Biological properties of aromatic plants used in folk medicine: a focus on the essential oil of *Thymus zygis*

Alexandra Teixeira Coimbra

Tese para obtenção do Grau de Doutor em
Biomedicina
(3º ciclo de estudos)

Orientadora: Professora Doutora Ana Paula Coelho Duarte
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Covilhã, agosto de 2023

UNIVERSIDADE BEIRA INTERIOR

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Covilhã, 21 de julho de 2023

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Dedicatory

Aos meus queridos pais.

“The most wasted day of a life is the day that we don't laugh”

- Charles Chaplin

“I am among those who think that science has great beauty”

- Marie Currie

“It always seems impossible until it’s done”

- Nelson Mandela

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List of Publications

Publications included in the thesis resulting from this Doctoral work

I. **Coimbra, A.**, Ferreira, S., Duarte, A.P., 2022. Biological properties of *Thymus zygis* essential oil with emphasis on antimicrobial activity and food application. Food Chemistry. 393, 133370. https://doi.org/10.1016/j.foodchem.2022.133370

II. **Coimbra, A.**, Miguel, S., Ribeiro, M., Coutinho, P., Silva, L., Ferreira, S., Duarte, A.P. 2022 Chemical composition, antioxidant, and antimicrobial activities of six commercial essential oils. Letters in Applied Microbiology. 76, 1–8. https://doi.org/10.1093/lambio/ovac042

III. **Coimbra, A.**, Miguel, S., Ribeiro, M., Coutinho, P., Silva, L., Duarte, A.P., Ferreira, S., 2022. *Thymus zygis* essential oil: Phytochemical characterization, bioactivity evaluation and synergistic effect with antibiotics against *Staphylococcus aureus*. Antibiotics. 11, 146. https://doi.org/10.3390/antibiotics11020146

IV. **Coimbra, A.**, Carvalho, F., Duarte, A.P., Ferreira, S., 2022. Antimicrobial activity of *Thymus zygis* essential oil against *Listeria monocytogenes* and its application as food preservative. Innovative Food Science and Emerging Technologies. 80, 103077. https://doi.org/10.1016/j.ifset.2022.103077

V. **Coimbra, A.T.**, Ferreira, S., Duarte, A.P., 2020. Genus *Ruta*: A natural source of high value products with biological and pharmacological properties. Journal of Ethnopharmacology. 260, 113076. https://doi.org/10.1016/j.jep.2020.113076

Publications not related to the thesis

I. **Coimbra, A.T.**, Luís, Â.F.S., Batista, M.T., Ferreira, S.M.P., Duarte, A.P.C., 2020. Phytochemical characterization, bioactivities evaluation and synergistic effect of *Arbutus unedo* and *Crataegus monogyna* extracts with amphotericin B. Current Microbiology. 77, 2143–2154. https://doi.org/10.1007/s00284-020-02125-w

II. Carvalho, F., **Coimbra, A.T.**, Silva, L., Duarte, A.P., Ferreira, S., 2023. *Melissa officinalis* essential oil as an antimicrobial agent against *Listeria monocytogenes* in watermelon juice. Food Microbiology. 109, 104105. https://doi.org/10.1016/j.fm.2022.104105

List of Scientific communications

Oral scientific communications of this Doctoral work

I. **Alexandra Coimbra**, Filomena Carvalho, Ana P. Duarte, Susana Ferreira. Atividade antimicrobiana do óleo essencial de *Thymus zygis* na conservação de alimentos - "O meu trabalho… 1 Slide", within the scope of the International Day of the Microorganism organized by the Health Sciences Research Center, University of Beira Interior, Covilhã, Portugal, 17th September 2021.

II. **Alexandra Coimbra**, Filomena Carvalho, Ana P. Duarte, Susana Ferreira. Antimicrobial activity of *Thymus zygis* essential oil and its application for food decontamination – XVI Annual CICS-UBI Symposium, Covilhã, Portugal, 30th September and 1st October 2021.

Poster presentations of this Doctoral work

I. **Alexandra Coimbra**, Susana Ferreira, Ana Paula Duarte. Screening of the bioactive activities of various plant essential oils used in the Mediterranean diet – Microbiotec'19 Congress of Microbiology and Biotechnology, Coimbra, Portugal, 5-7 December 2019.

II. **Alexandra Coimbra**, Susana Ferreira, Ana Paula Duarte. Antimicrobial activity of *Thymus zygis* essential oil against *Listeria monocytogenes* – Antimicrobial Chemotherapy Virtual Conference 2021 organized by the British Society for Antimicrobial Chemotherapy, online format, 2-3 February 2021.

III. **Alexandra Coimbra**, Filomena Carvalho, Ana Paula Duarte, Susana Ferreira. Effect of *Thymus zygis* essential oil against *Listeria monocytogenes* and its application on food – Microbiotec'21 Congress of Microbiology and Biotechnology, online format, 23-26 November 2021.

Other oral scientific communications

I. **Alexandra Coimbra**, Ângelo Luís, Maria T. Batista, Susana Ferreira, Ana Paula Duarte. Caracterização fitoquímica e avaliação das propriedades bioativas das plantas *Arbutus unedo* e *Crataegus monogyna* – IV Jornadas de Educação e Investigação em Saúde, Guarda, Portugal, 12 de dezembro de 2019.

II. Filomena Carvalho, **Alexandra Coimbra**, Ana P. Duarte, Susana Ferreira. Antimicrobial activity of *Melissa officinalis* essential oil against *Listeria monocytogenes* and application in food models – XVI Annual CICS-UBI Symposium, Covilhã, Portugal, 30th September and 1st October 2021.

Other poster presentations

I. **Coimbra A.**, Luís Â., Ferreira S., Duarte A.P. Phytochemical characterization and evaluation of antimicrobial properties of *Arbutus unedo* and *Crataegus monogyna* – VI ENEQUI, Encontro Nacional de estudantes de química, Covilhã, Portugal, 23 a 26 de março de 2018.

Resumo Alargado

Durante séculos, as plantas têm sido utilizadas numa ampla variedade de aplicações, tais como o tratamento de doenças, aromatização e conservação de alimentos e perfumaria. Os óleos essenciais são substâncias voláteis aromáticas, presentes em diferentes partes das plantas, como frutas, sementes, polpa, casca ou raiz e que têm sido usados como agentes terapêuticos desde a antiguidade. De facto, estes possuem uma ampla gama de atividades biológicas, podendo ser aplicados em diferentes áreas, como química, cosmética, indústria alimentar, perfumaria e farmacêutica. O *Thymus zygis* é uma planta amplamente distribuída, utilizada principalmente como aromatizante culinário e o seu óleo essencial tem demonstrado propriedades bioativas, como atividade antioxidante, antibacteriana, inseticida e antiparasitária. Além disso, a potencial aplicação como conservante de alimentos tem sido descrita em diferentes matrizes alimentares, principalmente devido à sua atividade antimicrobiana contra microrganismos patogénicos e deteriorantes de alimentos.

O aumento da resistência dos microrganismos aos antibióticos e o surgimento de novas doenças, exigem o desenvolvimento urgente de novos agentes antimicrobianos mais fortes e eficazes. Assim, as plantas medicinais são promissoras, pois oferecem fontes únicas e renováveis de novos compostos com propriedades bioativas.

Considerando as diferentes aplicações dos óleos essenciais, este trabalho teve como objetivos estudar a composição, propriedades antioxidantes, antimicrobianas e também a citotoxicidade dos óleos essenciais de *Foeniculum vulgare*, *Helichrysum stoechas*, *Mentha pulegium*, *Pinus pinaster*, *Ruta graveolens* e *Thymus mastichina* e avaliar a composição química e as propriedades bioativas do óleo essencial de *T. zygis* com foco na atividade antimicrobiana contra *Staphylococcus aureus* bem como o seu efeito anti-*Listeria monocytogenes* e aplicação em alimentos como conservante.

Através da cromatografia gasosa acoplada à espectrometria de massa (GC-MS) verificou-se que os óleos essenciais possuem composição diferente, identificando-se diversos compostos no óleo essencial de *F. vulgare* (12 compostos), *H. stoechas* (27), *M. pulegium* (8), *P. pinaster* (24), *R. graveolens* (8), *T. mastichina* (16) e por fim no EO de *T. zygis* 18 compostos com o anetol, acetato de neril, pulegona, α-pineno, 2-undecanona, 1,8-cineol e timol como compostos maioritários, respetivamente. Apenas os óleos essenciais de *H. stoechas, M. pulegium* e *T. zygis* sequestraram os radicais livres de DPPH exibindo uma atividade antioxidante muito forte, enquanto todos os óleos essenciais apresentaram atividade antioxidante atuando por meio da inibição da peroxidação lipídica de acordo

com o ensaio do β-caroteno. Assim, verificou-se que os óleos essenciais de *H. stoechas, M. pulegium* e *T. zygis* possuem atividade antioxidante através de pelo menos dois mecanismos de ação distintos, a sequestração de radicais livres e inibição da peroxidação lipídica. Os óleos essenciais de *M. pulegium* e *T. zygis* exibiram atividade antimicrobiana mais relevante com concentração mínima inibitória (MIC, do inglês *minimum inhibitory concentration*) entre os valores de 0,06 e 4 μL/mL, exceto para *Pseudomonas aeruginosa,* que foi a espécie mais resistente. As espécies de *Candida* foram as mais suscetíveis a estes óleos essenciais (halos de inibição entre 30,94 ± 5,34 e 64,35 ± 4,44 mm) e apenas os compostos voláteis destes dois óleos essenciais, libertados do disco durante a incubação, demonstraram atividade inibitória (halos de inibição entre 19,28 ± 3,45 e 65,18 ± 8.78 mm). Por outro lado, os óleos essenciais de *H. stoechas* e *R. graveolens* revelaram a atividade antimicrobiana mais fraca (halos de inibição entre 6,00 ± 0,00 e 11,82 ± 1,07 mm e MIC entre 16 e >256 μL/mL). Em relação à citotoxicidade dos compostos, esta foi avaliada considerando o seu efeito sobre uma linha celular de fibroblastos (NHDF), sendo que o efeito foi diretamente proporcional à concentração de óleos essenciais, obtendo-se maior atividade citotóxica com o óleo essencial de *R. graveolens*. Pelo contrário, com o óleo essencial de *T. zygis* com concentrações entre 0,0030 e 0,0125%, obteve-se viabilidade superior a 70% e tendo em conta a ISO 10993:5-2009, pode-se considerar que estas concentrações não são citotóxicas.

Tendo em atenção o potencial antimicrobiano do óleo essencial de *T. zygis* e uma vez que mostrou maior atividade que os restantes óleos essenciais, decidiu-se avançar com estudos com este óleo essencial, aprofundando assim as suas atividades bioativas, principalmente a atividade antibacteriana contra *S. aureus* e *L. monocytogenes*.

Considerando que *S. aureus* é uma bactéria Gram-positiva que causa vários tipos de infeções clínicas, e que em alguns casos pode estar associada a elevada morbilidade e mortalidade, as quais podem ser agravadas pela resistência a antibióticos, a atividade antibacteriana do óleo essencial de *T. zygis* foi avaliada contra estirpes desta espécie. O óleo essencial revelou atividade antimicrobiana contra estirpes de *S. aureus* com MIC de 0,05 e 0,1% (v/v) apresentando efeito bactericida. Para além disso, os compostos voláteis do óleo essencial de *T. zygis* também inibiram o crescimento destas estirpes. Relativamente ao efeito combinado com antibióticos, o óleo essencial de *T. zygis* demonstrou um efeito sinérgico em combinação com os antibióticos ampicilina e ciprofloxacina contra a estirpe *S. aureus* MRSA 12/08 e efeito aditivo com a vancomicina. Em relação às estirpes *S. aureus* ATCC 25923 e SA 03/10, a combinação do óleo essencial de *T. zygis* com os três antibióticos revelou efeito aditivo. Em alguns casos, a junção do óleo essencial de *T. zygis* alterou o fenótipo de resistente para suscetível ao antibiótico, de

acordo com os valores de corte do Clinical and Laboratory Standards Institute, como se pode verificar para a estirpe SA 03/10 aos três antibióticos testados e para a estirpe MRSA 12/08 à ampicilina e ciprofloxacina em que os valores de MIC reduziram consideravelmente restaurando a sensibilidade a estes. Adicionalmente, o óleo essencial de *T. zygis* também inibiu a formação de biofilmes formados pelas estirpes de *S. aureus* e eliminou parcialmente os biofilmes preformados mesmo em concentrações subinibitórias. A pré-exposição de *S. aureus* a concentrações subinibitórias do óleo essencial de *T. zygis* reduziu significativamente a atividade hemolítica de *S. aureus* SA 03/10 (única estirpe em estudo que provocou hemólise de eritrócitos) em comparação com os respetivos controlos, de forma dose-dependente. A comunicação entre células permite o controlo da regulação e libertação de fatores de virulência por parte das bactérias e utilizando *Chromobacterium violaceum* como um biossensor para avaliar o potencial do óleo essencial como inibidor deste processo, os resultados mostraram que concentrações subinibitórias do óleo essencial de *T. zygis* inibiram significativamente a produção do pigmento violaceína sem afetar o crescimento deste biossensor. Assim, verificou-se que este óleo essencial também reduz esse processo de regulação de virulência. Resumindo, as concentrações testadas de óleo essencial de *T. zygis* reduzem significativamente o crescimento de *S. aureus*, tanto de células planctónicas como de biofilmes, potenciam o efeito de antibióticos e inibem a capacidade hemolítica e a comunicação entre células. Portanto, esses resultados demonstram promissoras propriedades bioativas do óleo essencial de *T. zygis*, revelando o seu potencial para ser utilizado como agente antibacteriano e/ou potenciador do efeito de antibióticos.

Tendo em conta que *L. monocytogenes* é um dos agentes bacterianos causadores de doenças transmitidas por alimentos com mais elevada taxa de mortalidade e possui a capacidade de sobreviver e de se replicar em condições adversas vulgarmente associadas ao sector alimentar permitindo a sua ampla distribuição em diferentes ambientes e matrizes alimentares, foi avaliada a atividade antimicrobiana do óleo essencial de *T. zygis* contra estirpes desta bactéria. O óleo essencial de *T. zygis* apresentou também atividade antibacteriana contra *L. monocytogenes* com halos de inibição entre 41,55 ± 2,63 e 55,04 ± 3,64 mm, tal como os seus compostos voláteis (halos de inibição entre 36,13 ± 3,41 e 50,43 ± 2,59 mm), com um MIC de 0,05%, apresentando também efeito bactericida. Concentrações subinibitórias do óleo essencial reduziram significativamente a motilidade das três estirpes em estudo, reduzindo também a formação de biofilme com percentagens de inibição entre 16,85 e 89,86%, mesmo com concentrações subinibitórias. Uma pré-exposição de *L. monocytogenes* ao óleo essencial de *T. zygis*, apenas aumentou a robustez de *L. monocytogenes* quando sujeita a stress osmótico, não afetando a sua tolerância a outros fatores de stress, tais como a dessecação, pH ácido e elevadas temperaturas. Para

além disso, a pré-exposição a óleo essencial de *T. zygis* não induziu resistência cruzada a antibióticos de diferentes classes, como ampicilina, cefotaxima, eritromicina, gentamicina, tetraciclina e vancomicina. Como a capacidade de invasão desta bactéria permite a passagem através das células epiteliais do intestino, provocando infeções generalizadas no hospedeiro, o óleo essencial de *T. zygis* foi testado em relação à capacidade de invasão em células de adenocarcinoma colorretal humano (Caco-2). A incubação com concentrações subinibitórias do óleo essencial de *T. zygis* não afetou o poder de invasão de *L. monocytogenes,* sendo assim considerado um fator neutro. Uma vez que o uso de compostos antimicrobianos sintéticos como conservantes de alimentos tem despertado a preocupação dos consumidores e tendo em atenção a atividade antimicrobiana obtida para o óleo essencial de *T. zygis*, avaliou-se o uso deste óleo como um conservante e desinfetante de alimentos. Verificou-se que as duas concentrações mais elevadas do óleo essencial de *T. zygis* (2× e 1× MIC) reduziram significativamente as contagens de *L. monocytogenes* (inóculo inicial de ~10^6 UFC/mL) num modelo de suco de frango (1,53 log UFC/mL) e no modelo de alface Iceberg (abaixo do limite de deteção), após dois dias de armazenamento em refrigeração. Em relação às amostras de leite (leite magro e leite gordo), a diferença no conteúdo lipídico influenciou a atividade antibacteriana do óleo essencial de *T. zygis*, sendo que apenas se obteve uma redução significativa das contagens bacterianas com a concentração mais elevada e no leite magro. Para concentrações subinibitórias do óleo essencial de *T. zygis*, apenas a concentração 0,5× MIC mostrou uma redução significativa e apenas no modelo de alface, a partir do quarto dia de armazenamento. Em relação à desinfeção de vegetais frescos, o uso de 0,2% (v/v) de óleo essencial por 5 min de imersão, reduz a contagem de *L. monocytogenes* e da microbiota natural para valores abaixo do limite de deteção do método na lavagem das folhas de alface. Nas folhas de espinafre, as contagens de *L. monocytogenes* e da microbiota natural foram reduzidas para 4,35 log UFC/mL e no intervalo de 4,47 a 5,94 log UFC/mL, respetivamente, quando comparadas com a lavagem apenas com água. Assim, o óleo essencial de *T. zygis* demonstrou atividade antibacteriana promissora contra *L. monocytogenes* e estes resultados apontam para o potencial uso do óleo essencial de *T. zygis* como conservante ou desinfetante natural de alimentos para o controlo de *L. monocytogenes* e da microbiota natural em produtos alimentares.

No geral, o presente estudo revelou propriedades bioativas significativas de diferentes óleos essenciais, destacando o óleo essencial de *T. zygis,* a ser considerado em estudos futuros, devido às suas propriedades antioxidantes e antimicrobianas, bem como no controle e redução da patogenicidade de *S. aureus* e *L. monocytogenes*.

Palavras-chave

Óleos essenciais; GC-MS; atividade antioxidante; atividade antimicrobiana; citotoxicidade; *Thymus zygis*; *Staphylococcus aureus*; *Listeria monocytogenes*; atenuação da virulência; interação com antibióticos; desinfetante; vegetais

Abstract

For centuries, plants have been used for a wide variety of purposes, such as treating diseases, food flavouring and preservation and perfumery. The essential oils (EOs) are aromatic, oil-like volatile substances present in different plant materials that have a wide range of biological activities and can be used in different areas, such as chemical, cosmetic, food, perfumery, and pharmaceutical industries and have been used as therapeutic agents since ancient times. *Thymus zygis* is a widespread plant, mainly used as a culinary flavouring agent, which EO has demonstrated bioactive properties, such as antioxidant and antimicrobial, and that may even enhance the effect of certain antimicrobial agents. Furthermore, the potential application as a food preservative has been described on different matrixes mainly due to its antimicrobial activity against pathogenic and spoilage microorganisms in food.

Considering the different applications of EOs, this work aimed to study the composition, antioxidant, and antimicrobial properties and also the cytotoxicity of the EOs of *Foeniculum vulgare*, *Helichrysum stoechas*, *Mentha pulegium*, *Pinus pinaster*, *Ruta graveolens*, and *Thymus mastichina*. The chemical composition, and the bioactive properties of the *T. zygis* EO were also evaluated with a focus on antimicrobial activity against *Staphylococcus aureus* and *Listeria monocytogenes*, and application of the EO in food.

The *F. vulgare*, *H. stoechas*, *M. pulegium*, *P. pinaster*, *R. graveolens*, *T. mastichina* and *T. zygis* EOs showed antioxidant activity acting through inhibition of lipid peroxidation, while only the *H. stoechas*, *M. pulegium* and *T. zygis* EOs scavenged the free radicals of DPPH. *M. pulegium* and *T. zygis* EOs showed the strongest antimicrobial activity and only volatiles compounds from these EOs demonstrated inhibitory activity. Regarding the EO cytotoxicity on a fibroblasts cell line, it was observed that the effect was directly proportional to the EOs concentration, in which the highest cytotoxic effect was obtained with the *R. graveolens* EO.

Considering the antimicrobial potential of the EO from *T. zygis* and since it showed the greater activity among the tested EOs, it was decided to proceed with further evaluation of this EO, thus deepening its bioactive activities, mainly the antibacterial activity against *S. aureus* and *L. monocytogenes*.

Taking into account that *S. aureus* is a Gram-positive bacterium that causes a wide variety of clinical infections, from less severe to serious and life-threatening infections and

because these are aggravated by antibiotic resistance, the antibacterial activity of *T. zygis* EO against strains of *S. aureus* was evaluated. The *T. zygis* EO demonstrated antimicrobial activity against *S. aureus* strains with bactericidal effect. The EO of *T. zygis* also revealed a synergistic or additive effect in combination with the antibiotic's ampicillin, ciprofloxacin, or vancomycin against *S. aureus* strains and, in some cases, changed the antibiotic-resistance phenotype from resistant to susceptible. The *T. zygis* EO inhibit the formation of biofilms by *S. aureus* and partially eliminate the preformed biofilms even at subinhibitory concentrations. The EO reduced the haemolytic activity of *S. aureus* SA 03/10 (the only strain tested that causes haemolysis) as well the quorum-sensing in *Chromobacterium violaceum* biosensor. Therefore, these results demonstrate the good bioactive properties of the *T. zygis* EO, mainly the antimicrobial activity against *S. aureus*, revealing its potential to be used as an antibacterial agent and/or as an enhancer of the effect of antibiotics.

