activity against *S. cerevisiae* was not evidenced on HAE tests, where it significantly stimulated yeast growth after 48 h (Figure 4). Different studies [126], [188], [192] indicate that EOs are more efficient antifungals and antibacterials compared to the polar extracts, which is in accordance with our results. The antibacterial and antifungal activities of *Mentha* species have been studied on various bacteria and fungi [49], [68], [89], [91], [185], [190], [212], [213]. Park *et al.* [49] reported *M. pulegium* extract as having higher activity than *M. pipperita*, followed by *M. aquatica* and *M. spicata*, which is not in agreement with our current findings. Such differences can be due to distinct plant origins determining essential oil chemical composition, as well as different assay conditions and differences between strains [90], [214]. *Mentha* EOs show remarkable antimicrobial activity mainly due to the presence of oxygenated monoterpenes, such as menthone, menthol, carvone, pulegone and piperitone, in their chemical composition, but their concentrations are low in the crude extracts, which might account for the HAEs lower antimicrobial activity [49], [185], [188], [192], [215]. The amount of menthol in the essential oil appears to be correlated with EO antimicrobial activity [121]. Hence, the differences observed in the antimicrobial activity of the four mint EOs are likely related to varying amounts of compounds known by a high potential of activity, namely menthol, which might be in a lower quantity on the least active oils. Additionally, synergistic action of the various molecules contained in the EO might play a part in their activities.

Anethum graveolens (dill) and *C. sativum* (coriander) induced moderate inhibition of yeast growth. Opposed to results obtained by Delaquis *et al.* [216], dill EO was more effective than coriander, showing higher activity against *Z. parabailii,* as seen on Figure 13 and 14, respectively. Dill antimicrobial activity against the mould *A. niger* and the yeasts *S. cerevisiae* and *C. albicans* has been reported by Jirovetz *et al.* [172]. Freires *et al.* [217] and Silva *et al*. [218] found that coriander essential oil had a potent antifungal activity against *C. albicans*. Moreover, coriander oil has been shown to inhibit most fungi tested (*Aspergillus flavus*, two different strains of *Aspergillus niger*, *Cladosporium herbarum*, *Penicillium brevicompactum*, *Penicillium roquefortii*, and *Trichothecium roseum*) [48]. The high content of carvone and limonene might play an important role on the antimicrobial activity of dill EO [216], [219], [220]. Regarding coriander, alcohols and aldehydes, mainly linalool, have been described as the major components responsible for its activity [217], [221], [222].

Essential oils of *Salvia* spp. revealed weak action. After 48 h, it was observed a significant growth of *S. cerevisiae* with 100 to 500 µg/mL and 200 µg/mL of *S. officinalis* (Figure 15A) and *S. sclarea* (Figure 16A) EO, respectively. A similar result was obtained with *S. sclarea* HAE against *S. cerevisiae* with concentrations between 1000 and 4000 µg/mL (Figure 2A) and suggests a higher resistance of this yeast to both HAE and EO. *S. sclarea* EO could not completely inhibit yeast growth after 48 h and, thus, was least active than *S. officinalis*. In accordance with these results, the inhibitory activity of *S. officinalis* against 10 bacterial strains has been demonstrated [223] to be significantly higher than that of *S. sclarea* EO, which might be attributed to different amounts of the major compounds, such as camphor, thujone and 1,8-cineole, but also to the presence of synergy, antagonism or additive effects of these compounds or other compounds found in smaller amounts, due to its various activity potency [135], [189], [224], [225]. Increased MICs have also been reported in literature regarding *Salvia* oils. It was described [214], [224], [226], [227] that EOs from *S. officinalis* inhibited *E. coli* growth with 5000 and 10000 µg/mL; *S. sclarea* had MIC values between 7500 and 1000 µg/mL against bacteria and between 1500 and 10000 µg/mL against fungi.

*A*pium *graveolens* L. (celery) had the highest MIC after 24 h and could not completely inhibit yeast growth after 48 h, as evidenced in Figure 17, presenting the weakest activity among the 12 EO tested. However, it was capable of completely inhibiting growth after 24 h and, consequently, confirms that yeast can overcome the oil antimicrobial activity and resume its growth. Gupta *et al.* [228] and Kamdem *et al.* [229] reported inhibition against bacteria with 970-125000 µg/mL and against fungal strains with 1228-70000 µg/mL of celery oil, evidencing a need to test higher concentrations in order to conclude about its inhibitory capacity. Conner and Beuchat [199] reported weak inhibitory activity of celery oil on yeast as well. Nonetheless, other authors [186], [230] have demonstrated antifungal activity of *A. graveolens* oil, and thus additional studies are required to confirm celery antimicrobial activity.