Since *L. monocytogenes* is a foodborne Gram-positive bacterium with a high mortality rate and has the ability to survive and replicate in adverse conditions, allowing the wide distribution of this bacterium in different environments and matrices, such as water, soil, and food products, the antimicrobial activity of *T. zygis* EO against strains of *L. monocytogenes* was evaluated. The *T. zygis* EO presented good antibacterial activity against *L. monocytogenes* with MIC value of 0.05% while showing a bactericidal effect. The EO significantly reduced the biofilm formation with inhibition percentages from 16.85 to 89.86% and also the motility, while not inducing cross-resistance to antibiotics. The concentration of 2× MIC of *T. zygis* EO reduced the *L. monocytogenes* counts (initial inoculum of ~10^6 CFU/mL) in the chicken juice (1.53 log CFU/mL) and the lettuce model (to below the detection limit) after two days of storage. Regarding the sanitizing of fresh vegetables, the use of 0.2% (v/v) of EO for 5 min of immersion, reduce *L. monocytogenes* and natural microbiota counts for values below the detection limit of the method for iceberg lettuce. For the spinach leaves, *L. monocytogenes* and the natural microbiota counts were reduced to 4.35 log CFU/mL and in a range of 4.47 to 5.94 log CFU/mL, respectively, when compared with the washing with water. Thus, the *T. zygis* EO has demonstrated promising antibacterial activity against *L. monocytogenes* and these results point to the potential use of the *T. zygis* EO as a natural food preservative or sanitiser for controlling *L. monocytogenes* and the natural microbiota in food products.

Overall, the present study revealed significant bioactive properties of different EOs, highlighting *T. zygis* EO to be considered in further studies for potential use in food preservation, due to their antioxidant and antimicrobial properties as well as in the control and reduction of the pathogenicity of the *L. monocytogenes* and *S. aureus*.

Keywords

Essential oils; GC-MS; antioxidant activity; antimicrobial activity; cytotoxicity; *Thymus zygis*; *Staphylococcus aureus*; *Listeria monocytogenes*; virulence attenuation; interaction with antibiotics; sanitizer; vegetables

Thesis Overview

The research work that comprises this thesis was performed at the CICS-UBI, Health Sciences Research Centre from the Faculty of Health Sciences of the University of Beira Interior.

This thesis is structured in three main parts. The first part includes three chapters, chapter 1 with a general introduction consisting of a review of the literature regarding the essential oils of the plants under study, considering their geographical distribution, botanical characteristics, folk medicine use, phytochemistry and bioactive properties. The chapter 2 is a published review about the essential oil of *Thymus zygis,* the plant that was the main focus of this thesis, which complements the information in the Introduction chapter. Chapter 3 concerns to the aims of this thesis. The second part comprises three chapters corresponding to one accepted manuscript (chapter 4) and two original publications (chapter 5 and 6), that describe the experimental results obtained in this work. The last part, chapter 7, summarizes the main conclusions obtained in this work and refers to the future perspectives for further research in this field. In the annex, it is presented another published review on the genus *Ruta,* one of its species was studied in this thesis, where it can be found information about its main characteristics, traditional medicine, composition, and biological and pharmacological properties.

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List of Abbreviations and Acronyms

AA	Antioxidant activity
AAI	Antioxidant Activity Index
AAPH	2,2-Azobis (2-methylpropionamidine) dihydrochloride
ABTS	2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
AChE	Acetylcholinesterase
AD	Alzheimer's disease
AgNPs	Silver nanoparticles
AMP B	Amphotericin B
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BCCM	Belgian Coordinated Collections of Microorganisms
BChE	Butyrylcholinesterase
BHA	Butylated hydroxyanisole
BHI	Brain Heart Infusion
BHIA	Brain Heart Infusion agar
BHT	Butylated hydroxytoluene
BM	Buffered methanol
CFU	Colony-forming units
CNS	Central nervous system
COX	Cyclooxygenase
CRNPs	Rutin-loaded chitosan nanoparticles
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DW	Dry weight
EC_{50}	Half maximal effective concentration
EDTA	Ethylenediamine tetraacetic acid
EO	Essential oil
ESKAPE	*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.
FBS	Fetal Bovine Serum

FDA	U.S. Food and Drug Administration
FIC	Fractional inhibitory concentration
FICI	Fractional inhibitory concentration index
FV	*Foeniculum vulgare*
GAE	Gallic acid equivalents
GC-MS	Gas Chromatography coupled to Mass Spectrometry
GRAS	Generally recognized as safe
HCV	Hepatitis C Virus
HIV-1	Human Immunodeficiency Virus type 1
HS	*Helichrysum stoechas*
IC_{50}	Half maximal inhibitory concentration
IL-8	Interleukin-8
ISO	International Organization of Standardization
KI	Kovats index
LB	Luria-Bertani broth
LPS	Lipopolysaccharide
MA	Massachusetts
MAO	Monoamine oxidase
MHA	Müeller-Hinton agar
MHB	Müeller-Hinton broth
MIC	Minimum inhibitory concentration
MOPS	3-(N-morpholino)propanesulfonic acid
MP	*Mentha pulegium*
MRSA	Methicillin-resistant *Staphylococcus aureus*
MS	Mass spectrometer
MSD	Mass spectrometer detector
MTT	4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NA	No activity
ND	Not described
NF	Not found
NIST	National Institute of Standards and Technology
NO	Nitric oxide
NS	Not specified
OD	Optical density
PALCAM	Polymyxin acriflavine lithium chloride ceftazidime aesculin mannitol agar
PBS	Phosphate-buffered saline

PCA	Plate count agar
PCLNFs	Polycaprolactone nanofibers
PP	*Pinus pinaster*
PTZ	Pentylenetetrazole
RE	Rutin equivalents
RG	*Ruta graveolens*
RNA	Ribonucleic acid
ROS	Reactive oxygen species
Rpm	Rotations per minute
RPMI	Roswell Park Memorial Institute 1640
SDA	Sabouraud Dextrose Agar
SEM	Scanning Electron Microscopy
STZ	Streptozotocin
TAP	Triple antibiotic past (Metronidazole, Ciprofloxacin and Minocycline, ratio 1:1:1)
TBARS	Thiobarbituric acid reactive substances
TBHQ	Tertiary Butyl Hydroquinone
TET	Tetracycline
TM	*Thymus mastichina*
TSA	Tryptic soy agar
TSB	Tryptic soy broth
UFC	Unidades formadoras de colónias
UHT	Ultra-high-temperature
USA	United States of America
UV	Ultraviolet
VRBGA	Violet red bile glucose agar
WHO	World Health Organization
YCGA	Yeast extract glucose chloramphenicol agar

Chapter 1

Introduction

1. Introduction

Plants have been traditionally used in the healing processes of numerous diseases, with many of them presenting scientifically confirmed beneficial effects, thus diligent research in this matter should be imperative (Burlacu and Tanase, 2021). There are a wide range of plants and new molecules from plants that have been observed in countless studies and have many applications in different fields: (i) in pharmaceutical industries due to its therapeutic potential in the treatment of cancer (Burlacu and Tanase, 2021; Cheng et al., 2016; Davoodvandi et al., 2020; Karthika and Sureshkumar, 2021; Talib et al., 2022), respiratory diseases (Mitra et al., 2021), neurodegenerative diseases (Hassan et al., 2022; Naoi et al., 2022), in management of glycaemic response (Singh et al., 2022), and others, but also due its antimicrobial activity (Devillers and Devillers, 2019; Hochma et al., 2021) and use as drug delivery systems (Babich et al., 2020; Gong et al., 2022); (ii) in food industry as natural pigments, preservatives, additives among other uses (Azmin et al., 2022; Baindara and Mandal, 2022; Sharma et al., 2022; Zang et al., 2022); or (iii) for example in the relief of toxicity provoked by drugs and metalloids (Bjørklund et al., 2022; Koss-Mikołajczyk et al., 2021).

Several problems have been pointed as in need for the world to pay attention to, and antimicrobial resistance and foodborne diseases are some of the most relevant (Rahman et al., 2022; Saraiva et al., 2022; Verraes et al., 2013). The antimicrobial resistance can increase the morbidity and mortality associated with infectious diseases (Davies and Wales, 2019). In fact, World Health Organization (WHO) has declared that antimicrobial resistance is one of the top 10 global public health threats facing humanity, with drug-resistant infections contributing to nearly 5 million deaths every year (Murray et al., 2022; WHO, 2020). Furthermore, the long-time overuse and misuse of antibiotics have resulted in the dissemination of antibiotics all over the environment (Ogawara, 2019; Uluseker et al., 2021), which may associate with an adaptation of bacteria to environmental stresses, and the development of resistance to different antimicrobial agents (Olaimat et al., 2018; Varela et al., 2021).

According to the WHO an estimated 600 million people fall ill after eating contaminated food each year, with 420 000 deaths being caused by foodborne illnesses (WHO, 2022). Operators from the food chain and regulatory agencies faced a globalization and a changing consumer's habits that provides a new perspective on food safety (Strambu-Dima, 2022; Verraes et al., 2013). These scenarios may carry new challenges concerning foodborne pathogens and their increased resistance to antibiotics and sanitizers, with antimicrobial therapeutics becoming less effective and pathogens more adjusted and tolerant to

disinfectants, sanitizers and topical agents (Jones and Joshi, 2021; Meade et al., 2021; Tong et al., 2021).

The research for novel antibiotics has decreased and hardly any novel antibiotics have been introduced into the market (Langeveld et al., 2014). Thus, the development of alternatives to antibiotics and the discovery of adjuvants could be a good strategy to fight resistant bacteria and inhibit their spread (Ju et al., 2020; Langeveld et al., 2014). Knowing that the chemical compounds from plants can be useful for the discovery of new antimicrobial agents (Kooti et al., 2015; Korinek et al., 2021), the combination of available antibiotics with other nonantibiotic drugs or combining antibiotics with adjuvants or antimicrobials selected from natural sources, can be a promising path to deal with the antimicrobial resistance (Ayaz et al., 2019; Langeveld et al., 2014). Furthermore, finding compounds that present both antimicrobial and antioxidant properties show a great potential for its application as food preservatives (Carrasco et al., 2015; Ribeiro-Santos et al., 2018; Yousefi et al., 2020).

Among the possible solutions to solve these problems, plants and their active compounds have been highlighted.

1.1 Medicinal and aromatic plants

The use of medicinal and aromatic plants for the prevention and treatment of diseases is the oldest medicinal practice, being extremely important for the population living in developing countries (Arantes et al., 2019; Gahukar, 2018; Owen and Laird, 2018; Roosta et al., 2017). Due to the high shortage of health care, more than three billion humans depend on traditional remedies for their primary health care (Korinek et al., 2021). Furthermore, the medicinal and aromatic plants are a valuable source in the supply of herbal cosmetics, drug discovery, and food supplements, being used as alternative therapies to improve human health (Arantes et al., 2019; Gahukar, 2018; Roosta et al., 2017). The natural compounds from plants, such as carotenoids, flavonoids, terpenes, among others, have also been subject of interest in the production of different industrial products, including fertilizers, paints, surfactants, rubbers, textiles, perfumes, and others (Ferreira-Santos et al., 2020; Singh and Pandey, 2018).

Taking into account the awareness about a safe, green, and clean environment and the growing demand for green consumerism, the consumers have been requesting the reduction of synthetic and chemical products, such as pesticides, sanitisers and synthetic food preservatives (Angane et al., 2022; Rao et al., 2019; Silvestre et al., 2019; Van Haute et al., 2016). For these reasons, the food industries are reformulating and replacing artificial and synthetic products, with more natural alternatives and label-friendly increasing the study

and introduction of plant-derived substances (Hao et al., 2021; Nazzaro et al., 2017; Rao et al., 2019). Therefore, plants, by their biological potential, are promising natural sources for use in the industry sector and with an extensive economic growth potential (Kala, 2015). Due to their natural antimicrobial and antioxidant activities, and diverse therapeutic properties, essential oils (EOs) have been pointed as possible alternatives to preservatives, drugs or other products (Perumal et al., 2022; Silvestre et al., 2019).

1.2. Essential oils

According to the European Pharmacopoeia, EO is defined as an "odorous product, usually of complex composition, obtained from a botanically defined plant raw material by steam distillation, dry distillation, or a suitable mechanical process without heating. Depending on the monograph, the plant raw material may be fresh, wilted, dried, whole, broken or ground" (*European Pharmacopoeia*, 2019).

The EOs are lipophilic liquids, rarely coloured, limpid, volatile and composed of complex compounds characterized by a strong odour (Ahmad et al., 2021; Bakkali et al., 2008; Da Silva et al., 2021; De Martino et al., 2015; Rao et al., 2019). These natural products could be biosynthesized in different plant organs, such as bark, buds, flowers, fruits, leaves, roots, seeds, stems, twigs, and wood (Asbahani et al., 2015; Da Silva et al., 2021; Perricone et al., 2015; Ribeiro-Santos et al., 2018), being usually stored in canals, cavities, oil ducts, resin ducts, secretory cells, glandular trichomes or epidermal cells of the plants (Bakkali et al., 2008; De Martino et al., 2015; Raut and Karuppayil, 2014; Saeed et al., 2022).

The EOs are commonly extracted from plant raw material by conventional techniques, such as hydrodistillation, steam distillation, vapour-hydrodistillation, hydro diffusion, organic solvent extraction, Soxhlet extraction, cold pressing and distillation–extraction (Asbahani et al., 2015; Figueiredo et al., 2010; Raut and Karuppayil, 2014; Trifan et al., 2020). Innovative techniques have also been applied to perform the extraction of EOs, for example, supercritical fluid extraction, subcritical fluid extraction (H_2 and CO_2), ultrasound-assisted organic solvent extraction, microwave-assisted organic solvent extraction, solvent-free microwave extraction, microwave hydro diffusion and gravity, microwave steam distillation, microwave steam diffusion, instant controlled pressure drop, solid-phase microextraction, and pressurized liquid extraction (Asbahani et al., 2015; Figueiredo et al., 2010; Giacometti et al., 2018).

These complex natural mixtures of secondary metabolites (De Martino et al., 2015; Rao et al., 2019; Vázquez-Ucha et al., 2020), usually contain about 20–80 polar and non-polar compounds at different concentrations (Bakkali et al., 2008; De Martino et al., 2015; Diánez et al., 2018; Nazzaro et al., 2017; Rao et al., 2019). The EOs are characterized by two or three

major components at high concentrations compared to other compounds that are present in trace amounts (Bakkali et al., 2008; Diánez et al., 2018) and include mainly terpenes and terpenoids (Asbahani et al., 2015; Da Silva et al., 2021; Vázquez-Ucha et al., 2020).

In nature, plants naturally produce a wide diversity of secondary metabolites as protection against fungi, bacteria, insects or even herbivores (Bakkali et al., 2008; Nazzaro et al., 2017; Wu et al., 2019). In turn, the EOs may also attract some insects as advantage for the dispersion of pollens and seeds, or repel undesirable others (Bakkali et al., 2008; Nazzaro et al., 2017). In fact, the composition of the EOs can change with several biotic and abiotic factors, which can be the referred response to predators or other stress factors, but also the plant genetics and genotypes, environmental conditions, or season. Besides, during the EOs production process, the use of different extraction techniques or solvents, the harvest region or the climate, the drying process, the plant age, or the use of different plant parts also influence their composition (Piras et al., 2019; Ribeiro-Santos et al., 2018; Rota et al., 2008; Silvestre et al., 2019). Since the bioactive properties of the EOs are directly correlated with particular secondary metabolites produced by the plant and its quantities (Calo et al., 2015; Da Silva et al., 2021), the determination of their composition is very important (Ribeiro-Santos et al., 2018; Rota et al., 2008).

1.2.1 Bioactive activities of the EOs

EOs, in addition to playing a fundamental role in the biological processes of plants and in their own protection, have been used for centuries as aromatizers in perfume fragrances, culinary, and folk medicine. Due to their bioactive properties, the EOs have received considerable attention in the synthesis of chemicals, in the cosmetic, food, pharmaceutical, phytotherapy and textile industries, but also pest control (Ahmad et al., 2021; Owen and Laird, 2018; Ribeiro-Santos et al., 2018; Silvestre et al., 2019; Trifan et al., 2020).

Amongst phytochemicals, the EOs have been related to a wide range of remarkable biological activities, such as antifungal, anti-inflammatory, antioxidant, antineoplastic, and antiviral (Chen et al., 2020a).

The EOs are natural antioxidants that can prevent the oxidative deterioration of food products and can be useful for preventing oxidative stress disorders (Akbari et al., 2022; Arantes et al., 2019; Perumal et al., 2022). Many biological membranes are predisposed to lipid peroxidation because of their polyunsaturated fatty acid content and with ageing, there is an increase in the production of free radicals as well as a decrease in the activity of the antioxidant enzyme systems (Youdim et al., 2002). This imbalance can provoke damage in membranes, lipids and lipoproteins and can induce DNA mutation, leading to diverse

pathological disorders, such as atherosclerosis, cardiovascular diseases, carcinogenesis as well as Alzheimer's and other neurodegenerative diseases (Carrasco et al., 2015; Jordán et al., 2009; Youdim et al., 2002). Antioxidants can compete with free radicals, preventing oxidation reactions and acting differently depending on the reactive species present or the target substrate (Ribeiro-Santos et al., 2018). The EOs can act as hydrogen donors, reducing agents, and singlet oxygen quenchers and also have metal chelation properties (Jordán et al., 2009). The antioxidant capacity of the EOs is directly related to their composition, mainly with the high percentage of major compounds, but also with the presence of other components found in lower quantities or the synergy among them (Fkiri et al., 2019; Ribeiro-Santos et al., 2018).

In a similar way, the antimicrobial activity of the EOs can be related to one or two of their major constituents, with the ratio of the main active compounds, but also to the interactions between major and minor constituents in the EOs (Chouhan et al., 2017). The antimicrobial activity of the EOs has been associated with their lipophilicity. The interaction between the EOs hydrophobic components with the lipids of the microorganism's cell membrane allow the passage of the components through the microorganism's cell wall causing the loss of membrane integrity (Calo et al., 2015; Da Silva et al., 2021; Silva et al., 2019; Yousefi et al., 2020). Every functional group can interact with components of the target bacterial cell. Therefore, the mode of action of EOs against bacterial cells cannot be defined by a single mechanism. All the target operations inside the bacterial cell are interconnected, and some cellular mechanisms can be directly disrupted by the EOs, or it can interfere in one system consequently interfering in other cellular activities, thus causing an indirect disruption (Saeed et al., 2022). The EOs can induce changes in the functioning of the electron transport chain, in both protein and nucleic acid synthesis, in the absorption of nutrients, including glucose uptake, and in the inhibition of enzymes responsible for energy metabolism (Angane et al., 2022; Calo et al., 2015; Da Silva et al., 2021; Silva et al., 2019; Yousefi et al., 2020).

Gram-positive bacteria tend to be more susceptible to EOs than Gram-negative bacteria probably due to the differences in their cell wall (Angane et al., 2022; Calo et al., 2015; Da Silva et al., 2021). The Gram-negative bacteria have a cellular structure more complex, which consist of an outer membrane composed of lipopolysaccharide with hydrophilic character that covers a thin layer of peptidoglycan, acting as a selectively permeable barrier to prevent the penetration of hydrophobic compounds (Calo et al., 2015; Da Silva et al., 2021). The outer membrane limits the diffusion of the hydrophobic compounds, contrary to Gram-positive bacteria cell walls for which the high composition of peptidoglycan in the cytoplasmic membrane allows the penetration of the EOs increasing its antimicrobial efficiency (Angane et al., 2022; Da Silva et al., 2021).

Furthermore, it is also described that EOs have different mechanisms of action regarding their antifungal activity. The inhibition of the cell wall or cytoplasmic membrane biosynthesis, the interference with the cell wall remodelling and the disruption of the integrity of the cell membrane, leading to the leakage of cellular components, are the main mechanisms of action of the EOs (Herman and Herman, 2021). Additionally, the EOs can inhibit the budding yeast transformation into hyphae and the biofilm formation, affect enzymes and modify intracellular functions, alter the membrane permeability, over-express membrane transporters but also, increase the production of the intracellular reactive oxygen species levels, decreasing the cell viability, and consequently, leading to cell death (Herman and Herman, 2021; Ribes et al., 2018).

These biological activities associated with the replacement or reduction of the use of synthetic additives have encouraged several studies on the utilization of EOs, as an alternative in the food industry to enhance the safety and the shelf life of food products but also, in the pharmaceutical industry (Jordán et al., 2009; Ribeiro-Santos et al., 2018; Yousefi et al., 2020).

1.2.2 Safety, advantages, and disadvantages of the EOs

The safety of aromatic and medicinal plants and the preparation of herbal products with minimal toxicity and side effects is an intended requirement (Kooti et al., 2015; Rather et al., 2016). One of the main described advantages for use of phytotherapy is the minimal adverse effects and the eventual absence of toxicity risk associated with the synthetic products (Davoodvandi et al., 2020; Karthika and Sureshkumar, 2021; Rather et al., 2016). However, many plants contain materials that are toxic and irritating, can cause severe allergic reactions and the ingestion or topical applications of these natural compounds can cause oral toxicity and dermatitis. Therefore it is required to discover a balance between the effective dose of the EO and the risk of toxicity (Angane et al., 2022; Ribeiro-Santos et al., 2017).

Beyond the use of the EOs in the prevention and treatment of diseases, the EOs can also be used in the food industry and the European Commission and the Food and Drug Administration (FDA) have documented a variety of EOs and isolated compounds that are approved flavour additives without risks to human health and classified as generally recognized as safe (GRAS), such as the EOs of *Coriandrum sativum*, *Foeniculum vulgare*, *Helichrysum augustifolium*, *Melissa officinalis*, *Mentha* spp., *Thymus vulgaris*, *Thymus zygis,* or the compounds, β-caryophyllene, carvacrol, carvone, cinnamaldehyde, citral, citronellal, eugenol, isoeugenol, lavandulol, limonene, linalool, menthol, pinene, thymol,

vanillin and others (European Commission, 2008; FDA, 2021). Nonetheless, the direct application of EOs in food has limitations due to its volatile nature, chemical instability, strong sensory properties and high hydrophobicity (Perumal et al., 2022). In fact, the associated unappealing sensory attributes, even at low concentrations, limits its application in food (Da Silva et al., 2021; Perumal et al., 2022). Moreover, when applied in food systems, their efficiency and bioactivity tend to be reduced, because the EOs may bind to carbohydrates, lipid constituents, fat content, proteins, starch, and enzymes, but also due to the pH and water activity of the food, decreasing the EO availability to act against microorganisms (Angane et al., 2022; Calo et al., 2015; Da Silva et al., 2021; Saeed et al., 2022). Therefore, a higher concentration of EO is needed to achieve its antimicrobial effect, which in turn can alter the organoleptic properties of food products (Perumal et al., 2022).

As for other compounds, such as antibiotics or biocides, the exposure of bacteria to EOs or their individual components at sublethal concentrations may lead to antibiotic resistance and alter the physiological responses of microorganisms when exposed to some stresses (heat, cold, acid, etc) conducting to a potential adaptation of the microorganisms to these natural antimicrobial agents (De Souza, 2016; Giacometti et al., 2021; Liao et al., 2020). Despite, some natural compounds do not influence the increasing antibiotic resistance, a few studies have shown that the bacteria can development tolerance and/or resistance to antibiotics when exposure to sub-inhibitory concentrations of natural compounds (Reviewed by De Souza (2016) and Giacometti et al. (2021)) Therefore, it is very important to evaluate the effect of EOs on the tolerance and/or resistance of microorganisms when using these natural products as antimicrobial agents.

1.3 Aromatic and medicinal plants under study

1.3.1 *Foeniculum vulgare*

Foeniculum vulgare or *Foeniculum officinale*, generally recognized as fennel, is a hardy annual, biennial or perennial–umbelliferous medicinal and aromatic plant belonging to the Apiaceae (Umbelliferaceae) family (Chang et al., 2016; Chen et al., 2020a; Rather et al., 2016; Yaldiz and Camlica, 2019). *F. vulgare* is originated on the shores of the Mediterranean Sea and from Asia Minor, but it has become widely dispersed in many parts of the world mainly in Central, South, and West Europe, South-West Asia, and North Africa and especially on the river banks and dry soils near the sea coast (Crescenzi et al., 2021; Rather et al., 2016; Servi et al., 2021; Yaldiz and Camlica, 2019). *F. vulgare* is cultivated throughout the tropical and temperate regions of the world in arid, as well as, semi-arid zones, as it does not tolerate humid or cold climates (Chang et al., 2016; Crescenzi et al.,

2021; Tanveer et al., 2021). This plant grows to a height of 2.5 m with straight and hollow stems with an intense green-blue colour, yellow umbels with 5–15 cm wide flowers and feathery leaves 40 cm long composed of filiform segments (threadlike), and about 0.5 mm wide (Badgujar et al., 2014; Crescenzi et al., 2021; Rather et al., 2016; Singh, 2019). The fruits have ridged and oblong to ovoid shapes with sizes of 1.5–2.5 mm broad and 4–10 mm long (Crescenzi et al., 2021; Hao et al., 2021). These plants have a bloom period between July and August and the seeds ripen from September to October (Badgujar et al., 2014). The seeds are aromatic, anise-flavoured spice, and when fresh they are brown or green and slowly turn a dull grey as the seed ages (Rather et al., 2016). *F. vulgare* seeds are rich in EO, responsible for their aroma and taste (Servi et al., 2021; Sharma et al., 2021).

F. vulgare is a flavourful and aromatic plant used in culinary, folk medicine and cosmetic and pharmaceutical products being industrially and economically valuable herbs (Badgujar et al., 2014; Chen et al., 2020a; Hao et al., 2021; Rather et al., 2016). The seeds are used as flavourings in baked goods, meat and fish dishes, alcoholic beverages, ice cream, and herb mixtures. Furthermore, *F. vulgare* is mainly used medicinally as purgative due to its carminative properties (Badgujar et al., 2014; Rahimi and Ardekani, 2013). It has been used to cure cough, menstrual disorders, and gingival wounds, to reduce the gripping effect of laxatives and the EOs and plant extracts are also used to control mites of the stored food (Badgujar et al., 2014; Yaldiz and Camlica, 2019). *F. vulgare* is also used to treat digestive disorders, such as dyspepsia, stomach discomfort, stomach-ache, intestinal pain, flatulence, bloating, diarrhoea and intestinal worms, but also to treat colds and cough, as an expectorant, chest softener, urine enhancer, kidney stone healer, sedative, insomnia reliever, milk secretion enhancer, vasodilator and also to treat eye itching (Badgujar et al., 2014; Dahmani et al., 2022; Servi et al., 2021; Yaldiz and Camlica, 2019). *F. vulgare* water mixed with sodium bicarbonate and syrup, constitute the domestic 'gripe water', used to control the flatulence of infants (Rather et al., 2016). In Algeria, decoctions or infusions of the *F. vulgare* seeds, administered orally or topically, were used in anaemia, anxiety, bloating, constipation, hair loss, obesity, tonsillitis, or wrinkles (Aumeeruddy and Mahomoodally, 2021; Djahafi et al., 2021). There are some systematic reviews and meta-analyses that gather information on the use of *F. vulgare* women's menstruation diseases and menopause. It can be verified that *F. vulgare* improves menopausal symptoms, such as the relieving of vasomotor symptoms, dryness, dyspareunia, vaginal itching, sexual function and satisfaction, and sleep distribution, and it is used in the treatment of menstrual disorders, amenorrhea, menorrhagia, irregular menstruation, oligomenorrhea, premenstrual syndrome, and dysmenorrhea (Jiao et al., 2022; Khadivzadeh et al., 2018; Lee et al., 2021).

The major component found in the *F. vulgare* EO was *trans*-anethole, fenchone and estragole (Akhbari et al., 2019; Ashokkumar et al., 2021; Chang et al., 2016; Chen et al., 2020a; Hao et al., 2021; Mandras et al., 2021; Yaldiz and Camlica, 2019), followed by limonene, anisaldehyde, γ-terpinene, α-pinene (Mandras et al., 2021; Yaldiz and Camlica, 2019). *F. vulgare* EO can also reveal the presence of estragole and limonene as major components, while only 1.9% accounted for anethole (Korinek et al., 2021).

F. vulgare's antioxidant, anti-inflammatory, antimicrobial and antithrombotic effects and also cytotoxic activity have been reported (Akhbari et al., 2019; Chang et al., 2016; Chen et al., 2020a; Yaldiz and Camlica, 2019). In general, *F. vulgare* extracts showed radical scavenging activity (Barros et al., 2009; Bhatti et al., 2018; Chang et al., 2016; Crescenzi et al., 2021; Yaldiz and Camlica, 2019), and lipid peroxidation inhibition capacity (Barros et al., 2009). When considering the EO of *F. vulgare,* its antioxidant activity has been reported from weak to excellent (Chen et al., 2020a; Dahmani et al., 2022; Korinek et al., 2021; Servi et al., 2021). This considerable variation may be justified by the difference in several factors, such as the origin, climatic conditions, method and duration of extraction, and the chemical composition of EO (Dahmani et al., 2022). Evaluating the metal chelating activity of the EO, it interfered with the formation of ferrous and ferrozine complex suggesting that it has chelating activity (Chang et al., 2016).

Regarding antimicrobial activity, *F. vulgare* extracts showed strong antibacterial activity against food pathogens, such as *Escherichia coli*, *Listeria monocytogenes*, *Salmonella* Typhimurium, *S. aureus*, and also relevant activity against *Helicobacter pylori* and *Campylobacter jejuni* (Chang et al., 2016; Cwikla et al., 2010; Kaur and Arora, 2009; Mahady et al., 2005). In turn, studies on *F. vulgare* EO antimicrobial activity have shown diverse results, as it can display weak antibacterial activity against Gram-negative bacteria, and present no effectiveness on Gram-positive bacteria (Servi et al., 2021), or demonstrated to be active against bacteria, fungi or virus. In fact, the results of Dahmani et al. (2022) showed good antimicrobial activity, with yeasts being more sensitive to *F. vulgare* EO than bacteria, with *Pseudomonas aeruginosa* being the most resistant species (Dahmani et al., 2022). *F. vulgare* EO also showed good antifungal activity even against vegetable fungi, as a green fungicide and against fungi of food wastes, such as *Aspergillus niger* and *Fusarium oxysporum* (Chen et al., 2020a; Khaleil et al., 2021; Mandras et al., 2021; Martins et al., 2012). Likewise, the EO of the aerial parts and bulb of *F. vulgare* has manifested promising antiviral activity against the Hepatitis C and Hepatitis A viruses (Ibrahim and Moussa, 2021).

F. vulgare EO displayed good anti-lipoxygenase activity compared to the standard indomethacin (Servi et al., 2021) and had a high inhibitory effect, suppressing the activation

of human neutrophils, being a potent anti-inflammatory agent in the treatment of inflammatory diseases (Korinek et al., 2021).

F. vulgare has demonstrated a gastroprotective effect in a randomized placebo-controlled study, where it was observed a significant improvement in the colic of infants in the group treated with *F. vulgare* EO emulsion compared with the control group, while no reported side effects for infants during the trial have been reported (Alexandrovich et al., 2003). Furthermore, the hepatoprotective effect of *F. vulgare* EO was also shown, being used to treat rats with carbon tetrachloride-induced liver injury (Özbek et al., 2003).

Concerning the potential antidiabetic activity, *F. vulgare* EO presented weak α-glucosidase inhibitory activity compared to the reference inhibitor, acarbose (Servi et al., 2021). However, the ingestion of EO of *F. vulgare* seeds by diabetic rats showed good antidiabetic activity (El-Soud et al., 2011).

F. vulgare had demonstrated neuroprotective activities, with the EO showing antidepressant properties when tested in mice (Abbasi-Maleki and Maleki, 2021) and exhibiting a promising anxiolytic activity, with a higher dose of the EO seeming to be potentially sedative (Mesfin et al., 2014).

In terms of cardiovascular function, *F. vulgare* EO demonstrated a safe antithrombotic activity possibly due to their antiplatelet activity, clot destabilizing effect and vasorelaxant action. An additional advantage in the use of this EO is that the antithrombotic dosage has no haemorrhagic side effects, such as the ones associated with acetylsalicylic acid that was used as a reference drug (Tognolini et al., 2007). A randomized controlled trial in rabbits demonstrated that *F. vulgare* incorporated in the diet may have some role in the maintenance of haemoglobin levels and some cases also improving it, being these results important for possible natural treatment of anaemia (Abbas et al., 2021).

The *F. vulgare* EO can inhibit the oxytocin and prostaglandin E_2-induced contraction of the uterus, indicating the possible use of the *F. vulgare* EO in the relief of dysmenorrhea (Ostad et al., 2001).

Regarding its cytotoxic activity, the results demonstrated that the *F. vulgare* EO inhibited the growth of human gastric cancer (MGC-803) cells, human breast cancer (MCF-7 and MDA-Mb) cells, Henrietta Lacks cervical adenocarcinoma (HeLa) cells, human lung cancer (A549) cells, human hepatoma cancer (Huh-7 and HepG2) cells, and human colon cancer tumour (SW620) cells in a dose-dependent manner (Akhbari et al., 2019; Chen et al., 2020a). Furthermore, the *F. vulgare* EO exhibited relative safety in the human normal lung fibroblast (WI-38) cell line (Ibrahim and Moussa, 2021).

Besides, the different bioactivities, *F. vulgare* exhibited diverse pharmacological activities, as well as some environmental activities, which are important in the management of insects, nematodes, mosquitoes, and some harmful larvae (Badgujar et al., 2014). Moreover, as reviewed by Sousa et al. (2021), the *F. vulgare* EO have high fumigant toxicity against the red flour beetle *Tribolium castaneum* (Oviedo-Sarmiento et al., 2021), larvicidal and suicidal activities against house fly *Musca domestica* (Abdel-Baki et al., 2021), and acaricidal activity and repellency effects against *Rhipeciphalus annulatus* tick (Aboelhadid et al., 2021). Furthermore, the EO showed anthelmintic potential against gastrointestinal nematodes in sheep, having 100% effectiveness in the inhibition of egg hatchability (Štrbac et al., 2022).

1.3.2 *Helichrysum stoechas*

The *Helichrysum* genus of the Asteraceae family includes more than 500 species that are widespread around the world (Akaberi et al., 2019; Haddouchi et al., 2014; Kherbache et al., 2020), being originally from Africa, Madagascar, Australasia and Eurasia (Akaberi et al., 2019). Among the best-known and studied species of this genus are *Helichrysum arenarium*, *Helichrysum stoechas*, and *Helichrysum italicum* (Akaberi et al., 2019). Everlasting flower or shrubby everlasting is the common name of *H. stoechas,* which grows mainly in the Iberian Peninsula (Barros et al., 2010; Garcia-Oliveira et al., 2021). This species is an evergreen grey-cotton subshrub that grows on shallow or sandy soils (Ascensao et al., 2001; Kherbache et al., 2020), but also in light forest and scrubs, rocky areas, maritime zones and drylands in altitudes between 0 to 2000 m (Garcia-Oliveira et al., 2021).

Several species of this genus have been studied for different applications (Akaberi et al., 2019; Garcia-Oliveira et al., 2021; Lourens et al., 2008; Viegas et al., 2014). Ethnobotanical studies revealed that *H. stoechas* has been used to treat influenza, common cold, bronchitis, fever, digestive disorders, wound healing, mitigation of inflammatory complications, soothe toothache, manage urologic, nervousness, and pancreatic problems and it has also been employed as an expectorant, antipyretic, antimicrobial, antiviral, diuretic, and for snake bites and sciatica (Ascensao et al., 2001; Barros et al., 2010; Kherbache et al., 2020; Zengin et al., 2020). *H. stoechas* was used as an ingredient in a syrup based on honey and plant extracts and a clinical trial using this syrup was performed on 106 children with a persistent cough. The results of this study revealed that the administration of the syrup reduced the severity and the cough duration (Carnevali et al., 2021). Furthermore, for example, the patent number FR3040625A1 is about an active cosmetic principle from *H. stoechas* use to combat skin ageing and the patent number CN106038385A is for the cosmetic composition capable of stimulating collagen generation for wrinkle resistance.

Regarding the chemical composition, the *H. stoechas* EO presents as major constituents the β-caryophyllene, α-humulene, α-pinene and isoeugenol acetate (Roussis et al., 2002). The monoterpene hydrocarbon fraction was dominant in *H. stoechas* EOs from the flowers and leaves, with α-pinene being the major component in both oils, in higher amounts in the leaf's EO compared to the EO of the flowers. The second most representative component was limonene, but in this case in greater quantity in the flower's EO (Ascensao et al., 2001). In another sample of the *H. stoechas* EO, the major constituents were β-caryophyllene, followed by β-elemene and benzyl benzoate (Chinou et al., 1997).

Several biological activities have been described for *H. stoechas,* such as antioxidant, anti-inflammatory, antimicrobial, anticancer, and neuroprotective properties (Garcia-Oliveira et al., 2021), with *H. stoechas* extracts being more studied that its EO (Albayrak et al., 2010; Bremner et al., 2009; Carini et al., 2001; Haddouchi et al., 2014; Kherbache et al., 2020; Les et al., 2017; Zengin et al., 2020).

Concerning antimicrobial activity, the *H. stoechas* extracts have demonstrated activity against different bacteria and also fungi (Albayrak et al., 2010; Bogdadi et al., 2007; Kutluk et al., 2018). The EO of *H. stoechas* also showed good antimicrobial activity against *Bacillus subtilis*, *E. coli, Staphylococcus epidermidis, S. aureus* and different species of *Candida* (Chinou et al., 1997; Roussis et al., 2002).

The *H. stoechas* extracts have shown to reduce the formation and growth of kidney stones and to be beneficial for patients with recurrent stones (Orhan et al., 2015); to inhibit enzymes involved in glucose metabolism, the α-glucosidase, with a very similar effect to the reference inhibitor acarbose (Les et al., 2017); to exhibit a neuroprotective action, inhibiting enzymes related to neurotransmitter metabolism and the central nervous system, such as monoamine oxidase, acetylcholinesterase, and tyrosinase (Les et al., 2017; Silva et al., 2017; Zengin et al., 2020) and with regard to antitumor activity, the extracts of the *H. stoechas* inhibits the proliferation of cervical cancer HeLa cells *in vitro* in a dose-dependent manner (Les et al., 2017).

1.3.3 *Mentha pulegium*

The genus *Mentha* is a group of aromatic and medicinal plants belonging to the Lamiaceae family, which includes more than 60 species. These plants grow worldwide, especially in South Africa, Australia and temperate regions of Eurasia (El Hassani, 2020; Jebali et al., 2022; Piras et al., 2019; Singh and Pandey, 2018). Commercially, the most important species are corn mint (*Mentha canadensis*), peppermint (*Mentha piperita*), and spearmint (*Mentha spicata*) (Singh and Pandey, 2018). *Mentha pulegium,* commonly known as

pennyroyal or European pennyroyal, is a perennial endemic plant in Europe and North Africa and it is also found in Asia Minor and Middle East regions (Alimi et al., 2022; Baali et al., 2019; Domingues and Santos, 2019) and reaches a height of 40 to 60 cm (Bektašević et al., 2021). *M. pulegium* grows in bioclimatic zones ranging from humid to arid (Alimi et al., 2022), like the moist meadows with sandy soil, alluvial plains, riparian habitats, and freshwater wetlands (Bektašević et al., 2021; Caputo et al., 2021). The stems are procumbent to ascending, the leaves are narrowly ovate or elliptic and the flowers in well-spaced verticillasters in the leaf axils (Caputo et al., 2021). The blooming period of *M. pulegium* is from June to September with pink to blue flower clusters (Bektašević et al., 2021; Domingues and Santos, 2019) and the plant presents a prostate stance but during flowering it becomes upright (Domingues and Santos, 2019).

The flowers, leaves, and stems of these plants' genus are frequently used in traditional medicine, as infusions or as additives in food to offer aroma and flavour. *M. pulegium* is known since ancient times, in Greek, Medieval, and Roman cultures, in culinary and traditional medicine (Caputo et al., 2021). The infusion of *M. pulegium* has been used to treat diverse illnesses including the common cold, cough, bronchitis, whooping sore throat, gastrointestinal ailments, amenorrhea, gout, and skin itching and also as an emmenagogue, antiseptic, anti-inflammatory, antispasmodic, carminative, an aromatic stimulant, analgesic, antimicrobial, insecticide and insect repellent and abortifacient (Ahmed et al., 2018; Benahmed et al., 2019; Caputo et al., 2021; Domingues and Santos, 2019; Piras et al., 2019; Teixeira et al., 2012). The infusions with the dried aerial parts and EOs of *M. pulegium* are used as an analgesic, expectorant, antiseptic, carminative, diuretic, antispasmodic, antihypertensive, antioxidant, antimicrobial and anti-inflammatory (Baali et al., 2019). In Morocco regions, the aerial parts, leaves, flowers, EO and also the whole plant of *M. pulegium* in decoction, macerations, infusion, inhalant, and external application were used against diabetes, respiratory diseases, gastrointestinal disorders (carminative), hypertension, cardiac and urinary infectious and parasitic diseases, rheumatism, musculoskeletal illnesses (antispasmodic), migraine, infertility, pains, fever, haemorrhoids, obesity, nervous disorders, gynaecological and endocrinological injuries and skin pathologies (Reviewed by El Hassani (2020)).

M. pulegium has been commercialized for its usages as a drink and food flavouring and, for fragrances and alternative medicine practices (Domingues and Santos, 2019). The *M. pulegium* plant is typically characterized by a pungent minty scent (Bektašević et al., 2021; Domingues and Santos, 2019) due to the presence of pulegone which, generally, is very abundant in its EO (Caputo et al., 2021). The most important biologically active constituents of *M. pulegium* are present in the EOs (Benahmed et al., 2019).

The *M. pulegium* EO is rich in oxygenated monoterpenes, followed by monoterpene hydrocarbons (Ahmed et al., 2018; Baali et al., 2019; Teixeira et al., 2012). The pulegone is in a clear predominance (Alimi et al., 2022; Vieira et al., 2017; Yakoubi et al., 2021a), followed by piperitenone, iso-menthone and *cis*-isopulegone with the other components being present in trace amounts, such as neoisomenthol, 1,8-cineole, α-pinene, limonene, and 3-*p*-methanol (Benahmed et al., 2019; Luís and Domingues, 2021; Piras et al., 2019).

The *M. pulegium* presents diverse bioactive properties. Regarding the antioxidant activity, most of the information found points to the good activity of the different *M. pulegium* extracts and fractions through different evaluation methods (Abbou et al., 2022; Gülçin et al., 2020; Jebali et al., 2022; Teixeira et al., 2012; Yakoubi et al., 2021b). In turn, the *M. pulegium* EO showed very low antioxidant activity considering 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) scavenging activity, reducing power assay and also in the β-carotene/linoleic acid assay (Ahmed et al., 2018; Baali et al., 2019; Kamkar et al., 2010; Yakoubi et al., 2021a). Nonetheless, some studies demonstrated the good antioxidant of the *M. pulegium* EO, which can act as a free-radical scavenger by reducing DPPH and was classified as very strong, according to the scale implemented by Scherer and Godoy (2009) (Benahmed et al., 2019; Luís and Domingues, 2021). The evaluation of the inhibition of lipid peroxidation exhibits a significantly higher antioxidant activity than the control butylated hydroxytoluene (BHT) (Luís and Domingues, 2021).