The tested oils showed different levels of antimicrobial activity depending on tested species. *Z. parabailii* evidences higher susceptibility to most of the oils, displaying significant growth reduction for lower concentrations when compared with *S. cerevisiae*. As mentioned previously, susceptibility differences between these two yeast species might be related to different adaptation capacity to a potential antimetabolic effect of a given EO [197]–[199].

A temporary inhibition of *S. cerevisiae* and *Z. parabailii* in relation to contact time and oil concentration was evidenced. Despite the inhibitory effect of the essential oils in the first 24 h of incubation, in some cases yeasts resumed their growth and thus different MIC were obtained considering the 24 h or the 48

h reads, as presented on Table 1. As previously stated regarding HAE assays, yeast growth might be an adaptative response to the EO or a stimulation by some of its constituents. Albeit, the inhibition was definitive at the highest oil concentration after 48 h, with the exception of *Salvia sclarea*, and *Apium graveolens*, for which a MIC value could not be defined. Khalil and Li [231] obtained a similar result with *S. officinalis* EO treatment against different bacteria. Moreover, Behtoei *et al.* [232] reported a fungistatic action of cinnamon bark with 100 µg/mL of EO on some isolates and fungicidal action with 200 µg/mL for all studied isolates.

According to the present study, the ranking of oils by their anti-yeast activity is as follows: *Thymus vulgaris* > *Cymbopogon flexuosus > Cymbopogon citratus* > *Mentha piperita* > *Mentha spicata* > *Mentha aquatica = Mentha pulegium > Coriandrum sativum* > Anethum graveolens > *Salvia officinalis* > *Salvia sclarea > Apium graveolens*. These results suggest that differences in the chemical composition of essential oils might result in different antifungal activities, as different essential oils and/or compounds obtained from different plant species can exhibit different MIC for the same microorganisms [14]. The experiments presented prove that these volatile oils are effective against the microorganisms tested, as most of them prevented yeast growth completely for the concentrations used. This activity was concentration-dependent, with higher concentrations resulting in higher inhibition.

It is difficult to accurately compare results with those reported in the literature because of the naturally varying composition of the plant material even in the same species due to geographical environment, cultivar type, seasonality, time of harvesting, use of different parts of the plant, physiological age of the plant, genetic differences in plant species and the method of obtaining the essential oil and its purification [213], [233]. Moreover, the method used to assess antimicrobial activity and the choice of test organism(s) varies between publications [214], [234]. Differences in microbial growth, exposure of microorganisms to plant oil, the lack of solubility of oil or oil components, the high degree of volatility and the use and quantity of an emulsifier are also variable factors, which may account for the differences in MICs obtained [187], [234], [235].

Overall, it is shown that the plant material (HAE and EO) in the present investigation contain antimicrobial properties. Results show that the most active plant material were the EOs, which might be related to its concentrated nature. Several authors [191], [192], [201], [202] also reported more potent antimicrobial activities after EO treatment than those associated with methanolic, hydroalcoholic or ethanolic extracts, in agreement with our results. Chemical composition should be analysed in order to fully correlate the reported antimicrobial activities with their components. Moreover, *in vivo* application of

EOs may be required to confirm the validity of some of the results obtained and assess the practical applicability as botanical pesticides.

III.2. Cell death induced by *Thymus vulgaris* L. essential oil

Due to its pronounced anti-yeast activity, the effects of the essential oil of *T. vulgaris* (TVEO) were studied in more detail. Rasooli and Owlia [233] have shown that the main target of two thyme oils were the cell wall and cell membrane, as a consequence of its low polarity. Phenolic compounds such as thymol and carvacrol, two major components of TVEO, altered the microbial cell permeability enabling the loss of macromolecules from its interior [236]–[238]. When membranes become permeabilized, it leads to impairment of essential processes in the cell and eventually to cell death by apoptosis and necrosis as a result of activation of intrinsic cell death programs or passive disruption of membrane integrity [35], [239], [240]. Therefore, one of the best markers for cell death is membrane integrity [239]. Cell damage is often associated with the production of reactive oxygen species (ROS) in a wide variety of organisms, including yeast [68], [241], [242]. Mitochondria are cell organelles essential for maintaining cell life and represent a major source of ROS. The accumulation of ROS has a negative effect on cell viability and lead to enzyme inactivation and membrane disruption. Moreover, when the levels of ROS exceed the antioxidant capacity of the cells, homeostasis is disrupted and molecules such as lipids, proteins, and nucleic acids are oxidized, compromising survival [68], [243].

To address the type of cell death induced by TVEO used in this study, a kinetic analysis of cellular markers associated with apoptotic cell death was carried out, which included assessment of plasma membrane integrity and accumulation of ROS. Results are shown on Figure 18.

The assessment of cell viability yielded detailed information about the temporal progression of the antimicrobial activity of TVEO. Figure 18A shows the results of two different concentration of TVEO, 50 and 100 μg/mL, as a function of time. *S. cerevisiae* yeast cells not treated with EO (negative control) presented a significant increase in the counts after 120 min. Percentage of viable cells in treated cultures was statistically different from control at all periods of EO treatment, presenting lower viable cell counts over time.