M. pulegium has presented good antifungal and antibacterial activities (Baali et al., 2019; Benahmed et al., 2019; Jebali et al., 2022; Piras et al., 2019; Teixeira et al., 2012). The *M. pulegium* EO showed good antibacterial activity against *B. subtilis*, *E. coli*, *P. aeruginosa, S. aureus*, and *Streptococcus pneumoniae*, with the most resistant species being *P. aeruginosa* and also inhibitory activity against foodborne spoilage bacteria (Teixeira et al., 2012; Vieira et al., 2017). In another study, the *M. pulegium* EO had presented a good antibacterial activity, with the strains of *Acinetobacter baumannii* showing to be the most sensitive, presenting the highest inhibition zones and the lowest MIC values, when compared with different species of Gram-positive and Gram-negative bacteria and also yeasts (Luís and Domingues, 2021). Furthermore, the *M. pulegium* EO demonstrated anti-quorum sensing activity when evaluated by the biosensor strain *Chromobacterium violaceum* ATCC 12472. The EO has shown a significantly inhibitory capacity for the formation of the pigment violacein and higher activity than the positive control, resveratrol (Luís and Domingues, 2021). This has been further supported since when *M. pulegium* EO was evaluated against the quorum sensing-regulated bioluminescence of *V. campbellii,* it was able to block the *Vibrio campbellii* bioluminescence (Zheng et al., 2020). These results

can provide an important strategy for the reduction of some microorganisms' virulence mechanisms and also the pathogenicity of microorganisms (Luís and Domingues, 2021).

Concerning the anti-inflammatory activity of *M. pulegium* EO, its potential action was suggested, in an assay that evaluate the ability of the EO to inhibit protein denaturation, where the IC_{50} value obtained was very similar to the one obtained for acetylsalicylic acid used as control. This has been associated with the high content of pulegone of the EO (Luís and Domingues, 2021), a compound that has shown anti-inflammatory activity through different mechanisms (Hilfiger et al., 2021; Roy et al., 2018; Yang et al., 2019).

The EO of *M. pulegium* was tested for acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibition, with the results revealing that the EO contains compounds that, in addition to antioxidant activity, also have cholinesterase inhibition activity (Bektašević et al., 2021). The anticholinesterase activity of *M. pulegium* EO demonstrated moderate AChE inhibitory activity and higher inhibition against BChE when compared with the positive control, galantamine (Yakoubi et al., 2021a). Therefore, the *M. pulegium* EO may be relevant in the treatment and prevention of neurodegenerative disorders, such as Alzheimer's disease and others, as well as in other problems associated with oxidative stress (Bektašević et al., 2021).

Regarding the insecticidal activity, the EOs of *M. pulegium* was tested against the chickpea pest *Callosobruchus maculatus* and the insecticidal activity and the inhalation test showed a high efficacy with 100% of mortality, thus this EO can be an ecological alternative to eliminate *C. maculatus* from stored seeds (Aimad et al., 2021). The use of *M. pulegium* EO in fumigant and topical applications proved to be also effective in the mortality of the house fly, *M. domestica* L. (Pavela, 2008). The *in vitro* acaricidal effect of *M. pulegium* against *Hyalomma scupense* revealed that the EO was toxic to engorged females and had strong repellency against these ticks. However, the *M. pulegium* EO had low efficacy against the larvae when compared with the positive control amitraz (Alimi et al., 2022).

The potential damage of herbicides and chemical pesticides to human health has become a high concern and there is an increasing interest in new eco- and bio-sustainable products as alternative approaches (Domingues and Santos, 2019). To evaluate the toxicity of the EOs in order to use them as new bio-herbicides, the toxicological test on *Artemia salina* is a useful and rapid screening tool to use and investigate the eco-compatibility as well the toxicity in aquatic organisms (Caputo et al., 2021). Regarding the toxicity of *M. pulegium*, the results demonstrated that the *M. pulegium* EO was safe at low concentrations for aquatic organisms and it could be used safely as eco-sustainable bio-herbicides (Caputo et al., 2021). *M. pulegium* EO showed important allelopathic potential against the germination of *Medicago sativa* seeds, with fewer germination percentages than control (Ahmed et al.,

2018). The phytotoxic potential of *M. pulegium* EOs was demonstrated on dicotyledonous and monocotyledonous plant species, inhibiting the germination and the radical elongation of this species' seeds (Caputo et al., 2021).

Concerning other bioactivities of *M. pulegium* extracts, the results showed that the extracts and fractions of *M. pulegium* exhibited high inhibitory activity against both α-amylase and α-glucosidase enzymes and in some cases, had a higher effect than the standard acarbose (Abbou et al., 2022; Gülçin et al., 2020). The inhibition of both carbohydrate-hydrolysing enzymes allows for the reduction of postprandial blood glucose levels, and this can be an important therapeutic strategy to manage hyperglycaemia associated with diabetes (Gülçin et al., 2020). The extracts and fractions of the *M. pulegium* demonstrated a high photoprotective effect against exposure to ultraviolet (UV) rays, which can be associated with some skin disorders, such as inflammation, carcinogenesis, immunosuppression, wrinkles, and premature ageing of the skin (El Aanachi et al., 2021; Yakoubi et al., 2021b). Finally, the methanolic extract of *M. pulegium* also showed inhibition of proliferation in human glioblastoma (U87) cells (Jebali et al., 2022).

1.3.4 *Pinus pinaster*

Pinus is the most common genus of the conifers in the Pinaceae family with more than 250 species recognized (Khouja et al., 2021; Maimoona et al., 2011). *Pinus pinaster*, also known as maritime pine, is widespread in Europe, mainly in the Atlantic and Mediterranean regions, and in some north-western African countries (Ferreira-Santos et al., 2020; Fkiri et al., 2019). *P. pinaster* can reach 30 m tall and it has bright reddish-brown bark, the needles are paired and its cones are brown, oval, and up to 2 cm long (Maimoona et al., 2011).

In folk medicine, the EO of the *P. pinaster* needles is specially used because it has cardiovascular benefits, reduces cholesterol, enhances microcirculation by increasing capillary permeability, as well as in the treatment of respiratory infection, and as an antioxidant and anti-inflammatory (Fkiri et al., 2019).

The chemical composition of the *P. pinaster* revealed that the monoterpene hydrocarbons α-pinene and β-pinene were the dominant volatiles compounds found in the *P. pinaster* EO followed by δ-3-carene, β-caryophyllene, and germacrene D (Gonçalves et al., 2020; Hmamouchi et al., 2001; Rodrigues et al., 2017). Analysing the composition of the EO obtained from different *P. pinaster* parts, the major compound found was the α-pinene in cones, needles, and wood's EO, with a higher amount found in the wood's EO. In the needles EO, the major compounds found after α-pinene were β-pinene, β-myrcene, *trans*-caryophyllene, α-amorphene, rimuene, cupressene, abietatriene, and abietadiene. β-

pinene, δ-3-carene, limonene, junipene, and *trans*-caryophyllene were also found in cones' EO. The β-pinene, limonene, α-terpineol, and junipene were also present in high quantities in the EO of wood (Tümen et al., 2018). However, the EO composition of *P. pinaster* can suffer variations, accordingly to the varieties, for example the EO of *P. pinaster* needles of the var. Renoui and var. Maghrebiana revealed as major compounds the *trans*-caryophyllene, α-amorphene, β-cadinene, abietane, Δ-cadinene and sclarene (Fkiri et al., 2019).

When considering different extracts, *P. pinaster* bark extracts obtained with ethanol at 50% exhibited high DPPH and ABTS radical scavenging activities followed by the extracts obtained using ethanol 70%, ethanol 30%, water and ethanol 90%. The *P. pinaster* bark extracts also showed potent reducing power being the extract obtained with ethanol 50% also the extract with the higher activity and followed by the other extracts (Ferreira-Santos et al., 2020). Regarding the EO, different samples of *P. pinaster* EO have presented good antioxidant activity and the Renoui variety had the highest activity when compared to Magrebiana variety (Fkiri et al., 2019)

Few assays have been performed to assess the antimicrobial activity of *P. pinaster*, with its hydroethanolic and aqueous extracts demonstrating potent antibacterial activity against Gram-positive bacteria and weaker activity for Gram-negative bacteria and fungi (Ćurković-Perica et al., 2015; Ferreira-Santos et al., 2020). The *P. pinaster* EO showed to have also good antibacterial activity against several bacterial species, such as *E. coli*, *Klebsiella pneumoniae*, *L. monocytogenes* and *S.* Typhimurium (Fkiri et al., 2019; Hmamouchi et al., 2001), but with no antifungal activity against *Aspergillus flavus*, *A. niger* and *Candida albicans* (Fkiri et al., 2019).

The *in vivo* wound healing potential of the EOs of *P. pinaster* (cone, needle, and wood) revealed that the EO sample obtained from the cones of *P. pinaster* was more active than the other extracts in the enzyme inhibition of hyaluronidase, collagenase, and elastase enzymes. Keeping these enzymes at a minimal level is important to proper wound healing. The major cause of tissue damage is the increase of free radicals and consequently, the increase of oxidative stress and massive quantities of free radicals are produced in the wound area, damaging the healing process. These EOs of *P. pinaster* presented good antioxidant activity, nonetheless with some organic extracts showing better activity (Tümen et al., 2018).

The information found about the bioactive properties of *P. pinaster* extracts and EO is limited and is mainly about a commercial product, Pycnogenol®, a nutritional supplement based on an aqueous extract prepared from French marine pine bark. Regarding the anti-viral of *P. pinaster*, Pycnogenol® inhibited the replication *in vitro* of human

immunodeficiency virus type-1 (HIV-1), suggesting its potential as a new anti-HIV-1 agent (Feng et al., 2008). This extract can reduce the hyperglycaemia associated with diabetes mellitus, obesity-induced by diet and in the control of postprandial hyperglycaemia (Fernando et al., 2019; Ferreira-Santos et al., 2020), presenting higher activity than the control acarbose (Ferreira-Santos et al., 2020). Besides, Pycnogenol® showed protective effect at beginning of diabetic retinopathy, visual improvement, reduce the risk of cataract formation and it was effective in the treatment of diabetic ulcers in the lower limb (Belcaro et al., 2006; Kamuren et al., 2006). Regarding the effects on human skin, the extract from the *P. pinaster* bark, Pycnogenol®, is appropriate for photoprotection of human skin against UV radiation, but also the oral intake of this extract affects several basic skin functions that are of relevance for the maintenance of healthy skin as can be seen in the information reviewed by Grether-Beck et al. (2016). Pycnogenol® also demonstrated anti-inflammatory activity (Canali et al., 2009; Cho et al., 2001), anticancer activity (Becit and Aydin, 2020; Ferreira-Santos et al., 2020; Harati et al., 2015; Huang et al., 2005; Yang et al., 2014, 2016), neurodegenerative protection (Ishrat et al., 2009) and it has been used to treat cardiovascular problems (Cesarone et al., 2006a, 2006b; Zibadi et al., 2007). All these results suggest that *P. pinaster* extracts can be useful therapeutic agents in the treatment of many diseases.

1.3.5. *Ruta graveolens*

Ruta genus, common name rue, belongs to Rutaceae family plants and it is a strongly fragrant subshrubs native to the Mediterranean region (Hammami et al., 2015; Morton and Telmer, 2014; Wei et al., 2012, 2015). The genus *Ruta* includes ten species of perennial shrubs, of which *R. graveolens* is one of the most common (Meloni et al., 2013). *R. graveolens* is an evergreen subshrub mainly found in temperate and tropical regions that can reach 30-80 cm and are native to the Mediterranean region more specifically in Southern Europe and can be distributed in Europe and many Asian countries, including China, India, and Japan (Gentile et al., 2018; Hale et al., 2004; Kostova et al., 1999; Ratheesh et al., 2009; Salvo et al., 2008). The stems are much ramified. The leaves have 4-11 cm long and 3-7 cm wide, oblong, small, deeply divided, glandular dotted, pinnate, bluish-green, emit a powerful odour and have a bitter taste. The flowers are yellow, small, and not fringed with 4 petals, except for the central flower, which has 5 petals and possesses ten stamens. The flowering period in Algeria is in June and in Chine is from March to June. The fruits are brown, lobulated, rounded, and small (Farzaei et al., 2017; Gentile et al., 2018; Haddouchi et al., 2013; Hale et al., 2004; Harat et al., 2008; Khori et al., 2008; Pollio et al., 2008; Ren and Tang, 2012).

These plants are used in traditional medicine for different purposes, such as veterinarian uses (roundworms, tapeworms, treatment for endoparasites) (Lans et al., 2007). The fresh extract or infusion of the leaves are used in contraception, as anti-inflammatory, sedative, antifungal, analgesic, antispasmodic, antihelmintic and abortive agent (Harat et al., 2008). The whole plant is used as antidotes for some snake and scorpion venoms and to treat many infections and inflammation and also against stomachache, vomit, diarrhea, snake bite, wounds, cough, fever, headache, inflammation, nervousness, flu, toothache, body pain, ear pain, eczema, ulcers, arthritis, fibromyalgia, abortifacient drug, faintness, cramp, hysteria, diseases of the womb (Alonso-Castro et al., 2012; Ciganda and Laborde, 2003; Ghosh et al., 2014; Koblovská et al., 2008; Mancuso et al., 2015). The deccoctions of the whole plant were used in palpitations and heart protection (Seak and Lin, 2007).

The analysis of the composition of *R. graveolens* EO indicates that 2-undecanone, 2-nonanone and 2-decanone are the main constituents (Da Silva et al., 2014; Faria et al., 2013, 2016a,b; Laquale et al., 2015; Mancuso et al., 2015; Orlanda and Nascimento, 2015; Soares et al., 2016).

R. graveolens demonstrated several pharmaceutical properties, associated with their bioactive compounds, including the antioxidant activity. The *R. graveolens* EO was able to reduce the free radical DPPH with comparable activity to that of the control delta-tocopherol, but lower to butylated hydroxyanisole (BHA) and BHT, and also reduces the lipid peroxidation (Jianu et al., 2021).

In relation to antibacterial activity, it was described that the EO of *R. graveolens* has activity against Gram-positive and Gram-negative bacteria, such *Bacillus megaterium*, *B. cereus*, *E.* coli, *Enterobacter aerogenes*, *Legionella pneumophila, M. flavus*, *Micrococcus luteus*, *P. aeruginosa, Salmonella* Typhi, and *S. aureus*, and also fungi (Chaftar et al., 2016; Jianu et al., 2021; Nahar et al., 2021; Orlanda and Nascimento, 2015; Owlia et al., 2009). The *R. graveolens* EO demonstrated a strong anti-*Legionella* activity against isolates from Tunisian spas and also reference strains and the authors suggested that the anti-*Legionella* activity may be due to the high content of 2-undecanone (Chaftar et al., 2015). The EO from *R. graveolens* also inhibited all the isolates of *Staphylococcus* and *Candida* species from patients with acute otitis externa (Nogueira et al., 2008). The effect of *R. graveolens* EO was tested on the growth and aflatoxin production of *Aspergillus parasiticus* and the results showed that the EO of *R. graveolens* inhibited the aflatoxin production, even if the fungal growth inhibition was low (Soares et al., 2016). These, suggesting that the *R. graveolens* EO may interfere with the virulence factors of microorganisms, such as production of mycotoxins and biofilms (Donadu et al., 2021; Mahmoud et al., 2020). Further, the *R.*

graveolens EO showed synergic effects when combined with the antifungal compound amphotericin B against *Candida* species (Donadu et al., 2021).

Regarding anticancer activity, the *R. graveolens* EO were tested against several cancer cell lines, including human colon adenocarcinoma (HT-29) cell line, human T lymphocyte (Jurkat) cell line, HeLa cell line, and human bladder carcinoma (T24) cell line, as well as normal cells of human embryonic kidney (HEK-293) cell line. This EO showed the highest antiproliferative activity against HeLa cells, weak activity against HT-29, Jurkat, MCF-7, and T24 cells and no antiproliferative activity against the normal cell line. 2-Undecanone and 2-nonanone tested alone reveal better antiproliferative activity against all the cell lines than the EO (Mahmoud et al., 2020). Possibly the interaction between the compounds in the EO interfered with the effect of this compounds alone.

The *R. graveolens* leaves EO showed larvicidal activity against third-instar larvae of *Aedes aegypti*, fourth-instar larvae of *Culiseta longiareolata*, second-instar larvae of *Plutella xylostella*, and juvenile nematodes of *Meloidogyne incognita* (Bouabida and Dris, 2020; Da Silva et al., 2014; Song et al., 2022), repellent efficacy against *Sitophilus zeamais* (Perera et al., 2022), but also fumigant activity against insect pest of stored grains *Ephestia kuehniella* (Bouzeraa and Labed, 2019). The composition can change depending on several environmental factors and influence the biological activity of the plant. For example, when *R. graveolens* EO was investigated basis on seasonal variation (October 2009 to September 2010), the results showed that during dry periods the major compounds (2-undecanone and 2-nonanone) were present in higher levels enhancing the larvicidal and nematocidal activities of the EO (Da Silva et al., 2014).

1.3.6 *Thymus mastichina*

The genus *Thymus* has 214 species and 36 subspecies, for which the main habitat is the Mediterranean region with some species being endemic to the Iberian Peninsula (Araujo et al., 2021). *T. mastichina* (Lamiaceae), commonly known as mastic thyme, white thyme or Spanish marjoram (Arantes et al., 2019; Araujo et al., 2021; Gordo et al., 2012), is an endemic species from the Iberian Peninsula and can be found in uncultivated, ruderal, and rupicolous lands, clearings of cultivated fields, xerophilic grasses, thickets zones, cork oaks and pine forests, rocky and dry outcrops and berms of roads and it is strongly resistant to frost, pests and diseases (Arantes et al., 2019; Gordo et al., 2012; Rodrigues et al., 2020). *T. mastichina* is a semi-woody shrub that grows up to 50 cm tall, the leaves are simple and opposite, the flowers are bilabiate grouped in flower heads or capitula and inflorescences

with 10 mm in diameter and blossoms from April to June (Arantes et al., 2019; Cutillas et al., 2018b; Rodrigues et al., 2020).

T. mastichina leaves in fresh or dry form are traditionally used as condiments and spices, in seasoning dishes and salads, as a salt substitute, to aromatize olive oil and to preserve olives (Gordo et al., 2012; Rodrigues et al., 2020; Taghouti et al., 2020). Infusions with dry parts of the *T. mastichina* have been used to relieve cough, flu, colds, throat irritations, hoarseness, acute nasopharyngitis, to treat gastrointestinal problems, such as gastric ulcer, functional dyspepsia, abdominal and pelvic pain, nausea, and vomiting and to wash open wound of unspecified body region and localized oedema (Gordo et al., 2012; Rivera et al., 2019; Rodrigues et al., 2020). The infusions with fresh parts are used for indigestion and stomach pain (Rodrigues et al., 2020). The *T. mastichina* flowering aerial part when fried in oil has been used to wash burns and corrosions and other dermatologic disorders. The decoctions of *T. mastichina* are used to rub rheumatism areas (Rivera et al., 2019), while the EO has been used in the cosmetic and perfume industries (Rodrigues et al., 2020; Taghouti et al., 2020).

Regarding the chemical composition, *T. mastichina* EO is especially represented by oxygenated monoterpenes, followed by hydrocarbon monoterpenes, with 1,8-cineole as the main compound (Aazza et al., 2016; Arantes et al., 2019; Ballester-Costa et al., 2013; Delgado et al., 2014; Figueiredo et al., 2010; Hwa-Jung Choi, 2018; Vieira et al., 2017). Some monoterpene hydrocarbons, such as α-pinene, β-pinene, and camphene were also detected, but in fewer amounts (Araujo et al., 2021; Delgado et al., 2014; Figueiredo et al., 2010). The *T. mastichina* showed two chemical polymorphisms, the 1,8-cineole and linalool chemotypes, although, the relative amount of 1,8-cineole was always high (Faleiro et al., 2003; Figueiredo et al., 2010). Even *T. mastichina* EO samples obtained from different parts of the plant were characterized by their richness in 1,8-cineole and the percentages were higher in the EO obtained from the flowers than the leaves (Faleiro et al., 2003).

T. mastichina is recognised for its bioactive properties. Comparing extracts and EOs, extracts have shown greater antioxidant activity (Albano et al., 2012; Delgado et al., 2014; Taghouti et al., 2020). The antioxidant activity of the EO of *T. mastichina* have been screened by different methods. When evaluating the radical scavenging activity, the *T. mastichina* EO revealed weak activity (Aazza et al., 2016; Bentes et al., 2009; Delgado-Adámez et al., 2017; Miguel et al., 2004). Also, it showed weak or no activity in preventing lipid peroxidation, it was not able to chelate the Fe (II), scavenge superoxide radicals or act as reducing agents (Aazza et al., 2016; Arantes et al., 2017; Bentes et al., 2009). However, depending on the *T. mastichina* EO samples, this EO can also demonstrate high activity to

protect the lipid substrate in the β-carotene/linoleic acid system, ability to act as DPPH radical scavengers, and act as ferric reducers (Arantes et al., 2017, 2019; Araujo et al., 2021).

The *T. mastichina* extracts and EO have exhibited good antimicrobial activity (Arantes et al., 2019; Araujo et al., 2021; Ballester-Costa et al., 2013; Leal et al., 2013) even against pathogenic fungi of vegetables and mushrooms (Diánez et al., 2018). The EO of *T. mastichina* showed good antibacterial activity against *S. aureus*, *B. subtilis*, *E. coli*, *P. aeruginosa* and *S. pneumoniae* with the most resistant species being *P. aeruginosa* (Vieira et al., 2017). Comparing the EO of the leaves and flowers of the *T. mastichina*, the EO of leaves had higher activity than flower EO against *E. coli*, *L. monocytogenes, Proteus mirabilis*, *Salmonella* sp., *S. aureus*, and *C. albicans* (Faleiro et al., 2003). However, there are also some reports of weak antibacterial activity of the *T. mastichina* EO (Delgado-Adámez et al., 2017). When tested for its activity against the biofilm formation of *E. coli*, S. *aureus* and *P. aeruginosa*, it was found that the MIC value of *T. mastichina* EO was able to inhibit its formation. Furthermore, in the presence of the subinhibitory concentrations, 1/2× MIC and 1/4× MIC, the EO of *T. mastichina* EO was quite effective in the inhibition of the biofilm formation by *S. aureus* (Vieira et al., 2017).

The *T. mastichina* EO also has anti-influenza activity with this EO exhibiting a higher anti-influenza activity than the positive control oseltamivir and no cytotoxicity to the Madin-Darby canine kidney (MDCK) cell line used, at a concentration of 100 μg/mL, unlike oseltamivir which demonstrated cytotoxicity at this concentration (Hwa-Jung Choi, 2018).

The *T. mastichina* EO showed a low ability for inhibiting lipoxygenase, in contrast with the activity of water decoction extracts of *T. mastichina* (Aazza et al., 2016; Albano et al., 2012). However, *T. mastichina* EO revealed nitric oxide radical scavenging capacity and taking into account that nitric oxide free radical is implicated as a mediator of inflammatory responses, *T. mastichina* EO reveals good anti-inflammatory activity through this mechanism of action (Aazza et al., 2016).

Regarding the inhibition of enzymes, the EO of *T. mastichina* showed activity against problems associated with diabetes and neurodegenerative diseases. *T. mastichina* EO presented a weak ability for inhibiting the enzyme α-amylase, but good activity against the enzyme α-glucosidase. The inhibition of α-amylase and α-glucosidase slows and prolongs the release of glucose into circulation and can retard hyperglycaemia after the consumption of a meal (Aazza et al., 2016). The inhibition of AChE and BChE enzymes are important in the treatment of Alzheimer's disease since are considered a potential target, and the aqueous extracts and EO of *T. mastichina* showed high anticholinesterase activity with a high capacity to inhibit these cholinesterases (Albano et al., 2012; Arantes et al., 2017; Cutillas et al., 2018b).

With regard to anticancer activity, the *T. mastichina* EO decrease the cell viability of the human breast carcinoma (MDA-MB-231) cells in dose-dependent (Arantes et al., 2019). Moreover, when the tumour cell lines, HeLa cell line and free-floating cells from human histiocytic leukaemia (U937) cell line were exposed to *T. mastichina* EOs, the results evidenced a substantial statistically significant cytotoxic effect in a dose-dependent in both tumour cell lines (Delgado-Adámez et al., 2017).

The *T. mastichina* can also act as a natural insecticidal or repellent. In fact, the EO from this plant revealed good insecticidal efficacy against the western flower thrips (*Frankliniella occidentalis* Perg.), one of the most economically important pests of greenhouse plants (Stepanycheva et al., 2019). The fumigant activity of the *T. mastichina* EO is highly toxic to the third instar of *Spodoptera littoralis* larvae even after topical application (Pavela, 2005), and also proved to be effective in the mortality of the house fly (*M. domestica* L.) (Pavela, 2008).

Chapter 2

Biological properties of *Thymus zygis* essential oil with emphasis on antimicrobial activity and food application

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Abstract

The *Thymus* plants have been used for centuries in traditional medicine and as a food spice, among this genus, *Thymus zygis* (red thyme) is a widespread plant, vastly used as a culinary flavouring agent. Its essential oil has demonstrated diverse bioactive properties, such as antimicrobial, insecticidal, larvicidal and antiparasitic activities. Numerous studies have characterized this essential oil showing that it possesses a broad antimicrobial spectrum and may even enhance the effect of certain antimicrobial agents. Its potential application as a food preservative has been analysed on different matrixes pointing to its antimicrobial activity against spoilage and pathogenic microorganisms in food. This review provides an insight in the chemical composition, antimicrobial, insecticidal, larvicidal and antiparasitic activities and toxicity of *T. zygis* essential oil, as well as its potential application in food as a preservative.

Keywords: *Thymus zygis*; essential oil; antimicrobial activity; food preservative

2.1. Introduction

Foodborne spoilage and pathogenic bacteria are major concerns in the food sector contributing to food quality and safety issues, with foodborne diseases constituting a global health problem (Munekata et al., 2020; Pateiro et al., 2021; Takó et al., 2020). Moreover, the resistance of food pathogens to adverse conditions, such as heat, cold, acidic, or high salt, and their capacity to form biofilms on biotic or abiotic surfaces, can facilitate their growth, persistence and spread on food contact surfaces (Takó et al., 2020).

Further, the short shelf life of fresh food, that causes heavy losses from harvesting to final consumption, is one of the most important problem of the food industry (Sapper et al., 2019; Van Haute et al., 2016). To maintain the safety and prolong the shelf-life of food, the food industry uses synthetic preservatives widely applied to eliminate bacteria or molds and also to control the lipid oxidation (Ballester-Costa et al., 2017). Synthetic chemicals like aromatic hydrocarbons, sterol biosynthesis inhibitors, benzimidazoles, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butyl hydroquinone (TBHQ), calcium propionate, sorbate, benzoate and others have been used as preservatives agents for many years (Álvarez et al., 2012; Debonne et al., 2018b; Moghaddam et al., 2015; Van Haute et al., 2016). Although synthetic antioxidants and antimicrobials are widely used in foods, their presence is associated with suspected carcinogenic problems over a long-term period of consumption. Therefore, the rejection by the consumers of synthetic food additives is growing (Ballester-Costa et al., 2017; Ghabraie et al., 2016; Nieto et al., 2010).

Increasing interest for the use of natural products safer for human health has been observed, promoting the search for alternatives to synthetic compounds aiming to limit their use, and so plant-based essential oils and extracts have been suggested to control pathogenic and spoilage microorganisms (Ballester-Costa et al., 2017; Hernández et al., 2015; Moghaddam et al., 2015). In fact, the study of the antimicrobial properties has been directed to different sources, such as spice extracts, plant and microbial metabolites, food subproducts, among others, considering its potential for food application (Ghabraie et al., 2016). The traditional application of spices and aromatic plants to preserve food has been observed for centuries, providing them special attention in recent years, not only for their antioxidant power but also for their antimicrobial properties (Álvarez et al., 2012; Ghabraie et al., 2016).

Essential oils (EOs) consist of secondary metabolites that can protect plants from environmental threats, pathogenic microorganisms, among others, being mixtures of phenols, monoterpenes, sesquiterpenes, and other compounds from aromatic plants (Ballester-Costa et al., 2017; Sangha et al., 2017). EOs are aromatic oily liquids derived from plant materials like leaves, buds, flowers, seeds, bark, roots, twigs, herbs, wood, and fruits (Ghabraie et al., 2016; Lee et al., 2018). They have been traditionally used for natural therapeutics, food preservation, and complementary medicines and as culinary flavouring agents since their aroma and flavour are familiar and widely accepted by consumers (Ballester-Costa et al., 2017; Fratianni et al., 2010). Nowadays, approximately 3000 EOs are known and some of them are commercially important, being employed in agronomic, cosmetic, chemical, perfume, fragrance, pharmaceutical and food industries due their potential bioactivities (Cutillas et al., 2018a; Ghabraie et al., 2016; Lagha et al., 2019). EOs have been generally recognized as safe (GRAS) (Ballester-Costa et al., 2016), and some are approved by the U.S. Food and Drug Administration (FDA) to be used as food additives, such as lemon balm, basil, coriander, clove, thyme and vanilla EOs (FDA, 2019). Thereby, they are gaining interest for their potential as preservatives and as decontamination agents (Ballester-Costa et al., 2016), while their potential as antimicrobial and antioxidant agents provide the basis for many applications in processed and raw food preservation (Ballester-Costa et al., 2013).

Therefore, the present review aims to provide the available information on the biological properties of *T. zygis* EO, with focus in its antimicrobial activity and the application as food preservative, as well as on the effect on animal with diets supplemented with this EO.

2.2. *Thymus* plants

Thymus plants belong to the *Lamiaceae* family and are a botanical genus predominantly found in the Mediterranean region (Afonso et al., 2018), North Africa, Asia (Ballester-Costa et al., 2013; Cutillas et al., 2018a) and Southern Europe, growing wild mostly in the Iberian Peninsula (Gonçalves et al., 2010; Pérez-Sánchez et al., 2007). As these plants present a high capacity to adapt to extreme climate conditions, like temperature and water supply, they can be often found in stones or rocks growing in arid and cold conditions (Afonso et al., 2018).

The genus *Thymus* comprises around 350 species (Ballester-Costa et al., 2013; Cutillas et al., 2018a) of perennials, shrubs or subshrubs, and aromatic herbs with 10-30 cm tall, with simple and small leaves, a quadrangular stem, prostrated and ramified branches and big clusters of small white, pink, purple or cream flowers (Afonso et al., 2018; Ballester-Costa et al., 2013). There are different ecotypes of these plants, which differ in their composition of EOs and their morphological characteristics, although all of them are characterized by a moderate odour and sometimes a very pronounced spicy and balsamic flavour (Cutillas et al., 2018a).

The *Thymus* species have been widely applied in food seasoning and flavouring, for perfumery, cosmetic as well as in medicine (Ballester-Costa et al., 2013; Schött et al., 2017), but only a few species are considered economically important, particularly those used as spices, such as the leaves of *T. zygis* and *T. vulgaris* (Schött et al., 2017). Moreover, *Thymus* EO is also used in many industries such as food, pharmaceuticals, detergents, personal health care, and insecticides (Afonso et al., 2018).

As other herbal medicines that are used in folk medicine in diverse countries, and play a vital role in treatment of diseases (Moghaddam et al., 2015), *Thymus* species have been used to treat respiratory and throat ailments (Afonso et al., 2018; Aminkhani et al., 2019), gastrointestinal problems and as effective therapies for irritating coughs and bronchitis due their expectorant and antitussive properties (Santoyo et al., 2014). These plants also have been used to treat skin problems, such as oily skin, acne, dermatitis, eczema, parasite affections, fungal infections, and insect bites (Afonso et al., 2018). Dried *Thymus* leaves are used for infusions, as well as in other products such as bath soap, lotion, and toothpaste (Afonso et al., 2018).

As reviewed by Figueiredo et al. (2008), different species of the genus *Thymus* have been used to the treatment of various problems, with *T. zygis* being mainly used as tea against colds and sore throat and as digestive tonic for medical purposes, for cosmetic use in hair and skin creams and lotions and as condiment, to season food (Figueiredo et al., 2008).

Aerial parts, stem, flowers, branches, leaves, and even the whole plant were used to make infusions and decoctions, for oral administration or external application (El Yaagoubi et al., 2021). The leaves of *T. zygis* have been used as a decoction for diarrhea, as carminative and colds and influenza (Benlamdini et al., 2014; El Azzouzi and Zidane, 2015; El Hafian et al., 2014; Hachi et al., 2016), while the infusion of the leaves can be used to treat gastrointestinal infections (El Hafian et al., 2014). The infusions with flowers and leaves have been applied against gastralgia, in case of colds (El Azzouzi and Zidane, 2015), and decoctions of flowers was consumed to treat pain in the digestive tract and flu (El Hafian et al., 2014).

2.3. Phytochemical composition of *Thymus zygis* essential oil

Regarding the chemical composition of *T. zygis*, studies regarding it essential oil, describe more than 60 bioactive compounds (Table 2.1), with the most common chemotype of this plant being thymol and linalool (Gonçalves et al., 2010; Lagha et al., 2019; Rota et al., 2008). Thymol is one of the major constituents of *T. zygis* EOs (Ballester-Costa et al., 2013; Dorman and Deans, 2004; Ghabraie et al., 2016; Machado et al., 2010; Marinković et al., 2020; Nieto et al., 2010; Peñalver et al., 2005; Pina-Vaz et al., 2004; Schött et al., 2017; Van Haute et al., 2016), and has been pointed as one component of great interest, due to its recognized properties, such as antimicrobial activity (Hernández et al., 2015). *p*-Cymene is also an important compound in the different samples, being one of the most common compounds, after thymol (Ghabraie et al., 2016; Gonçalves et al., 2010; Machado et al., 2010; Van Haute et al., 2016).

Table 2.1. Phytochemical composition of *Thymus zygis* essential oil.

Compound[a]	**Area (%)**	**Reference**
Thymol	0.52 – 55.91	(Ballester-Costa et al., 2013; Dorman and Deans, 2004; Lagha et al., 2019; Machado et al., 2010; Marinković et al., 2020; Pina-Vaz et al., 2004; Schött et al., 2017; Van Haute et al., 2016)
Linalool (β-linalool)	2.9 – 40.14	(Ballester-Costa et al., 2013; Dorman and Deans, 2004; Kim et al., 2016; Lagha et al., 2019; Machado et al., 2010; Marinković et al., 2020; Pina-Vaz et al., 2004; Schött et al., 2017; Van Haute et al., 2016)
p-Cymene	2.2 – 36.6	(Ballester-Costa et al., 2013; Dorman and Deans, 2004; Kim et al., 2016; Lagha et al., 2019; Machado et al., 2010; Marinković et al., 2020; Pina-Vaz et al., 2004; Schött et al., 2017; Van Haute et al., 2016)

Table 2.1. Phytochemical composition of *Thymus zygis* essential oil (continuation).

Compound[a]	Area (%)	Reference
Carvacrol	0.08 – 25.0	(Ballester-Costa et al., 2013; Dorman and Deans, 2004; Gonçalves et al., 2010; Kim et al., 2016; Lagha et al., 2019; Machado et al., 2010; Marinković et al., 2020; Pina-Vaz et al., 2004; Schött et al., 2017; Van Haute et al., 2016)
γ-Terpinene	3.13 – 21.0	(Ballester-Costa et al., 2013; Dorman and Deans, 2004; Kim et al., 2016; Lagha et al., 2019; Machado et al., 2010; Pina-Vaz et al., 2004; Schött et al., 2017; Van Haute et al., 2016)
Geraniol	19.8	(Gonçalves et al., 2010)
1,8-Cineol (terpan, eucalyptol)	0.3 – 12.0	(Dorman and Deans, 2004; Gonçalves et al., 2010; Kim et al., 2016; Marinković et al., 2020; Schött et al., 2017; Van Haute et al., 2016)
Terpinen-4-ol (terpinene-4-ol; terpineol-4)	0.2 – 11.7	(Ballester-Costa et al., 2013; Dorman and Deans, 2004; Kim et al., 2016; Lagha et al., 2019; Machado et al., 2010; Marinković et al., 2020; Pina-Vaz et al., 2004; Schött et al., 2017; Van Haute et al., 2016)
Myrcene (β-myrcene)	0.97 – 8.6	(Ballester-Costa et al., 2013; Dorman and Deans, 2004; Kim et al., 2016; Lagha et al., 2019; Machado et al., 2010; Marinković et al., 2020; Pina-Vaz et al., 2004; Schött et al., 2017; Van Haute et al., 2016)
Terpinene (α-terpinene)	0.4 – 5.8	(Ballester-Costa et al., 2013; Dorman and Deans, 2004; Kim et al., 2016; Lagha et al., 2019; Machado et al., 2010; Marinković et al., 2020; Pina-Vaz et al., 2004; Schött et al., 2017; Van Haute et al., 2016)
Cis and *trans*-thujan-4-ol (sabinene hydrate; *cis* and *trans*-4-thujanol; *cis* and *trans*-sabinene hydrate)	0.3 – 5.35	(Kim et al., 2016; Lagha et al., 2019; Machado et al., 2010; Marinković et al., 2020)
Borneol (isoborneol)	0.4 – 4.5	(Ballester-Costa et al., 2013; Dorman and Deans, 2004; Lagha et al., 2019; Machado et al., 2010; Marinković et al., 2020; Pina-Vaz et al., 2004; Schött et al., 2017; Van Haute et al., 2016)
Camphene	0.2 – 4.5	(Dorman and Deans, 2004; Gonçalves et al., 2010; Kim et al., 2016; Lagha et al., 2019; Machado et al., 2010; Marinković et al., 2020; Pina-Vaz et al., 2004; Van Haute, Raes, Van der Meeren, et al., 2016)
Camphor (L-camphor)	0.1 – 4.2	(Dorman and Deans, 2004; Kim et al., 2016; Lagha et al., 2019; Machado et al., 2010; Marinković et al., 2020; Pina-Vaz et al., 2004; Schött et al., 2017; Van Haute et al., 2016)

Table 2.1. Phytochemical composition of *Thymus zygis* essential oil (continuation).

Compound[a]	Area (%)	Reference
α-Pinene	0.81 – 3.8	(Ballester-Costa et al., 2013; Dorman and Deans, 2004; Kim et al., 2016; Lagha et al., 2019; Machado et al., 2010; Pina-Vaz et al., 2004; Van Haute et al., 2016)
Cis-caryophyllene (E-caryophyllene; β- caryophyllene)	1.0 – 3.6	(Ballester-Costa et al., 2013; Dorman and Deans, 2004; Kim et al., 2016; Lagha et al., 2019; Machado et al., 2010; Marinković et al., 2020; Pina-Vaz et al., 2004; Schött et al., 2017; Van Haute et al., 2016)
Limonene (D-limonene)	0.29 – 2.77	(Ballester-Costa et al., 2013; Dorman and Deans, 2004; Kim et al., 2016; Lagha et al., 2019; Machado et al., 2010; Marinković et al., 2020; Pina-Vaz et al., 2004; Schött et al., 2017)
Thujene (α-thujene)	0.1 – 2.6	(Ballester-Costa et al., 2013; Dorman and Deans, 2004; Lagha et al., 2019; Machado et al., 2010; Marinković et al., 2020; Pina-Vaz et al., 2004; Schött et al., 2017; Van Haute et al., 2016)
Terpineol (α-terpineol)	0.17 – 2.30	(Ballester-Costa et al., 2013; Dorman and Deans, 2004; Kim et al., 2016; Lagha et al., 2019; Machado et al., 2010; Pina-Vaz et al., 2004; Schött et al., 2017; Van Haute et al., 2016)
Terpinolene (α-terpinolene)	0.14 – 2.0	(Ballester-Costa et al., 2013; Dorman and Deans, 2004; Kim et al., 2016; Lagha et al., 2019; Machado et al., 2010; Marinković et al., 2020; Pina-Vaz et al., 2004; Van Haute et al., 2016)
β-Terpineol	0.3 – 1.9	(Dorman and Deans, 2004; Schött et al., 2017)
Pinene	0.2 – 1.9	(Marinković et al., 2020)
Bornyl acetate	0.07 – 1.9	(Dorman and Deans, 2004; Lagha et al., 2019; Marinković et al., 2020; Schött et al., 2017)
β-Pinene	0.1 – 1.3	(Ballester-Costa et al., 2013; Dorman and Deans, 2004; Gonçalves et al., 2010; Kim et al., 2016; Lagha et al., 2019; Machado et al., 2010; Pina-Vaz et al., 2004; Schött et al., 2017; Van Haute et al., 2016)
Geraniol acetate (geranyl acetate)	1.0	(Schött et al., 2017)
Caryophyllene oxide	0.4 – 1.0	(Marinković et al., 2020; Pina-Vaz et al., 2004; Schött et al., 2017)
Carvacrol methyl ether	0.4 – 0.9	(Marinković et al., 2020; Schött et al., 2017)
Sabinene	0.05 – 0.84	(Ballester-Costa et al., 2013; Lagha et al., 2019; Machado et al., 2010; Schött et al., 2017)
Ledol	0.8	(Schött et al., 2017)

Table 2.1. Phytochemical composition of *Thymus zygis* essential oil (continuation).

Compound[a]	Area (%)	Reference
β-Bisabolene	0.1 – 0.8	(Machado et al., 2010)
p-Menth-2.4(8)-diene	0.7	(Schött et al., 2017)
δ-Cadinene	0.2 – 0.7	(Marinković et al., 2020; Pina-Vaz et al., 2004; Schött et al., 2017)
Thymol methyl ether	0.6 – 0.62	(Marinković et al., 2020; Schött et al., 2017; Van Haute et al., 2016)
Linalyl acetate	0.5 – 0.62	(Kim et al., 2016; Lagha et al., 2019)
1-Octen-3-ol (oct-1-en-3-ol)	0.3 – 0.6	(Machado et al., 2010; Pina-Vaz et al., 2004; Schött et al., 2017)
p-Cymene-8-ol	0.2 – 0.6	(Pina-Vaz et al., 2004)
Sylvestrene (iso-sylvestrene)	0.1 – 0.53	(Marinković et al., 2020; Van Haute et al., 2016)
p-Thymol	0.5	(Schött et al., 2017)
Farnesol	0.5	(Schött et al., 2017)
Phellandrene (α-phellandrene)	0.1 – 0.48	(Lagha et al., 2019; Machado et al., 2010; Marinković et al., 2020; Pina-Vaz et al., 2004; Schött et al., 2017; Van Haute et al., 2016)
Spathulenol	0.4	(Schött et al., 2017)
Thuja-2,4(10)-diene	0.4	(Marinković et al., 2020)
Dihydrocarvone (*cis* and *trans*-dihydrocarvone)	0.17 – 0.4	(Gonçalves et al., 2010; Lagha et al., 2019; Marinković et al., 2020; Schött et al., 2017)
α-Caryophyllene (humulene; α-humulene)	0.1 – 0.4	(Marinković et al., 2020; Pina-Vaz et al., 2004; Schött et al., 2017)
γ-Cadinene	0.1 – 0.4	(Marinković et al., 2020; Pina-Vaz et al., 2004; Schött et al., 2017)
Alloaromadendrene (aromadendrene)	0.1 – 0.4	(Marinković et al., 2020; Schött et al., 2017)
E-β-Ocimene (β-*trans*-ocimene)	0.1 – 0.4	(Machado et al., 2010; Pina-Vaz et al., 2004; Schött et al., 2017)
Linalool oxide (*trans*-linalool oxide)	0.1 – 0.4	(Pina-Vaz et al., 2004; Schött et al., 2017)
Thymol acetate	0.1 – 0.4	(Marinković et al., 2020; Schött et al., 2017)
α-Amorphene	0.3	(Schött et al., 2017)
β-Phellandrene	0.3	(Machado et al., 2010)
Geranyl-isobutyrate	0.3	(Schött et al., 2017)
Thymoquinone	0.3	(Schött et al., 2017)
Viridiflorene	0.3	(Schött et al., 2017)
δ-Terpineol	0.1 – 0.3	(Gonçalves et al., 2010)
Trans-verbenol	0.1 – 0.3	(Gonçalves et al., 2010)

Table 2.1. Phytochemical composition of *Thymus zygis* essential oil (continuation).