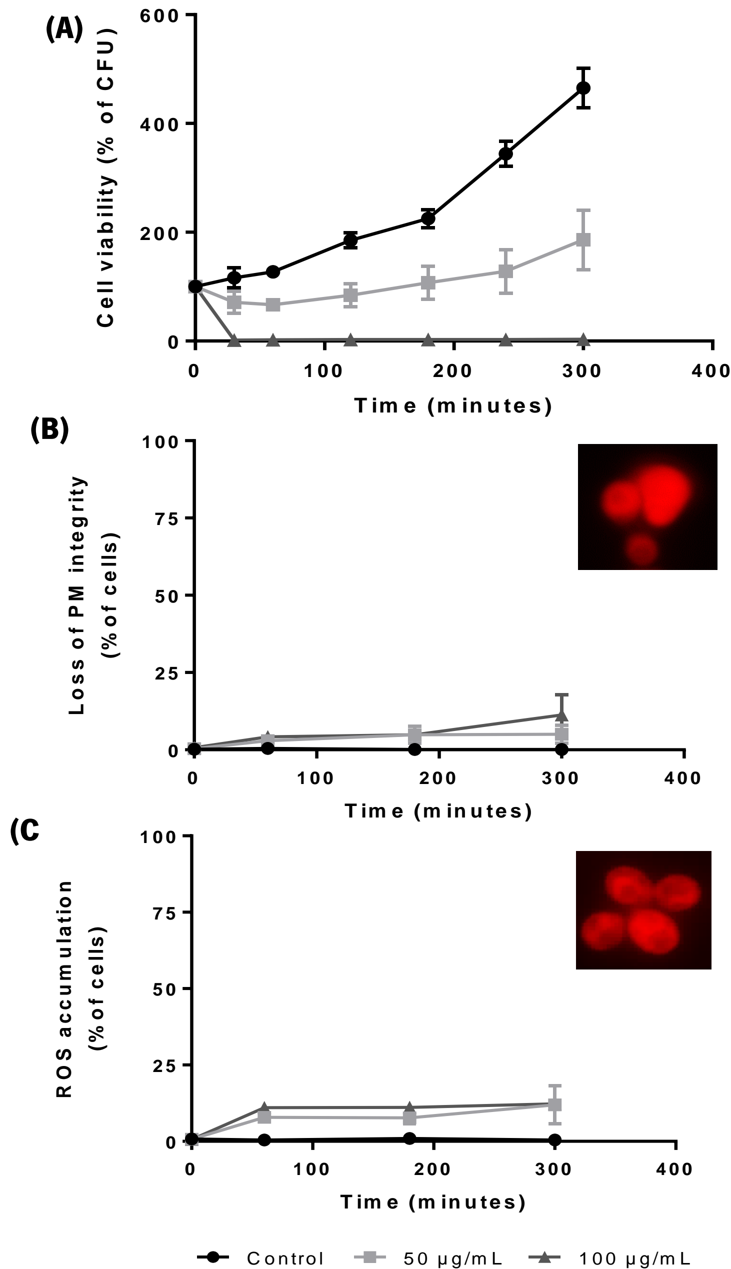


Figure 18 - **Cell death induced by *Thymus vulgaris* L. essential oil.** Kinetic analysis of the cellular events associated with cell death of *Saccharomyces cerevisiae* BY4741 after exposure to 50 and 100 µg/mL of *T. vulgaris* essential oil over a period of 300 min. (a) Cell viability (%) assessed by the number of colony forming units (CFU). (b) Percentage of cells exhibiting loss of plasma membrane integrity assessed by Propidium Iodide (PI) staining. The insert shows a photomicrography of a stained cell, an example of a PI-positive cell. (c) Percentage of cells exhibiting ROS accumulation monitored by MitoTracker Red CM-H2Xros staining. The insert shows a photomicrography of a stained cell, as an example of ROS accumulation. The data represent mean values with standard deviations of at least three independent experiments (n=3).

At 50 µg/mL, there was an initial decrease of the percentage of viable cells after which yeast growth was observed with no significant decrease or increase in the viable cell counts of *S. cerevisiae* in the first 240 min. Nevertheless, there was still a highly significant difference in yeast viability between the negative control and the culture sample exposed to the oil. When TVEO was added at 100 µg/mL, a rapid loss of cell viability was observed, and 30 min of incubation was sufficient to reduce the number of living cells to nearly zero throughout the evaluated interval. Thus, at 100 µg/mL, TVEO exhibited prolonged antimicrobial activity with a higher reduction in CFU counts compared with the starting inocula and control cultures throughout time. Moreover, results show that thyme oil induced drastic cell death at a concentration lower than its respective MIC (500 µg/mL), which can be attributed to different incubation times of the assays [244].