Compound[a]	**Area (%)**	**Reference**
Cis and *trans*-*p*-menth-2-en-1-ol	0.1 – 0.25	(Lagha et al., 2019; Machado et al., 2010; Pina-Vaz et al., 2004)
2,5-Dimethylstyrene	0.2	(Pina-Vaz et al., 2004)
3-Octanol	0.2	(Schött et al., 2017)
α-Gurjunene	0.2	(Schött et al., 2017)
γ-Gurjunene	0.2	(Schött et al., 2017)
τ-Cadinol	0.2	(Schött et al., 2017)
3-Carene (δ-3-carene; Δ-3-carene)	0.1 – 0.2	(Machado et al., 2010; Marinković et al., 2020; Schött et al., 2017)
Trans-*p*inocarveol	0.1 – 0.2	(Gonçalves et al., 2010)
Tricyclene	0.1 – 0.2	(Gonçalves et al., 2010)
Cis and *trans* piperitol	0.08 – 0.18	(Lagha et al., 2019)
Bicyclogermacrene	0.16	(Lagha et al., 2019)
α-Copaene	0.1	(Pina-Vaz et al., 2004)
α-Cubebene	0.1	(Schött et al., 2017)
α-Muurolene	0.1	(Marinković et al., 2020; Pina-Vaz et al., 2004)
β-Bourbunene	0.1	(Pina-Vaz et al., 2004)
β-*Cis*-ocimene	0.1	(Schött et al., 2017)
β-Cubebene	0.1	(Schött et al., 2017)
Cymenene	0.1	(Machado et al., 2010)
Germacrene D	0.1	(Machado et al., 2010)
Germacrene-4-ol	0.1	(Schött et al., 2017)
Guaiol	0.1	(Schött et al., 2017)
Terpinyl-acetate	0.1	(Schött et al., 2017)
Verbenone	0.1	(Marinković et al., 2020)

[a] In parentheses are some synonyms of the names of the compounds or the *cis*/*trans* conformations.

Lagha et al. (2019) analysed the composition of *T. zygis* EO showing that its major constituents were linalool (39.7%), terpinen-4-ol (11.7%), β-myrcene (8.6%) and γ-terpinene (7.6%) (Lagha et al., 2019). In case of Kim et al. (2016), linalool (40.14%) was also identified as the most abundant component in *T. zygis* EO and terpinen-4-ol (8.83%), myrcene (5.88%), sabinene hydrate (5.35%), and *p*-cymene (4.14%) were identified as main components (Kim et al., 2016). The variability in the chemical composition of *T. zygis* EOs can depend on several factors, including local climatic and environmental conditions (rain,

temperature, sun, day length, etc.), geology, geographical location, season, nutrients, part of the plant and the method used to obtain the EO (Ballester-Costa et al., 2013).

In fact, the influence of the harvest location of the plants was demonstrated by Dandlen et al. (2011), who showed that when plants were collected, during the flowering period, on mainland Portugal and on Azores, the chemical composition of the essential oils varies. *p*-Cymene was present in considerable amounts in all EOs samples, whereas carvacrol dominates in three samples, and thymol appears as the second major component in the other two samples. γ-Terpinene and camphene were two other major components in these essential oils (Dandlen et al., 2011). Cutillas et al. (2018a) also showed the effect of the harvest in the composition of the EO (Cutillas et al., 2018a). These variations on the chemical composition of the essential oils clearly influence their bioactive properties.

2.4. Biological activities of *Thymus zygis* essential oil

The widespread application of *Thymus* plants has long been associated with their highly valuable products and it is closely associated with their high nutritional value and/or predominance of bioactive compounds (Afonso et al., 2018; Aminkhani et al., 2019). *Thymus* species have been described as possessing several pharmaceutical properties, associated to their bioactive compounds. Amongst these, *Thymus zygis*, also known as red thyme, is one of the aromatic medicinal plants widespread in the Iberian Peninsula (Ballester-Costa et al., 2013; Cutillas et al., 2018a) and it is vastly used as a culinary flavouring agent, with its flavour and aroma being familiar and widely accepted by consumers (Ballester-Costa et al., 2013). Furthermore, it has been recognized for its bioactive properties.

2.4.1 Antibacterial and antifungal activities

Currently, there has been a number of studies performed for the development of safer antimicrobial agents, such as plant-based EOs, to control pathogenic microorganisms (Moghaddam et al., 2015), with some relevance being given to *T. zygis*. In fact, antibacterial and antifungal activities have been described for the EO of *T. zygis* against a wide range of pathogens, including phytopathogens (Table 2.2). Accordingly, to several authors, *T. zygis* EO has a stronger activity against Gram-positive than Gram-negative bacteria (Cutillas et al., 2018a; Dussault et al., 2014; Ghabraie et al., 2016), possibly associated with the presence of lipopolysaccharide in the cell wall of Gram-negative bacteria that allows them to be more resistant to EOs (Lagha et al., 2019; Trombetta et al., 2005). Lipopolysaccharide present in the surface of the Gram-negative bacteria repels EOs (Dussault et al., 2014), while in the case of Gram-positive bacteria, the hydrophobic molecules can easily enter the cells and act

on the cell wall and within the cytoplasm (Lagha et al., 2019). However, not all the studies present results consistent with this difference amongst bacteria (Table 2.2).

The *in vitro* antibacterial effect of *T. zygis* EO was further supported by analysis of its bactericidal activity, showing that *T. zygis* EO affects the growth of *Streptococcus mutans*, reducing the culturability of this bacterium (Schött et al., 2017).

Among the variety of compounds that constitute EOs, volatile compounds may also have a relevant antimicrobial activity, in fact, this has been observed for volatile compounds of *T. zygis* EO totally or strongly inhibiting the growth of *L. monocytogenes* and *S. aureus* (78.0 ± 0.0 mm), respectively, by using a micro-atmosphere diffusion assay. The volatile compounds of the EO also showed high antibacterial effect against *E. coli*, presenting an inhibition diameter of 47.0 ± 1.4 mm and being less effective against *S.* Typhimurium (25.4 ± 1.1 mm), while presenting no activity against *P. aeruginosa*. Further, this work provides an example of possible application of the volatile compounds of the EOs as air decontaminants in storage rooms or they can be good candidates to be used in active packaging, while not changing the organoleptic properties of foods (Ghabraie et al., 2016).

The biological activity of EOs depends on their chemical composition that is determined by the genotype and influenced by environmental and agronomic conditions, which will affect their mode of action and bioactivity (Rota et al., 2008). Similarly, *T. zygis* EO shows a high degree of variability, depending on seasonal, phenological or climatic conditions (Cutillas et al., 2018a). In fact, the different *T. zygis* EO chemotype used influence its bioactivity, and for example, the MIC value obtained against *Aspergillus niger* ATCC 16404 with linalool type *versus* carvacrol type was 1.25 and 0.32 µL/mL, respectively (Gonçalves et al., 2010). Further, the species *S. aureus*, *E. coli* and *L. monocytogenes* were more susceptible to *ch.* thymol than to the *ch.* linalool (Cutillas et al., 2018a; Lee et al., 2018; Rota et al., 2008). Regarding *Candida* species, also the *ch.* thymol were more efficient than the *ch.* linalool (Cutillas et al., 2018a; Gonçalves et al., 2010).

It must also be noted that the antimicrobial activity may also change among subspecies. When the antimicrobial activity of *T. zygis* subsp. *zygis*, *T. zygis* subsp. *sylvestris*, *T. zygis* subsp. *gracilis* was evaluated against Gram-positive and Gram-negative bacteria and two fungi, a variation in the MIC of EO was observed, and for example, the MIC value found against *B. cereus* was 1.25, 0.62 and 0.31 mg/mL, respectively (Stanković et al., 2017). The same differences can be verified in another study, being the subsp. *zygis* more active in relation to the subsp. s*ylvestris* against seven of the nine bacterial species studied. In the case of yeast species, the subsp. s*ylvestris* was more efficient (Dandlen et al., 2011).

Table 2.2. Results of antimicrobial activity of *T. zygis* EO in disc/well diffusion assay and minimum inhibitory concentration (MIC).

Microorganism	Assays		Part of plant	Chemotype/ Subspecies/ Variety	Origin/ Supplier	Reference
	Disc diffusion	MIC				
Acinetobacter calcoaceticus	30.7 ± 0.5 mm		Aerial parts	NS	Commercial supplier	(Dorman and Deans, 2004)
Aeromonas hydrophila	complete inhibition					
Alcaligenes faecalis	53.8 ± 1.2 mm					
Bacillus subtilis	23.4 ± 1.2 mm					
Benecka natriegens	complete inhibition					
Brevibacterium linens	complete inhibition					
Brocothrix thermosphacta	complete inhibition					
Citrobacter freundii	complete inhibition					
Clostridium sporogenes	complete inhibition					
Enterobacter aerogenes	41.8 ± 0.8 mm					
Enterococcus faecalis	15.2 ± 0.7 mm					
Erwinia carotovora	35.8 ± 4.4 mm					
Escherichia coli	32.4 ± 0.1 mm					
Flavobacterium suaveolens	complete inhibition					
Klebsiella pneumoniae	31.8 ± 0.5 mm					
Lactobacillus plantarum	26.3 ± 0.4 mm					
Leuconostoc cremoris	complete inhibition					
Micrococcus luteus	complete inhibition					
Moraxella sp.	29.0 ± 5.6 mm					
Proteus vulgaris	complete inhibition					
Pseudomonas aeruginosa	33.5 ± 2.0 mm					
Salmonella pullorum	complete inhibition					
Serratia marcescens	39.1 ± 0.8 mm					
Staphylococcus aureus	complete inhibition					

Table 2.2. Results of antimicrobial activity of *T. zygis* EO in disc/well diffusion assay and minimum inhibitory concentration (MIC) (continuation).

Microorganism	Assays		Part of plant	Chemotype/ Subspecies/ Variety	Origin/ Supplier	Reference
	Disc diffusion	MIC				
Yersinia enterocolitica	25.5 ± 2.9 mm					
E. coli 01 (poultry origin)		40 µL/mL	NS	Thymol chemotype	Natural origin provided by different suppliers	(Peñalver et al., 2005)
Salmonella enteritidis RG2 (poultry origin)		20 µL/mL				
Salmonella Essen (poultry origin)		5 µL/mL				
E. coli enterotoxigenic (pig origin)		40 µL/mL				
Salmonella Choleraesuis (pig origin)		20 µL/mL				
Salmonella Typhimurium (pig origin)		20 µL/mL				
Salmonella enteritidis CECT 4155	10.0 to 32.2 mm	< 0.5 µL/mL	Aerial parts	Thymol chemotype	Experimental crop of the Murcian Institute of Investigation and Agricultural Development, Spain	(Rota et al., 2008)
S. Typhimurium CECT 443	28.3 to 30.3 mm	< 0.2 µL/mL		39% linalool chemotype		
E. coli (serovar O157:H7 CECT 4267 and CECT 516)	8.0 to 20.3 mm	< 0.2 to 1.5 µL/mL		82% linalool chemotype		
Listeria monocytogenes (serovar 4b CECT 935 and serovar ½c CECT 911)	11.0 to 44.0 mm	< 0.5 µL/mL				
Yersinia enterocolitica serotype O:8; biotype 1 CECT 4315	27.6 to 46.6 mm	< 0.2 to 0.5 µL/mL				
Shigella flexneri serovar 2a CECT 585	22.6 to 36.0 mm	< 0.2 to 1.0 µL/mL				
Shigella sonnei CECT 457	18.6 to 30.0 mm	< 0.2 to 1.0 µL/mL				
S. aureus CECT 239	18.3 to 25.0 mm	< 0.2 to 1.2 µL/mL				

Table 2.2. Results of antimicrobial activity of *T. zygis* EO in disc/well diffusion assay and minimum inhibitory concentration (MIC) (continuation).

Microorganism	Assays		Part of plant	Chemotype/ Subspecies/ Variety	Origin/ Supplier	Reference
	Disc diffusion	MIC				
Helicobacter pylori (two clinical isolated strains)	7.33 to 20.83 mm		Aerial parts	Subsp. *zygis* Subsp. *sylvestris*	Portugal and on the Azores Islands Pico, S. Jorge and Terceira	(Dandlen et al., 2011)
S. aureus CFSA2	6.67 to 8.67 mm					
L. monocytogenes (two cheese isolated strain and one clincal)	9.17 to 12.67 mm					
S. Typhimurium ATCC 14028	7.00 to 11.83 mm					
Haemophilus influenza ATCC 49247	11.33 to 16.67 mm					
Streptococcus pneumoniae D39	15.00 to 20.00 mm					
Candida albicans (one reference and one clinical strains)	0.00 to 13.17 mm					
Listeria innocua CECT 910	39.52 ± 0.22 mm	3.75 µl/mL	Leaves, stems, and flowers	NS	Supplied by Esencias Martinez Lozano (Murcia, Spain)	(Ballester-Costa et al., 2013)
Aeromonas hydrophila CECT 5734	17.26 ± 0.06 mm	3.75 µl/mL				
Achromobacter denitrificans CECT 449	15.82 ± 0.08 mm	3.75 µl/mL				
Alcaligenes faecalis CECT 145	51.35 ± 0.21 mm	3.75 µl/mL				
Enterobacter amnigenus CECT 4078	13.20 ± 0.23 mm	7.5 µl/mL				
Enterobacter gergoviae CECT 587	10.62 ± 0.13 mm	7.5 µl/mL				
Serratia marcescens CECT 854	16.43 ± 0.00 mm	3.75 µl/mL				
Shewanella putrefaciens CECT 5346	17.54 ± 0.33 mm	1.87 µl/mL				
Pseudomonas fragi CECT 446	12.04 ± 0.33 mm	3.75 µl/mL				
Pseudomonas fluorescens CECT 844	11.94 ± 0.11 mm	3.75 µl/mL				

Table 2.2. Results of antimicrobial activity of *T. zygis* EO in disc/well diffusion assay and minimum inhibitory concentration (MIC) (continuation).

Microorganism	Assays		Part of plant	Chemotype/ Subspecies/ Variety	Origin/ Supplier	Reference
	Disc diffusion	MIC				
L. monocytogenes HPB 2812		833 ppm	Leaves	Var. *gracilis* Boissier	Food ingredient supplier (BSA Food ingredients s.e.c/l.p, Montreal, Canada)	(Dussault et al., 2014)
S. aureus ATCC 29213		313 ppm				
Bacillus cereus LSPQ 2872		417 ppm				
S. Typhimurium SL 1344		2083 ppm				
E. coli O157:H7 EDL 933		1250 ppm				
P. aeruginosa ATCC 15442		3333 ppm				
L. monocytogenes HPB 2812	13.2 ± 3.1 mm	10000 ppm	Aerial part	NS	Spain	(Ghabraie et al., 2016)
S. aureus ATCC 29213	70.8 ± 3.1 mm	1250 ppm				
E. coli O157:H7 EDL 933	64.3 ± 4.9 mm	1250 ppm				
S. Typhimurium SL 1344	34.1 ± 6.4 mm	> 10000 ppm				
P. aeruginosa ATCC 15422	16.8 ± 2.6 mm	> 10000 ppm				
S. aureus ATCC 6538		0.01 to 0.03 mg/mL	NS	Subsp. *zygis* Subsp. *sylvestris* Subsp. *gracilis*	Spain, Caceres, La Garganta Spain, Badajoz, Guadajira Spain, Badajoz, Badajoz	(Stanković et al., 2017)
Sarcina lutea ATCC 9431		0.07 to 0.62 mg/mL				
B. cereus ATCC 11778		0.31 to 1.25 mg/mL				
E. coli ATCC 8739		2.50 to 5.00 mg/mL				
P. aeruginosa ATCC 9027		5.00 to 10.00 mg/mL				
S. enteritidis ATCC 13076		1.25 mg/mL				
C. albicans ATCC 10231		1.25 to 2.50 mg/mL				
Aspergillus niger (ATCC 16404		10.00 to 20.00 mg/mL				
S. aureus ATCC 6538		0.2 µL/mL to 1.3 µL/mL	NS	Thymol chemotype	Murcia (Spain)	(Cutillas et al., 2018a)
E. coli ATCC 8739		1.3 µL/mL to 2.5 µL/mL		Linalool chemotype		
P. aeruginosa ATCC9027		> 10 µL/mL				
C. albicans ATCC 1023		1.3 µL/mL to 2.5 µL/mL				

Table 2.2. Results of antimicrobial activity of *T. zygis* EO in disc/well diffusion assay and minimum inhibitory concentration (MIC) (continuation).

Microorganism	Assays		Part of plant	Chemotype/ Subspecies/ Variety	Origin/ Supplier	Reference
	Disc diffusion	MIC				
Five-strain cocktail of *L. monocytogenes* isolated from food		78.1 to 156.3 µL/L	Leaves Flowers	Thymol chemotype Linalool chemotype	Purchased from Neumond-Düfte der Natur GmbH (Raisting, Germany)	(Lee et al., 2018)
50 clinical *E. coli* isolates	Strong inhibitory activity on 90% of the isolates (≥22 mm)	0.19 mg/mL to 0.78 mg/mL	NS	NS	Purchased from Laboratoires OMEGA Pharma (Groupe Perrigo) – Phytosun Arôms (France)	(Lagha et al., 2019)
S. aureus DSM 1104 *E. coli* DSM 1103		0.05 mg/mL to 0.8 mg/mL 0.05 mg/mL to 0.4 mg/mL	Blooming herb	NS	Provided by three different German EO manufacturers	(Thielmann et al., 2019)
30 clinical MRSA isolates	Strong inhibitory activity on 80% of the isolates (≥22 mm)	0.39 mg/mL to 0.78 mg/mL	Aerial parts of flowering stage	Subsp. *zygis*	Purchased from Laboratoires OMEGA Pharma (Groupe Perrigo) – Phytosun Arôms (France)	(Abdallah et al., 2020)
Colistin-susceptible and colistin-resistant isolates of *Acinetobacter baumannii* and *Klebsiella pneumoniae*		256 mg/L to 1024 mg/L	NS	NS	Purchased from Acofarma	(Vázquez-Ucha et al., 2020)
Clinical isolates of *Streptococcus mitis*, *Streptococcus sanguinis*, and *Enterococcus faecalis*		1 mg/mL to 2 mg/mL	NS	NS	Purchased from Ayus GmbH, Bühl, Deutschland	(Marinković et al., 2020)

Table 2.2. Results of antimicrobial activity of *T. zygis* EO in disc/well diffusion assay and minimum inhibitory concentration (MIC) (continuation).

Microorganism	Assays		Part of plant	Chemotype/ Subspecies/ Variety	Origin/ Supplier	Reference
	Disc diffusion	MIC				
56 *Streptococcus suis* isolates belonging to pigs (n = 49) and humans (n = 6)		156.25 to 625 µg/mL	NS	NS	Purchased from AromiumTM (Barcelona, Spain)	(De Aguiar et al., 2021)
Leuconostoc citreum	22.2 ± 0.1 mm *	1.25 µL/mL	Flowering herb	NS	Purchased from Neumond-Düfte der Natur GmbH (Raisting, Germany)	(Lee et al., 2020)
C. albicans (one reference and two clinical strains)		0.16 – 0.32 µL/mL	Aerial parts	Subsp. *zygis*	Mirandela, Portugal	(Pina-Vaz et al., 2004)
Candida glabrata (two clinical isolates)		0.32 µL/mL				
Candida guillermondii MAT 23 (clinical isolated)		0.16 µL/mL				
Candida krusei H9 (clinical isolated)		0.16 – 0.32 µL/mL				
Candida parapsilosis ATCC 90018		0.32 µL/mL				
Candida tropicalis (one reference and one clinical strains)		0.16 – 0.32 µL/mL				

Table 2.2. Results of antimicrobial activity of *T. zygis* EO in disc/well diffusion assay and minimum inhibitory concentration (MIC) (continuation).

Microorganism	Assays		Part of plant	Chemotype/ Subspecies/ Variety	Origin/ Supplier	Reference
	Disc diffusion	MIC				
C. albicans ATCC 10231 (reference strain)		0.32 to 1.25 µL/mL	Aerial parts at the flowering stage	Subsp. *Sylvestris*	Central part of Portugal (Rabaçal, Eiras, Degracias and Covão do Feto)	(Gonçalves et al., 2010)
C. guillermondii MAT23 (clinical strain)		0.32 to 0.64 µL/mL		Geraniol chemotype		
C. krusei H9 (clinical strain)		0.32 to 1.25 µL/mL		Thymol chemotype		
C. parapsilosis ATCC 90018 (reference strain)		0.32 to 1.25 µL/mL		Linalool chemotype		
C. tropicalis ATCC 13803 (reference strain)		0.32 to 1.25 µL/mL		Carvacrol chemotype		
Cryptococcus neoformans CECT 1078		0.16 to 0.64 µL/mL				
Trichophyton mentagrophytes FF7		0.16 to 0.32 µL/mL				
Trichophyton rubrum CECT 2794		0.16 to 0.32 µL/mL				
Microsporum canis FF1		0.16 to 0.32 µL/mL				
Microsporum gypseum CECT 2908		0.16 to 0.32 µL/mL				
Aspergillus niger strains (clinical strain F01 and two reference strains ATCC 16404 and CECT 2574)		0.32 to 1.25 µL/mL				
Aspergillus fumigatus strains (three clinical strain F05, F07 and F17 and two reference strains ATCC 46645 and CECT 2071)		0.16 to 0.64 µL/mL				
A. flavus F44 (clinical strain)		0.64 to 1.25 µL/mL				

* Agar well diffusion assay; NS – not specified.