The results obtained here do not show a progressive increase in antifungal effect with increased concentration, but rather a sudden significant reduction in viable counts once a certain (critical) concentration is applied. Juven *et al.* [243] hypothesized that the phenolic active compounds sensitize the membrane and, when saturation of the site(s) of action occurs, there is an evident damage and a sudden collapse of the integrity of the cytoplasmic membrane and leakage of vital intracellular constituents. Rasooli *et al.* [245] have reported complete elimination of *Listeria monocytogenes* within 20 min of exposure to essential oils of *Thymus eriocalyx* and *Thymus x-porlock* in culture broth with 250 µg/mL of oil, confirming the high efficacy of thyme oils.

To investigate the relationship between cell reduction and membrane permeability, a propidium iodide (PI) staining was used. PI cannot permeate intact membranes and so, only penetrates cells showing severe membrane lesions [178]. As shown in Figure 18B, fluorescence due to PI was negligible and did not change in the control suspension of *S. cerevisiae* cells throughout the incubation period. Addition of 50 and 100 ug/mL of TVEO resulted in a higher percentage of fluorescent cells relative to control suspensions, however such increase was uniquely significant, compared with control, after 300 min exposure to the oil ($p<0.05$ and $p<0.0001$, respectively). Similar patterns of PI values were observed for 50 and 100 µg/mL of EO treatment, except at the last time point. At this time point, a significant increase of PI-stained cells was observed for the incubation with 100 µg/mL of EO ($p<0.05$), when compared with the 180 min of treatment, suggesting that dead cells increase over time in a concentration- and time-dependent effect.

Overall, loss of plasma membrane integrity was maintained at reduced levels even when cell viability was almost lost and attained a maximum of 11.26% of the cells, following treatment with 100 μg/mL EO at the last time point. However, because PI only penetrates dead cells showing severe membrane lesions, the concentrations tested might have had caused damage on membrane integrity which were not severe enough to allow PI to enter the cell and, consequently, permeability of the membrane was not detected through the membrane integrity assay. These results indicate that a different mechanism of action might be involved in TVEO at low concentrations, with cell death occurring without any severe membrane damage. Prindle and Wright [246] mentioned that the effect of phenolic compounds, such as thymol and carvacrol, is concentration-dependent. At low concentration, phenols affected enzyme activity, especially of those enzymes associated with energy production, while at greater concentrations, caused protein precipitation.

We further evaluated the accumulation of ROS by *S. cerevisiae* in response to exposure to TVEO through MitoTracker Red CM-H2Xros staining. This fluorescent probe is a specific mitochondrial stain in live cells and its accumulation depends on the membrane potential [180]. However, once incorporated in the mitochondria, the reduced version of this probe is oxidized by ROS to a red fluorescent compound and then sequestered in the mitochondria. The percentage of ROS positive cells observed in TVEO-treated cultures was significantly higher than the non-treated cells as shown in Figure 18C, indicating a high level of ROS generated in *S. cerevisiae* cells upon exposure to TVEO. A significant increase in the percentage of stained cells was observed from the earliest time point, before membrane permeability was significantly increased. Hence, ROS accumulation might be caused by other factors besides damage to the plasma membrane, which on the other hand might be caused by that accumulation.

ROS accumulation was extremely significant ($p<0.0001$) in cells treated with 100 μg/mL throughout time relative to untreated cells and attained a maximum of 12.33% of ROS-positive cells. Levels of ROS between the two concentrations tested were similar at all time points and remained constant until the end of the experiment. In accordance with our results, Ferreira *et al.* [68] reported a significant accumulation of ROS at the earliest time point after exposure to 720 μg/mL of *M. piperita* EO, before any loss of cell viability could be detected, which then remained constant throughout time. Moreover, Hou *et al.* [247] found that carvacrol and thymol can increase cell membrane permeability and cause mitochondrial damaged and ROS accumulation in *Botrytis cinerea*. Accumulation of ROS may result from mitochondrial disruption related to mitochondrial membrane potential, as reported by Tian *et al.* [248]

and Monzote *et al.* [249]. Increased levels of ROS could induce oxidative stress in yeast cells which consequently cause damage to cell membrane, vital proteins, DNA and respiratory system [250].

Collectively, it was demonstrated that TVEO exhibited strong antimicrobial activity against *S. cerevisiae in vitro* by decreasing cell viability (Fig. 18A). Determination of the mechanism of action of essential oils is not easy because the antimicrobial activity is not generally the result of a single mechanism but of a cascade of reactions involving different cell targets such as cell wall degradation, membrane damage, dissipation of the proton motive force, inactivation of essential enzymes, disturbance of genetic material functionality, etc. [238], [251], [252]. In this study, cell death was associated with the production of ROS from the earliest time point (Fig. 18C). However, membrane permeability was not significantly reported, as inferred by reduced number of PI-stained cells, and did not appear to play a major role in decrease of CFUs (Fig. 18B). According to the review of Nazzaro *et al.* (2017), thymol and p-cymene, two major compounds of our TVEO sample, have multiple mechanisms of antifungal action, such as cell membrane disruption, inhibition of cell wall formation, inhibition of efflux pump, changes in mycelium morphology, and production of ROS and nitric oxide [69], whereas γ-terpinene, also present in a significant amount in our sample, is responsible for the protein and lipid leakage in fungi [253]. Some of the mentioned targets require further research to gain more insight into the mechanism underlying the antifungal effect of TVEO and its components.

III.3. Evaluation of the effects of hydroalcoholic extracts on the chronological longevity of yeast

Aging is a complex biological phenomenon involving multiple actors and controlled by a variety of genetic and/or environmental factors, leading to loss of function and increased vulnerability to disease and death [254], [255]. *S. cerevisiae* is one of the most studied model organisms for the identification of genes and mechanisms that affect aging [183], [256].

Hydroalcoholic extracts (HAE) displayed low antimicrobial activity, where significant increase in yeast growth was induced with diverse concentrations of certain plant species extracts. Hence, a chronological lifespan (CLS) study was conducted with two HAE, from *M. piperita* and *S. sclarea*, on *S. cerevisiae*, in order to examine whether extracts would affect chronological longevity – longevity of cells after they stop dividing. Yeast cells were grown in synthetic complete medium and then washed and incubated in water. Incubation in water, which is also referred to as “extreme calorie restriction”, is known to increase yeast

longevity indicating that they have the potential to modulate the length of their life span according to the environment [183]. Supplementation with 2,000 µg/mL HAE during the time of cell cultivation or during caloric restriction, for a prolonged period of time (up to 28 days), was compared so as to access whether a potential protective effect of HAEs could depend on the effects of the extract on the cells during the propagation phase and not on aging. The choice of concentration employed considered the possible *in vivo* applicability, as higher concentrations would be of limited interest.

Results presented in Figure 19 and 20 showed that viability of the cell suspensions was not completely loss during 28 days of aging experiments. Supplementation with *S. sclarea* HAE during cultivation (Figure 20A) was the only condition where a percentage of viable cells lower than the control was reported after 28 days, displaying the lowest percentage of viable cells (34.57%) at that time point. Nevertheless, treated cells show higher percentages of viable cells in comparison to untreated control, indicating a protective effect on yeast viability.

When HAE was supplemented during caloric restriction, *S. sclarea* revealed significant differences along time. This result was not evidenced with *M. piperita*, HAE whose differences between treated and untreated cells were not significant. Howbeit, when the extract was supplemented during cultivation of cells, *M. piperita* had a significant increase on day 8, which was not observed for *S. sclarea*. Together, both results might suggest that the protective effects of these two plant extracts are not due to the same mechanism. Lutchman *et al.* [193] screened 37 plant extracts using yeast and observed that 6 of these extracts induced a dramatic increase in mean CLS, alongside with increased O_2 consumption, mitochondrial membrane potential and low level of ROS in early stages. However, their following functional study [257] with the knockout mutants showed that not all these extracts induced their effect through same mechanisms, in agreement with the present results. Moreover, Georgieva *et al.* [258] studied methanol extracts from the plant *Haberlea rhodopensis* and observed an increase in proliferative potential and a delay in apoptotic processes.