Furthermore supporting the relevance of plant' cultivation conditions, Dandle et al. (2011) studied the inhibitory activity of *T. zygis* EOs collected in different places on mainland Portugal and on the Azores islands, and verified differences in the results obtained for each oil depending on the place where the plant was harvested (Dandlen et al., 2011).

The relation of the bioactivity with the composition of *T. zygis* EO was additionally demonstrated by Pérez-Sanchez et al. (2007) who showed that the fungitoxic activity of the EO against phytopathogenic fungi was related to synergic effect between compounds, such as 3-octanol and α-terpinene, instead of with the concentration of more active compounds, like carvacrol or thymol. This was supported by the study of six different populations of *T. zygis* EO against the phytopathogenic fungi (*Colletotrichum acutatum*, *Fusarium oxysporum*, *Pythium irregulare*, *Rhizoctonia solani*, and *Sclerotinia sclerotiorum*) showing a clear inhibition in the poisoned food test and presenting EC_{50} ranging from 86 ppm to 577 ppm. These results encourage the evaluation of the activity of the isolated compounds as well as the interaction between the compounds (Pérez-Sánchez et al., 2007). Therefore, studying the composition of EO is essential, in order to understand which compounds may be associated with the bioactive activity.

2.4.1.1 Antibiofilm activity

Bacteria can bind to surfaces and form a matrix of extracellular polymeric substances, globally known as biofilms (Abdallah et al., 2020; Carrascosa et al., 2021). Biofilms are considered major virulence factors and contribute to more than 80% of human infections (Abdallah et al., 2020).

In the food industry, biofilms are a major problem because they can bind to different surfaces, such as polypropylene, plastic, rubber, glass, stainless steel and even food products (Carrascosa et al., 2021). Nevertheless, another problem associated with biofilms is their resistance ability towards the antibiotics and other antimicrobial agents, mainly to disinfectants used in many wide-ranging food industries (Carrascosa et al., 2021; Suresh et al., 2019).

The antibiofilm efficacy of *T. zygis* EO was tested against single species and multispecies oral biofilms of *Streptococcus mitis*, *Streptococcus sanguinis*, and *Enterococcus faecalis* (Marinković et al., 2020). The results showed that the potential of *T. zygis* EO was high in the treatment against both planktonic and biofilm embedded cells, inducing a higher bacterial reduction comparing to positive control TAP (Triple antibiotic paste, Metronidazole, Ciprofloxacin and Minocycline, ratio 1:1:1) (Marinković et al., 2020). Further, *T. zygis* EOs showed antibiofilm activity on 63.6% of the *E. coli* associated with urinary tract infection isolates (14 out of 22 strains) with a percentage of inhibition between

17.8% and 85.8%. In this study, the authors concluded that the addition of EOs prior to biofilm formation decreases the cell adhesion to the abiotic surface and may contribute to eliminating planktonic cells (Lagha et al., 2019).

When considering biofilm inhibition and eradication activities of *T. zygis* EO against methicillin-resistant *Staphylococcus aureus* clinical isolates, *T. zygis* present antibiofilm activity against 62.1% of the isolates (18 strains). The biofilm eradication percentage ranged from 12.6 to 94.4% of the isolates for *T. zygis* EOs (Abdallah et al., 2020).

Furthermore, the prevention of biofilm formation by *Candida tropicalis* was tested using biodegradable polycaprolactone nanofibers (PCLNFs) with different concentrations of *T. zygis* EO. The biofilm formation of *C. tropicalis* strains on EO-PCLNFs decreased when using 4% and 8% *T. zygis* EO-PCLNFs, demonstrating it potential use as biofilm inhibitive agents (Sahal et al., 2019).

It can be concluded that *T. zygis* EOs have antimicrobial activities that can be used to prevent the formation of biofilms, thus reducing the use of synthetic antimicrobial agents with the same effect.

2.4.1.2 Antimicrobial activity potentiation by interaction

A common strategy used to enhance the potential of antibiotics and thus increase the susceptibility of resistant strains of microorganisms is to use combined treatments between antibiotics and non-antibiotic compounds. It has been suggested that EOs could be used as antibiotic adjuvants to help restore the effectiveness of antibiotics used in first-line treatments against multidrug-resistant microorganisms (Owen and Laird, 2018; Trifan et al., 2020; Vázquez-Ucha et al., 2020). Moreover, the combined use of different EOs with weak activity may enhance their effect (Mittal et al., 2018), and a similar trend may be obtained by using combinations of isolated compounds from the EOs (Mittal et al., 2018; Pina-Vaz et al., 2004).

Pina-Vaz et al. (2004) evaluated the antifungal activity of the major compounds of the *T. zygis* EO (carvacrol, thymol, *p*-cymene and 1,8-cineole) when combined, against *C. albicans* M1 and *C. krusei* H9, by using the checkerboard method. The results showed that a higher synergistic interaction (Fractional inhibitory index (FICI) of 0.125) was observed for thymol/1,8-cineole and thymol/*p*-cymene, with the MIC values decreasing around three dilutions (Pina-Vaz et al., 2004). Once again pointing to the interaction between compounds constituting this EO.

The synergistic effects between *T. zygis* EOs and colistin was studied against different *A. baumannii* and *K. pneumoniae* strains resistant and susceptible to colistin (Vázquez-Ucha

et al., 2020). Colistin is a polycationic lipopeptide antibiotic that was used mainly against Gram-negative bacteria, since it targets the polyanionic LPS cell membrane of these bacteria. Colistin have high toxicity and pharmacokinetic/pharmacodynamic properties that are a challenging and initially was rejected for use, however, this antibiotic has been reintroduced in clinical practice (Kaye et al., 2017), and the use of adjuvants as EOs may potentiate its activity. In fact, the addition of *T. zygis* EOs to colistin significantly reduced the MIC of the antibiotic even in the most resistant strains, showing a synergistic interaction between EO/antibiotic (FICI ≤ 0.5) for most of the strains (Vázquez-Ucha et al., 2020). Indeed, the most active EO/antibiotic combinations were tested against *K. pneumoniae* and *A. baumannii* strains to evaluate their bactericidal activities, and it was shown that the combination of subinhibitory concentrations of colistin and *T. zygis* EO were effective. Thus, this combination may allow to reduce the dose administered of colistin, and as its toxicity is dose-dependent, reduce the adverse effects (Vázquez-Ucha et al., 2020).

Similarly, the combination of enrofloxacin with *T. zygis* EO had an additive or indifferent effect against multiple drug-resistant strains of *Salmonella enterica* from animal source. In case of ceftiofur, in one of the five tested strains, the MIC was reduced from 8 to 0.24 μg/mL when combined with *T. zygis* EO. Ceftiofur showed a synergistic effect when combined with *T. zygis* EO in two of the five tested strains. Lastly, the combination of trimethoprim–sulfamethoxazole with *T. zygis* EO led to a partial synergism in three of the five tested *S. enterica* strains (Solarte et al., 2017).

The interaction of combinations between *T. zygis* EO and other EOs have also been tested (Lee et al., 2020). When evaluating the potential synergistic interaction between *T. zygis* and *Origanum vulgare* or *Cinnamomum zeylanicum* EOs, against *Leuconostoc citreum*, the highest synergistic activity (FICI = 0.375) was obtained with the combination of *Origanum vulgare* and *T. zygis* EOs (Lee et al., 2020). Ghabraie et al. (2016) also evaluated the antimicrobial activity of combinations of EOs, with *T. zygis* demonstrating to be one of the five most active plant EO. *T. zygis* and *Cinnamomum cassia* showed better antimicrobial efficiency with an additive effect against *S. aureus*, *E. coli* and *S.* Typhimurium and no interactive effect against *L. monocytogenes* (Ghabraie et al., 2016).

Enterocin AS-48 is an antimicrobial peptide produced by different *Enterococcus* species and it is effective against foodborne Gram-positive and Gram-negative pathogenic and spoilage bacteria (Sánchez-Hidalgo et al., 2011). This bacteriocin makes pores in the bacterial cytoplasmic membrane, which leads to cell death (Burgos et al., 2014; Sánchez-Hidalgo et al., 2011). To developing future applications for this bacteriocin, enterocin AS-48 has been tested in combination with other antimicrobial compounds (Burgos et al., 2014), namely with *T. zygis* EO against *L. monocytogenes* in Russian-type salad stored at

10 ˚C (Molinos et al., 2009). The EO alone showed very low inhibitory effects at 0.5% of concentration, but in combination with enterocin AS-48 the antilisterial activity of treatments increased significantly. The viable cell counts were reduced to below detection levels by combined treatments of enterocin AS-48 and 1% of the EO, with the antilisterial activity being strongly enhanced (Molinos et al., 2009).

These studies are some examples that may represent an interesting starting point for designing new combination therapies or approaches. Selected combinations of EOs and other antimicrobials agents as food preservatives could be useful to increase the control of foodborne pathogens and spoilage microorganisms, while at the same time increase the shelf life of food products.

2.4.2 Antiparasitic activities

One of the most common human gastrointestinal parasites in the world is *Giardia lamblia,* which is associated with about 180 million infections annually in adults and children (Capewell et al., 2021; Fekete et al., 2021). This is a flagellated protozoan parasite that causes the diarrheal disease giardiasis in vertebrates, and can be vastly found in contaminated soil, food, or water (Capewell et al., 2021; Fekete et al., 2021). The anti-*Giardia* activity EO obtained from *T. zygis* was evaluated on parasite growth, cell viability adherence, and morphology. *T. zygis* EO decreased the number of *Giardia lamblia* trophozoites in a concentration-dependent manner, and after 30 min of incubation, the EO was able to kill almost 50% of the parasite population. The first hour of incubation with the *T. zygis* EO showed a decline of adhered trophozoites and after 7 h of incubation, only about 10% of cells were attached. The exposure to *T. zygis* EO caused the trophozoites to become completely misshapen, with the authors concluding that the anti-*Giardia* effect of *T. zygis* EO may result from the destabilization of protozoan plasmatic membrane, leading to alterations on membrane properties, modifications on organelles and cytoplasmic structure, that leads to cell death (Machado et al., 2010).

2.4.3 Insecticidal, larvicidal and antiparasitic activities

Pesticides have greatly contributed to the management of plagues population; however, they are associated with several side effects, like the development of resistance, toxicity for animals and humans, and environmental pollution (Barbosa et al., 2010; Kim et al., 2016). Consequently, the development of safer alternatives for conventional pesticides is urgently needed and plants are seen as good alternatives (Kim et al., 2016), and eco-friendly alternatives for chemical pesticides (Park et al., 2016). In fact, some organic pesticides based on EOs are already commercialized (Durán-Lara et al., 2020).

Many EOs derived from aromatic plants have demonstrated toxicity and insecticidal activity, but also a number of sublethal effects, including disruption of development, growth, behaviour, and reproduction of the insects (Sangha et al., 2017). EOs are considered low-risk alternatives to synthetic insecticides (Sangha et al., 2017) and *T. zygis* EO and its constituents have received considerable attention for their insecticidal properties.

Fumigant and contact toxicities of the *T. zygis* EO and their major compounds were evaluated against males and females spotted wing drosophila. After 24 h of fumigation at 11.76 mg/L air, EOs showed high mortalities of 93.9% and 72.0% against males and females, respectively. Regarding contact toxicity, after 24 h treatment with 7.5 μg/fly, *T. zygis* EO exhibited 93.7% mortality against males, and 77.1% mortality against females. In this study, *p*-cymene, thymol and carvacrol were identified as the major components of the EO, and *p*-cymene showed small contact toxicity even at the highest concentration of 20 μg/fly, while thymol and carvacrol showed relatively stronger activity than the EO. The difference in the activity may be related to the fact that *p*-cymene, thymol and carvacrol have the same carbon skeleton, but the last two have positional isomers, each with a phenolic hydroxyl group (Park et al., 2016).

Another similar study on the fumigant toxicity of *T. zygis* EO and their constituents was performed on adult rice weevil *Sitophilus oryzae*, where the EO showed a strong fumigant toxicity, at 12.5 mg/L air concentration, with 96% of mortality against *S. oryzae* adults. The major compound of this EO was linalool, which at 12.5 mg/L air concentration, exhibited 100% fumigant toxicity. The authors concluded that within the composition identified in *T. zygis* EO, linalool can been the major contributor to the fumigant toxicity of the EO (Kim et al., 2016).

Ovicidal and larvicidal effects, larval feeding and adult oviposition deterrence testing experiments were done with *T. zygis* EO using concentrations of 1, 2.5, and 5% v/v against diamondback moth, *Plutella xylostella*. *T. zygis* EO had some level of bioactivity against certain *P. xylostella* life stages, being toxic for larvae and eggs, as well as presented deterrent effects on larval feeding and settling behaviour, and adult oviposition (Sangha et al., 2017).

Additionally, *T. zygis* EO nematocidal activity was screened against the pinewood nematode *Bursaphelenchus xylophilus*. The results demonstrated that an exposure of 2 mg/mL solution of *T. zygis* EO for 24 hours, led to mortality of about 86.6 % (Barbosa et al., 2010).

These results point to the effective toxicity activity and potential for management of pests, as alternatives to conventional insecticides of the *T. zygis* EO and some major components.

2.4.4 Cytotoxicity

The determination of cytotoxicity of EOs is an important prerequisite for their application because depending on the cases, the toxicity can limit their use (Gonçalves et al., 2010; Marchese et al., 2016; Raut and Karuppayil, 2014). EOs can exert cytotoxic effects because may contain different concentrations of potentially toxic constituents, which should be taken into account when considering maximum safe dose (Raut and Karuppayil, 2014; Ribeiro-Santos et al., 2018).

The cytotoxicity of the EO of *T. zygis* was evaluated against different cell lines. Gonçalves et al. (2010) evaluated the cytotoxicity in foetal mouse skin dendritic cell line (FSDC) cells at concentrations showing significant antifungal activity, by the 4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The results demonstrated that the EO, with high amounts of carvacrol, showed no cytotoxic effect, at concentrations ranging from 0.08 to 0.16 μL/mL, for as long as 24 h, while having a stronger antifungal activity against dermatophyte strains in the tested concentrations. Only for higher concentrations of the *T. zygis* EO a strong decrease in the MTT reduction by FSDC was observed (Gonçalves et al., 2010). When tested on normal foetal lung fibroblasts (MRC-5 cell line), *T. zygis* significantly decreased cell viability after 24 h treatment, however, its cytotoxicity was comparable to the positive control Triple antibiotic paste (Metronidazole, Ciprofloxacin and Minocycline, in the ratio 1:1:1) (Marinković et al., 2020). Further, EO of *T. zygis* did not cause a significant alteration in the viability or induced morphology alterations of the macrophages (ATCC, RAW 264.7) and bovine aortic endothelial cells when compared to controls (Machado et al., 2010).

Through these studies we can verify that depending on the cell line used, the *T. zygis* EO may present varying levels of cytotoxicity. Further studies should be carried out in order to verify the mode of action of the EO and what are the safe quantities for the *T. zygis* EO.

2.5. *T. zygis* EO as antimicrobial preservative in foods

Many foods and food products are naturally perishable, being important during product preparation, storage, and distribution, the protection against biochemical and microbial deterioration (Fratianni et al., 2010; Ghabraie et al., 2016). Furthermore in the food industry, the elimination of foodborne pathogens is also of major importance, in order to avoid one of the biggest concerns of public health, foodborne diseases caused by the consumption of contaminated food (Ghabraie et al., 2016). The shelf life, nutrition and microbial quality and safety of food products are important aspects that food companies critically consider (Ghabraie et al., 2016).

The food quality problems associated with thermal and non-thermal preservation processes, the safety risks associated with chemical preservatives, pesticides and other compounds usually used in the food chain, as well as the demand for the use of natural preservatives, have potentiated the rise of the use of antimicrobials from natural origins, such as the EO of plants, which are allowed for use in organic foods (Fratianni et al., 2010; Gouveia et al., 2016; Lee et al., 2020). The interest in *T. zygis* EO has been increasing and there are several studies on its effectiveness as a food preservative.

Various approaches for evaluating the potential of *T. zygis* EO as a food preservative have been taken (Table 2.3). When using the agar diffusion method on culture plates prepared with extracts from meat homogenates (minced beef, cooked ham, or dry-cured sausage) against bacteria commonly associated with refrigerated foods, it was observed that in minced beef extract, *T. zygis* EO showed growth inhibitory activity against all strains tested (13.9 - 45.4 mm, including 9 mm disc), being the largest inhibition halos obtained against *Listeria innocua* followed by *Alcaligenes faecalis*. The antibacterial activity of *T. zygis* EO was also observed with cooked ham extract plates, where its activity was classified as moderately active to active against all bacteria strains tested (13.0 - 25.4 mm), with the highest inhibition halos against *A. faecalis* followed by *L. innocua*. When the activity was assessed with the medium from dry-cured sausage extract, the antimicrobial activity of the *T. zygis* EO was classified as moderately active against the bacteria tested (16.6 - 20.6 mm), except for *L. innocua* and *Aeromonas hydrophila* that presented the highest inhibition halos (51.0 and 40.7 mm). It was only with this medium that *T. zygis* EO proved to be inactive (9 mm) against some strains (*A. faecalis*, *Enterobacter amnigenus* and *Enterobacter gergoviae*). From these results, it is clear, that in complex food matrices the fat content, pH, water activity, proteins, and enzymes can potentially influence the efficacy of EOs. For example, the high levels of fat and protein in food can possibly protect the bacteria from the action of EOs (Ballester-Costa et al., 2017).

One possible use of *T. zygis* EO could be its application in marinades on meat and fish. In fact, when meat (pork and chicken), salmon or scampi were immersed for 2 minutes in marinade solution with *T. zygis* EO (1 % w/w), increased shelf life on pork filet and scampi was observed. On pork back-fat, total coliforms were reduced for at least 6 days with the marinade with EO, and on scampi, the growth of lactic acid bacteria and total aerobic psychrotrophs were also reduced. On the chicken matrices, the immersion was only moderately effective, and a small reduction of yeasts and moulds was achieved on chicken breast filet after 6 days. The marinade itself did not inhibit microbial growth of the different food matrices, but the addition of *T. zygis* EO slowed the growth of some spoilage microorganisms and extended the shelf life of meat/fish products (Van Haute et al., 2016).

Table 2.3. The use of *T. zygis* EO in food preservation.

Tested food	Microorganisms		Observations	Reference
	Name	Origin		
Ready-to-eat salad	*L. monocytogenes* CECT 940 (serotype 4d) and *L. monocytogenes* CECT 4032	Food inoculation	The combined treatments of enterocin AS-48 and *T. zygis* EO showed a significantly increased antilisterial activity	(Molinos et al., 2009)
Chicken meat	*Salmonella* sp., *E. coli* and Lactic acid bacteria	Natural microbiota	*T. zygis* EO treatment reduced the total microbial count in chicken meat	(Fratianni et al., 2010)
Meat model (ham)	*S. aureus*, *L. monocytogenes*, *B. cereus*, *E. coli*, *S.* Typhimurium, and *P. aeruginosa*	Food inoculation	Growth rate for bacteria in ham containing *T. zygis* EO was not significantly different from the control ham	(Dussault et al., 2014)
Marinade of fish and meat	Total coliforms, *E. coli*, yeasts and molds	Natural microbiota	Marinade did not inhibit microbial growth on the food matrices, but the addition of *T. zygis* EO to the marinade at low concentrations, showed potential to extend the shelf life on meat/fish products	(Van Haute et al., 2016)
Beef	*L. monocytogenes* ATCC 679	Food inoculation	Samples treated with *T. zygis* EO showed a lower population of *L. monocytogenes* than the control at 2 ˚C after 28 days	(Gouveia et al., 2016)
Meat model medium (minced beef, cooked ham, or dry-cured sausage)	*Listeria innocua*, *Serratia marcescens*, *Pseudomonas fragi*, *P. fluorescens*, *Aeromonas hydrophila*, *Shewanella putrefaciens*, *Achromobacter denitrificans*, *Enterobacter amnigenus*, *Enterobacter gergoviae*, *Alcaligenes faecalis*	Plate inoculation	*T. zygis* EO, in all culture media, had large inhibition halos against all tested bacteria, while the efficacy as antibacterial agent depends on food composition	(Ballester-Costa et al., 2017)
Par-baked wheat and sourdough bread	*Aspergillus niger* P1118 and *Penicillium paneum* Frisvad IHEM6652	Food inoculation	No clear shelf-life extension was observed when *T. zygis* EO was added to par-baked bread, but when added to sourdough, it resulted in more promising shelf-life extending properties	(Benlamdini et al., 2014)
Radish sprouts	*L. monocytogenes* isolated strains F8027, F8369, F8385, G1091 and LCDC 81861	Food inoculation	*T. zygis* EO gases showed antilisterial activity on the surface of radish sprouts	(Lee et al., 2018)
Dough/bread	*Penicillium paneum* IHEM 6652	Food inoculation	Fungal growth was inhibited for more than 45 days by using *T. zygis* EO	(Debonne et al., 2019)
Tomato juice	*Leuconostoc citreum*	Food inoculation	The combination of *Origanum vulgare* and *T. zygis* EOs showed synergistic antimicrobial activity	(Lee et al., 2020)

Table 2.3. The use of *T. zygis* EO in food preservation (continuation).