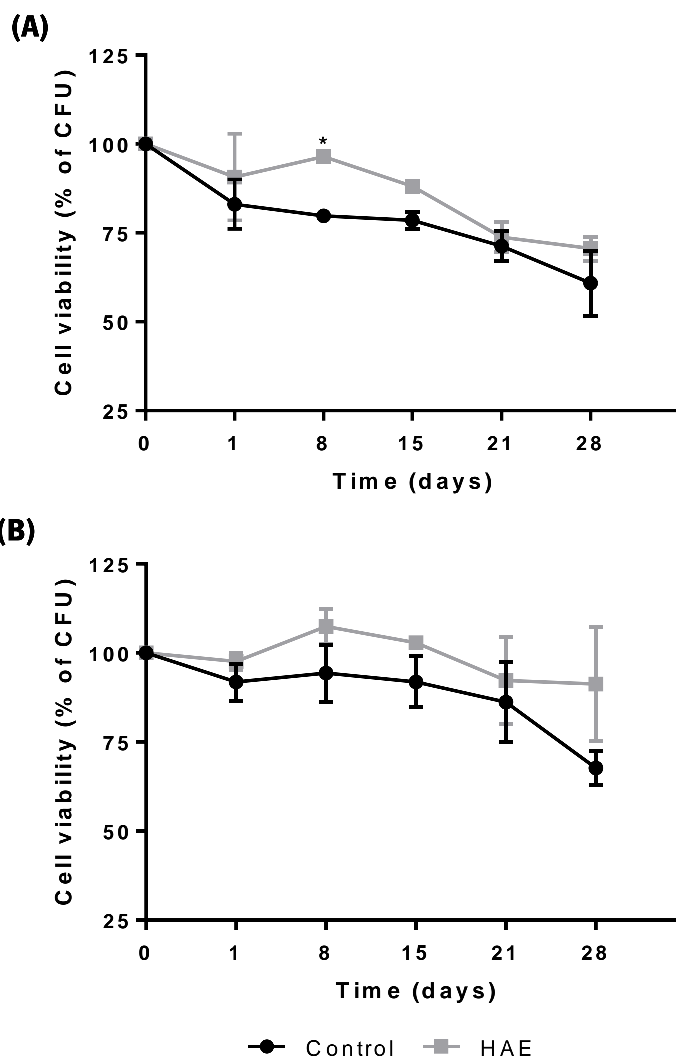


Figure 19 – Effect of *Mentha piperita* L. hydroalcoholic extract (HAE) on the chronological longevity of yeast. Survival (CFU counts, %) of *Saccharomyces cerevisiae* BY4741 incubated under extreme caloric restriction conditions for 28 days. (A) Supplementation of HAE during cultivation, but not aging. (B) Supplementation of HAE during aging. Day zero represents the beginning of aging experiments. The data represent mean values with standard deviations of at least two independent experiments (n=2). * indicates $p \leq 0.05$ compared with control.

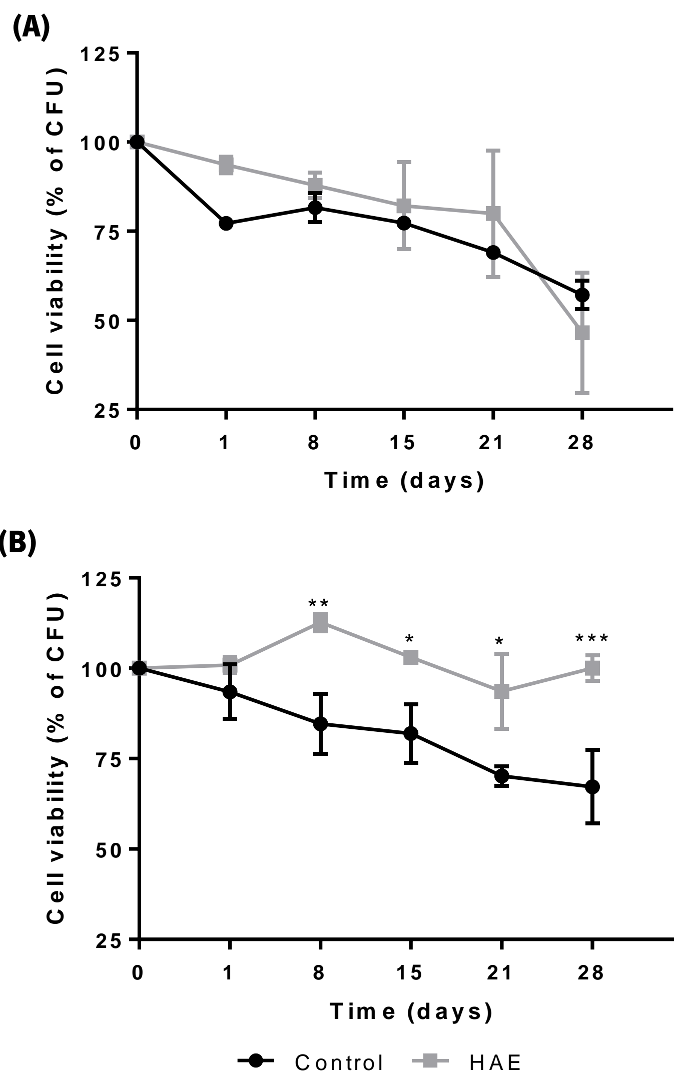


Figure 20 – Effect of *Salvia sclarea* L. hydroalcoholic extract (HAE) on the chronological longevity of yeast. Survival (CFU counts, %) of *Saccharomyces cerevisiae* BY4741 incubated under extreme caloric restriction conditions for 28 days. (A) Supplementation of HAE during cultivation, but not aging. (B) Supplementation of HAE during aging. Day zero represents the beginning of aging experiments. The data represent mean values with standard deviations of at least two independent experiments (n=2). *, ** and *** indicate $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively, compared with control.

The most promising results were observed with *S. sclarea* HAE supplementation during caloric restriction, where prolonged aging was continuously significant for 28 days. Moreover, protective effects appear to be mainly on aging since its supplementation during propagation phase (Figure 20A) did not induce significant differences in cell viability between treated and untreated cells and even caused a significant drop in cell viability after 28 days. Studies have shown that various plant derived chemicals, in

particular polyphenols and their subspecies flavonoids, could have a therapeutic potential for aging and age-related diseases [254], [259]–[261]. Most of these compounds function as antioxidants, which ameliorates one of the most critical aspects in the aging process: loss of mitochondrial function and accumulation of oxidative stress [254], [262]. Therefore, the increased proliferative potential of *S. sclarea* could be explained with the composition of this extract, mainly enriched with antioxidants [263], [264]. Wu *et al.* [265] found that dried roots of *Salvia miltiorrhiza* have significant anti-aging activity due to tanshinones isolated from the plant, which can greatly extend *S. cerevisiae* CLS. A chemical composition analysis would have to be performed to confirm if similar compounds were present in our extract, being responsible for its activity as well.

To our knowledge, this is the first experiment to show the potential properties of *M. piperita* and *S. sclarea* on CLS of yeasts. Nonetheless, additional experiments would have to be performed to confirm these results. Moreover, it would be of particular interest to access how HAE protect cells and whether they can delay the onset and progression of chronic diseases associated with aging. Additionally, the protective effect of HAE could be tested on different pathogens, which could be of interest on agriculture to improve the longevity of biocontrol agents, which are an alternative and sustainable way of protecting plant crops.

IV. CONCLUSIONS

Natural extracts are in increasing demand, not only from the manufacturers of foods, cosmetics, and pharmaceuticals but also from consumers, who have become more concerned about their health and the safety of the products they consume. Hence, the importance of conducting studies on plant extracts and essential oils lies not only in the chemical characterization but also in the study of particular biological properties, which could be potentially useful for pharmaceuticals, nutraceuticals, agriculture and cosmetic applications. Following this idea, this study was taken to investigate antimicrobial properties, which may play a major role in agricultural products, and thus highlight possible application as biological pesticides.

Antimicrobial properties of medicinal plants are being increasingly reported from different parts of the world. Plant extracts contain a very complex mixture of compounds with active ingredients present in the form of natural organic molecules, which can be extracted for further application. In our study, the hydroalcoholic extracts (HAE) of the tested plants did not demonstrate relevant anti-yeast activities. However, further studies using higher concentrations would be required to better evaluate their potential effectiveness as antimicrobial agents. Essential oils (EO) were more effective and had greater inhibitory effect, which might be related to its concentrated nature. In this context, *Thymus vulgaris* EO gave the most interesting results, displaying strong antifungal activity.

Another relevant point of the antimicrobial activity is the mechanism of action. Therefore, *S. cerevisiae* was used as a model organism to test antifungal activity of *T. vulgaris* EO with the specific aim to contribute to the elucidation of its mode of action. Generally, terpenes, particularly monoterpenes such as thymol and carvacrol, have hydrophobic properties that allows them to deposit on the lipophilic structures of membranes and increase permeability. However, other action mechanisms may be involved. In the present study, the yeast cell death induction effect of thyme EO at low concentrations was not related to its ability to disrupt the integrity of the plasma membrane. Nevertheless, thyme EO significantly increased ROS accumulation of *S. cerevisiae in vitro*, which might be indirectly related to cell death. These results provide an increase in understanding of the antifungal effects of thyme EO on an industrially important yeast.

Furthermore, certain chemical compounds of plant origin can delay the chronological aging and extend longevity of *S. cerevisiae*, such as resveratrol, caffeine, quercetin, curcumin, spermidine, methionine sulfoxide and cryptotanshinone [254], [260]–[262], [265], [266]. In search of protective effects of HAE on yeast lifespan, we have screened two plant extracts and observed that these extracts were able to

decrease loss of cell viability over a period of 28 days. Our results add valuable insights on the effects of natural compounds on ageing. Although compounds with anti-aging activity might not be of particular interest in the development of antimicrobial compounds, they could increase the overall quality of life by extending the healthy lifespan while delaying the onset of aging-associated diseases, such as cardiovascular disease, cancer, osteoporosis, diabetes, hypertension, and Alzheimer's disease. Yeast viability and vitality are also essential for different industrial processes, particularly to produce alcoholic beverages, where the yeast *S. cerevisiae* is used as a biotechnological tool [267], [268]. Nevertheless, the anti-aging effects of HAE should be confirmed with additional experiments.

Overall, the present results further confirmed the possibility of using plant material as antifungal agents to control spoilage. Due to yeast ability to produce substantial crop losses and generate food waste, these results are of particular importance. Our data form the basis for selection of plant species for future investigation in the potential discovery of new natural bioactive compounds. However, additional research is required to clearly define the conditions under which plant extracts and essential oils can be applied and studies *in vivo* are required to identify the effective dose of the extract or oil on the microorganisms and better understand their mode of action, safety, efficacy and properties. Furthermore, biosafety studies need to be conducted to ascertain their toxicity to humans, animals, and crop plants.

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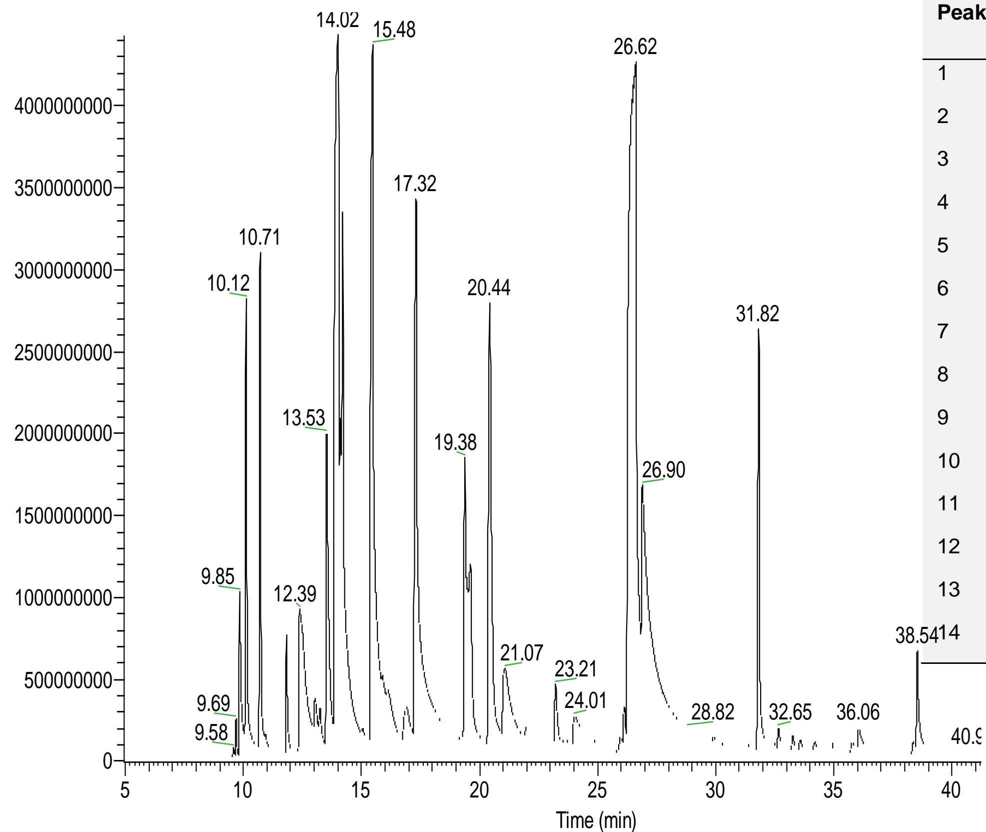
VI. APPENDIX

Appendix A - Analysis of *Thymus vulgaris* essential oil. Results of the qualitative and quantitative analysis of *T.vulgaris* essential oil performed by GC-MS and GC-FID by Rose Marie Sousa (Biology Dpt, Faculty of Sciences, University of Porto) within the scope of the EOIS-CropProt project.

GC-MS equipment: Thermo Scientific TraceTM 1300 GC coupled to a Single Quadrupole ISQ MS equipment and automatic injector. Operating conditions were as follow: injection and detector temperature, of 300 and 300°C respectively; split ratio of 1:13; Helium as a carrier gas with constant flow rate of 1.5 mL min^{-1}. Oven temperature program was 60 to 300°C achieved at a first rate of 3°C/min from 60 to 200 °C, and then at a rate of 8°C/min up to 300 °C, TG5-MS capillary column (60 m x 0.25 mm; 0.25 µm film thickness). Mass spectrometer conditions were: ionization potential of 70 eV; detection of mass m/z ranging from 40 to 400 m/z. Data acquisition was performed in positive mode (40-400 m/z). Partial identification of major compounds based on mass spectral analysis and comparison with reference mass spectra library (NIST; Mainlib; TR). RT-Retention time.

EOIS-CropProt project (POCI-01-0145-FEDER-031131)



Peak	RT	Compound	Relative content (%)
1	9.85	α-Thujene	0.64
2	10.12	α-Pinene	1.12
3	10.71	Camphene	1.28
4	12.39	β-Myrcene	2.28
5	13.53	α-terpinene	1.89
6	14.02	*O*-Cymene	17.32
7	14.21	1,8-Cineole	0.2
8	15.48	γ-Terpinene	11.7
9	17.32	Linalool	4.46
10	19.38	Camphor	1.31
11	20.44	Borneol	1.18
12	26.62	Thymol	45.85
13	26.9	Carvacrol	3.22
14	31.82	Caryophyllene	2.62