Tested food	Microorganisms		Observations	Reference
	Name	Origin		
Dry-fermented sausages	Gram-positive catalase positive cocci, *Enterobacteriaceae*, aerobic total viable count, lactic acid bacteria, mold/yeast	Natural microbiota	Chitosan alone or in combination with *T. zygis* EO presented antifungal activity against superficial fungi	(Soncu et al., 2020)
Microcapsules of starch-agave fructans with EO and mango	*Fusarium pseudocircinatum*, *Alternaria alternata*, *Neofusicocum kwambonambiense*, *Cladosporium pseudoclodosporioides*, and *Colletotrichum gloeosporioides*	Plate and food inoculation	The mycelial growth of all microorganisms was inhibited 100% with the sachets that contained *T. zygis* EO microcapsules during all the time tested (12 days). Antifungal sachets developed with *T. zygis* EO microencapsulated in modified starch/agave fructans controlled microorganisms associated with mango decay and in mango controlled the growth of *C. gloeosporioides*	(Esquivel-Chávez et al., 2021)
Salmon and seaweed burgers	Mesophilic bacteria and *Enterobacteriaceae*	Natural microbiota	The *T. zygis* EO slows down the mesophilic growth. Regarding the *Enterobacteriaceae*, the counts suffered some fluctuations during storage but with no clear trend.	(Dolea et al., 2018

The direct effect of *T. zygis* EO on fresh chicken breast meat storage for 3 weeks at 4 °C was evaluated. *T. zygis* EO reduced the deterioration of chicken meat and was able to extend the shelf life of the products when stored at 4 °C. Indeed, a bactericidal effect was observed during the first week of storage, and after 21 days of storage, the total microbial count increased to 4.4×10^4 CFU/mL in the EO-treated samples and to 1.17×10^5 CFU/mL for untreated meat (Fratianni et al., 2010).

Similar behaviour was found when the EO of *T. zygis* was applied in beef inoculated with *L. monocytogenes* and processed by sous vide cook-chill. After processing, rapid chilling, and chilled storage, samples treated with *T. zygis* EO showed a lower population of *L. monocytogenes* (3.60 log CFU/g) than control (4.17 log CFU/g), after 28 days at 2 °C. However, this trend was not observed when samples were conserved at 8 °C, showing that storage temperature was determinant on the growth of *L. monocytogenes* (Gouveia et al., 2016).

The application of *T. zygis* EOs has also been proposed to control bacterial pathogens in ready-to-eat food, like ham. When ham inoculated with *L. monocytogenes* was stored at 4 °C during 35 days under aerobic conditions, the activity of *T. zygis* EO was weaker since the growth rate of the bacterium in hams containing EOs was not significantly different compared with the control. The difference in relation to the control was only 0.008 ln CFU/g/day, considering that the control had a growth rate of 0.404 ln CFU/g/day and the *T. zygis* sample had 0.396 ln CFU/g/day (Dussault et al., 2014).

Moreover, the biopreservative potential of *T. zygis* EO in par-baked wheat and sourdough bread has also been investigated (Debonne et al., 2019, 2018a, 2018b). Despite *T. zygis* EO has shown promising antifungal activity against *Aspergillus niger* and *Penicillium paneu in vitro*, it was not observed a clear shelf-life extension for par-baked bread. However, the use of *T. zygis* in sourdough resulted in more promising shelf-life extending properties (Debonne et al., 2018a). In another study, the use of *T. zygis* EO increased the shelf-life of dough and bread. Nonetheless, *T. zygis* had a negative impact on dough rheology and in baker's yeast (*Saccharomyces cerevisiae*) activity (Debonne et al., 2018a). The growth of the fungus *P. paneum* was inhibited for more than 45 days when the par-baked bread was stored under modified atmosphere, with 0.3 mL *T. zygis* EO/100 g dough (Debonne et al., 2019). The *T. zygis* EO was able to biopreserve bread presenting good antifungal activities, however, it also interferes with the bread yeast resulting in deformed low-leavened breads (Debonne et al., 2018a).

Leuconostoc citreum is a Gram-positive lactic acid bacterium and is one of the major species present in the preparations of kimchi and sauerkraut from the initial to the middle stage of fermentation (Kim et al., 2018). However, this species is also known to cause spoilage of

beverages and foods leading to sliminess in fruit juices, such as citrus and tomato (Lee et al., 2020). *T. zygis* EO showed to significantly reduce the growth of *Leuconostoc citreum* in tomato juice within 48 h. This activity was further potentiated by the combination of *T zygis* EO with *Origanum vulgare* EO (Lee et al., 2020), supporting the previous enhancement of activity described *in vitro* and validating its application in food models.

The inhibitory activity of *T. zygis* EO against *L. monocytogenes* was additionally evaluated on the surface of radish sprouts, showing a significant reduction on the number of *L. monocytogenes* after exposure to EO at concentrations higher than 156 μL/L comparing with untreated samples. These results indicate that the *T. zygis* EO antilisterial activities observed *in vitro* were efficiently transposed to application on the surface of radish sprouts (Lee et al., 2018).

The effect of *T. zygis* EO on the quality and shelf life of fish and seaweed burgers was evaluated by incorporating 0.05% (v/w) of EO, followed by storage for 17 days at 4 ˚C in vacuum packaging. The authors found that the texture, moisture content, and pH of the product were not affected by the *T. zygis* EO, which in turn had a marked influence on the inhibition of burger oxidation. *Enterobacteriaceae* growth was not affected by the presence of *T. zygis* EO at the concentrations studied, while the addition of *T. zygis* EO caused a decrease in mesophilic growth (Dolea et al., 2018). Nonetheless, the EO had to be applied at a higher concentration to extend the product shelf life; however, higher concentrations will have an influence in the sensorial properties of the product (Froiio et al., 2019).

2.5.1 Food technological application of *T. zygis* essential oil

Different techniques have been utilized for enhancing the shelf-life and quality of foods, and the demand for healthy and nutritious foods triggered the search for more natural options (Dhumal and Sarkar, 2018). The direct application of EOs in foods may have limitations due to the high volatility of the compounds and the preservative effect can be lost during storage. Also, the EO can react with various food components and even change the organoleptic properties of the products (Ballester-Costa et al., 2016; Lee et al., 2020; Sapper et al., 2019). Thus, efforts have been made to reduce these limitations by the incorporation of these compounds in edible coatings or films, in active packaging, or by its encapsulation, while maintaining all its preservatives properties. Incorporation techniques of the EOs offers many advantages that can overcome the problems related to their use, like for example, protecting the EOs from degradation, suppressing the aroma of EOs that could alter the taste of food and the encapsulation of hydrophobic EOs into hydrophilic polymers that makes them soluble (Froiio et al., 2019). Thus, these food technologies can incorporate different components with preservative capacity, which are slowly released to the food

products, to improving their chemical and physical properties (Ribeiro et al., 2021).

Various approaches have been followed with these aims using *T. zygis* EO (Figure 2.1).

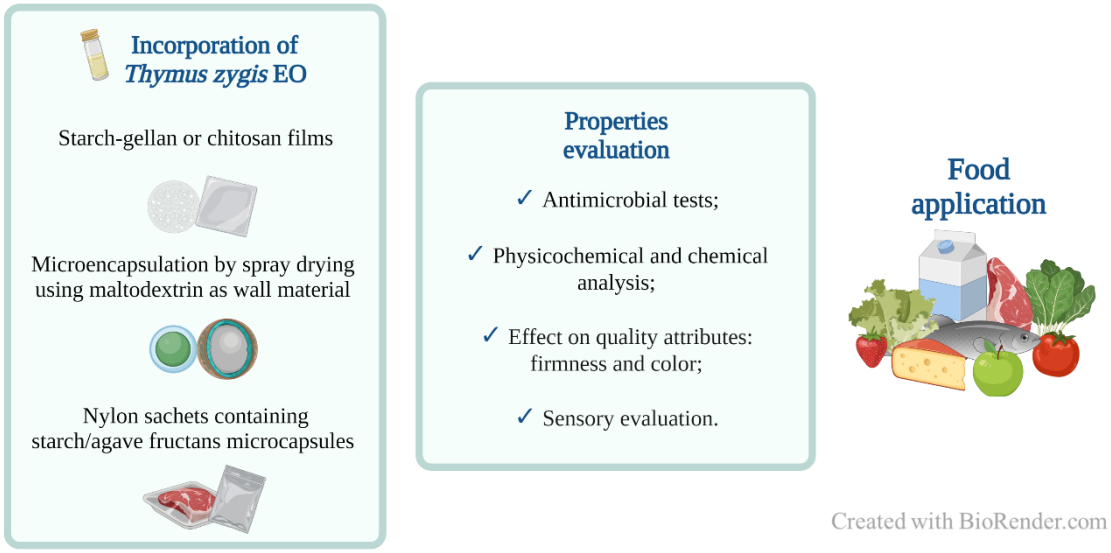

Figure 2.1. Food application of *Thymus zygis* EO using different methods of incorporation.

It was incorporated in films based on starch-gellan and the *in vitro* antifungal properties against *Alternaria alternata* and *Botryotinia fuckeliana* were evaluated. The *T. zygis* EO demonstrated a total fungicide action against *B. fuckeliana* with a concentration of 0.074 g/g dried film, showing a more effective antifungal action than against *A. alternata*. To ensure a good antifungal action against *A. alternata*, the films needed higher contents of EO. This study showed that films with EO were effective at controlling fungal growth, while still exhibiting adequate functional properties as coating/packaging materials (Sapper et al., 2018). The antifungal activity of this films proved to be relevant, however, there is a lack of studies on effective food matrices.

Undesirable microorganisms can negatively alter the sensory properties of fermented sausages and the growth on the sausage surface can represent health risks in foods for consumption. To understand the effect of chitosan enriched with *T. zygis* EO, the growth of superficial microorganisms in fermented sausages during storage at 4 ˚C for 3 months was investigated. During storage time, the counts of Gram-positive, catalase positive, cocci decreased in sausages treated with chitosan-*T. zygis* EO over the entire storage period. Regarding *Enterobacteriaceae*, significantly lower counts were determined in sausages dipped into chitosan-*T. zygis* when compared to those treated with distilled water (control). As for antifungal activity, the chitosan-*T. zygis* EO treated sausages had significantly lower mould and yeast counts than the ones treated with distilled water in the last month of storage. The results showed that chitosan-*T. zygis* effectively controlled the growth of microorganisms during refrigerated storage (Soncu et al., 2020).

Additionally, the *T. zygis* EO was also studied for the control of phytopathogens associated with mango decay (*Fusarium pseudocircinatum*, *Alternaria alternata*, *Neofusicocum kwambonambiense*, *Cladosporium pseudocladosporioides*, and *Colletotrichum gloeosporioides*). When these microorganisms were exposed to *T. zygis* EO microencapsulated with modified starch/agave fructans and placed inside nylon sachets, the mycelial growth was inhibited by 100%, on day 6, for all microorganisms with the sachets that contained 0.15 g of *T. zygis* EO microcapsules. The sachets with a higher dose (0.20 g) of EO microcapsules produced 100% of mycelial growth inhibition during all the periods of the test (12 days). *T. zygis* EO microcapsules were compared with the commercial antifungal sodium metabisulfite (0.10 g), commonly used in active packaging, and similar results were obtained. The effect of active sachets on the quality attributes of mango was also evaluated and the volatile compounds of the active package did not affected any physiological mechanistic relationship with the development of colour, maturity, firmness of mango (Esquivel-Chávez et al., 2021).

The antibacterial potential of microcapsules of *T. zygis* EO, produced by spray drying technique, was also evaluated against *Vibrio alginolyticus* and *Vibrio parahaemolyticys*. *T. zygis* EO microcapsules exhibited antimicrobial activity against both bacteria and the spray drying process did not have any negative effect on the antimicrobial activity of *T. zygis* EO. Consequently, these microcapsules can be used as a delivery form of this compound (Tomazelli Júnior et al., 2018). The *T. zygis* EO microencapsulated showed good antibacterial activity, however, missing testing in food matrices.

Through these studies, it can be suggested that the *T. zygis* EO has excellent antimicrobial activity, and it can be used as a food preservative, whether directly used in the food or when encapsulated or incorporated in films based on starch-gellan or chitosan, nonetheless, the type of food production needs to be considered.

2.6. Effect of animal diets supplemented with *T. zygis*

The animal feed with diets supplemented with *T. zygis* EO was studied with the objective of evaluating the effects of *T. zygis* on the by-products obtained from these animals.

The effect of diet with different concentrations of *T. zygis* EO on the shelf life and quality of gilthead seabream (*Sparus aurata*) was also studied (Álvarez et al., 2012; Hernández et al., 2015). The presence of *T. zygis* EO in the diet of gilthead seabream demonstrated a dose-dependent effect on the thiobarbituric acid reactive substances (TBARS) assay, colour, and total volatile basic nitrogen during ice storage on days 7 and 21. At high doses of the EO present in the diet, the microbiological counts were lower for *Enterobacteriaceae* and

coliforms in the fish fillets. Thus, the gilthead seabream feed with *T. zygis* had extended the shelf life from 17 to 18 days for all doses (Álvarez et al., 2012; Hernández et al., 2015).

A mixture of phytobiotics, such as volatile oils of three plants including *T. zygis* were incorporated into the diet of juvenile *Nile tilapia* to improve growth performance, oxidative stress, immune, hematological responses and resistance against *Aeromonas hydrophila* (De Rezende et al., 2021). The results suggest that the mixture of phytobiotics as a dietary supplement has benefits and it can replace the antibiotic enrofloxacin. The supplementation with the mixture of phytobiotics for 20 days provided greater antioxidant protection in *Nile tilapia*, mitigated the impacts of stressors and modulated immunity. Considering the haematological and immunological results obtained, the authors suggested that the supplement feed strengthens the immune response of *Nile tilapia* by activation of the fish innate immune system. This facilitates the phagocytosis and bacterial death, providing greater resistance to disease and protection against pathogens. These results reinforce the benefits of adopting dietary strategies and the use of a blend of phytobiotics containing the volatile oils as food supplement, which can be an alternative to synthetic antibiotics (De Rezende et al., 2021).

These results showed that feeding animals with a diet supplemented with natural compounds with antioxidant and antimicrobial properties, such as *T. zygis* EO, may improve the quality of the by-products obtained from these animals, decreasing the indices of oxidation, delaying *post-mortem* deterioration, and also allowing protection against diseases caused by microorganisms. Thus, diets containing *T. zygis* EO could be an alternative to synthetic additives found in animal feed. However, additional studies are needed, namely concerning parameters of supplementation of the diets and also on the mechanism of action behind these results.

2.7. Conclusion

The essential oil obtained from *Thymus zygis* may be used, by the food industry as a potential natural or “green” additive to replace or reduce the use of synthetic compounds, since they show significant antibacterial properties alone and in food models. Furthermore, this EO show antibiofilm activity as well as potentiate the effect of other antimicrobial agents. This essential oil showed very promising results in the food preservation, increasing its shelf life and reducing the growth of some spoilage microorganisms, as well as of foodborne pathogens. The findings of the reported studies may be considered as a basis for detailed investigation on the antimicrobial properties of *T. zygis* EOs and for possible applications in the most diverse foods such as meat, fruit, and vegetables.

Chapter 3

Aims of the thesis

Aims of the thesis

Plants have always played an important role in the control and treatment of various diseases, as well as, in the preservation and conservation of food. In fact, plants are a valuable source of compounds with bioactive properties that can be used in the most diverse products and fields. Antimicrobial resistance is a growing problem that urgently needs new solutions and considering the biological activities of plants, these natural options can contribute to the outcome of these problems. Therefore, the main goal of this work was to evaluate the antioxidant and antimicrobial activities of selected commercial essential oils, with focus on *Thymus zygis*.

The following specific objectives were pursued, and constitute the subject of each of the following chapters:

I. To screen the chemical composition, the antioxidant, and antimicrobial properties and to evaluate the biocompatibility of the EOs of *Foeniculum vulgare*, *Helichrysum stoechas*, *Mentha pulegium*, *Pinus pinaster*, *Ruta graveolens*, *Thymus mastichina* and *Thymus zygis*.

II. To characterize the chemical composition and to evaluate the bioactive properties of the *Thymus zygis* EO, with a focus on antimicrobial activity against *Staphylococcus aureus*, also considering the attenuation of this bacterium's virulence and the interaction of the EO with different antibiotics.

III. To study the anti-*Listeria monocytogenes* activity of *Thymus zygis* EO, evaluating the attenuation of this bacterium's virulence, its tolerance to adverse conditions and the cross-resistance to antibiotics, and the possible application of the *T. zygis* EO in food.

Chapter 4

Chemical composition, antioxidant, and antimicrobial activities of six commercial essential oils

This chapter corresponds to a manuscript accepted for publication with the following reference:

Coimbra, A., Miguel, S., Ribeiro, M., Coutinho, P., Silva, L., Ferreira, S., Duarte, A.P., 2022. Chemical composition, antioxidant, and antimicrobial activities of six commercial essential oils. Letters in Applied Microbiology. 76, 1–8. https://doi.org/10.1093/lambio/ovac042

Abstract

Essential oils (EOs) and their components extracted from medicinal and aromatic plants are used in several areas, such as perfumery and chemical, cosmetic, food, and pharmaceutical industries. Considering the different applications of EOs, this work aimed to screen the composition and the bioactivities properties of the EOs of *Foeniculum vulgare*, *Helichrysum stoechas*, *Mentha pulegium*, *Pinus pinaster*, *Ruta graveolens* and *Thymus mastichina*.

GC-MS revealed the presence of different compounds in EOs *F. vulgare* (12), *H. stoechas* (27), *M. pulegium* (8), *P. pinaster* (24), *R. graveolens* (8) and *T. mastichina* (16). All the EOs showed antioxidant activity acting through inhibition of lipid peroxidation, while only two EOs (*H. stoechas* and *M. pulegium*) scavenged the free radicals of DPPH. *M. pulegium* and *T. mastichina* EOs showed the strongest antimicrobial activity. Also, the effect on the fibroblast's viability was directly proportional to the EOs concentration, and the highest cytotoxic effect was registered with *R. graveolens* EO.

The present study revealed significant bioactive properties of different EOs, highlighting *M. pulegium* and *T. mastichina* EOs to be considered in further studies for potential use in the food and pharmaceutical industries, due to their antioxidant and antimicrobial properties.

Keywords: Essential oil; GC-MS; antioxidant activity; antimicrobial activity; cytotoxicity

4.1. Introduction

Medicinal and aromatic plants can be cultivated or found as spontaneous plants, being used for various health purposes mainly in traditional herbal preparations, like decoctions and infusions (Gahukar, 2018; Miara et al., 2019). Considering their therapeutically active substances, these plants may be extremely important for the healthcare needs of the world's population living in developing countries (Roosta et al., 2017). Moreover, these plants also play an important role in the industry, mainly cosmetic, food and phytotherapy (Gahukar, 2018; Roosta et al., 2017), presenting a vast economic growth potential as natural matrices (Kala, 2015). In the food industry, the consumers' request for more sustainable and healthy products allowed the expansion of clean-label foods. So, the food industry is reformulating the products and replacing the artificial food additives, such as flavors, colors, and preservatives, with more natural alternatives (Rao et al., 2019). In fact, essential oils (EOs) and their components emerged as natural alternatives to synthetic products, such as food preservatives, and pesticides (Silvestre et al., 2019; Van Haute et al., 2016).

EOs are natural products characterized by a strong odor and constituted by complex mixtures containing approximately 20 to 80 different molecular species, typically phenolic terpenes as major constituents (De Martino et al., 2015; Rao et al., 2019). EOs are used as aromatizers and flavors or in the synthesis of chemicals, and present diverse bioactive properties (Silvestre et al., 2019). Their antioxidant activity and fungicidal, bactericidal and virucidal properties trigger the interest of its use for the preservation of foods, and in pharmaceutical and cosmetic industries (Abuga et al., 2022; De Martino et al., 2015; Silvestre et al., 2019). In fact, the diverse bioactivity of the secondary metabolites found in EOs chemical composition, points to their possible application in different approaches. Therefore, the present study aimed to evaluate the chemical composition of six commercial EOs from plants belonging to different genera and species, and to provide a better understanding of their antioxidant and antimicrobial activities, as well as their cytotoxicity.

4.2. Materials and Methods

4.2.1 Essential oils

Six commercial EOs obtained by steam distillation from *Foeniculum officinale* (also known as *Foeniculum vulgare,* aerial parts), *Helichrysum stoechas* (aerial parts), Mentha *pulegium* (leaves) and *Thymus mastichina* (aerial parts) were achieved from the company Pharmaplant (Algarve, Portugal). The EO *of Pinus pinaster* (aerial parts) was purchased at the company Aromas do Valado (Idanha-a-Nova, Portugal) and the EO of *Ruta graveolens* (aerial parts) from the company Ternenic (Barcelona, Spain). The EOs were stored at 4 ˚C and protected from light until use.

4.2.2 GC-MS analysis of the essential oils

The EOs were characterized by Gas Chromatography coupled to Mass Spectrometry (GC/MS), on an Agilent Technologies 7890A GC-System apparatus equipped with a split–splitless injector (split ratio of 1:50), a DB-5^{MS} fused silica capillary column with a length of 30 m × 0.25 mm inner diameter and film thickness 0.25 µm (J&W Scientific Inc., part number: 122-5032) connected to a Mass spectrometer detector (MSD) from Agilent Technologies 5975C, Inert XL with Triple-Axis and data processing system (GC/MSD ChemStation). The temperature of the injector was kept at 250 ˚C. The oven temperature was programmed to increase from 60 ˚C to 250 ˚C at 5 ˚C/min. The carrier gas, helium, was applied at a flow rate of 1 mL/min and the injection volume was 1 µL. Dilutions of the EO were performed in GC vials with 10% (v/v) of dichloromethane. The operation conditions of MSD were an ion source temperature of 250 ˚C, ionization voltage of 70eV, and an interface temperature of 280 ˚C. The method of identifying the EO components was

achieved by comparison with the retention index and mass spectra from the data system library NIST and Wiley and some of the components were also compared with the pure standard compounds. The retention indexes were determined by the normalization method, without using correction factors, according to ISO 7609, calculated retention index relative to C7-C25 *n*-alkanes.

4.2.3 Antioxidant activity

4.2.3.1 DPPH radical scavenging assay

The antioxidant activity was evaluated using the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) method as described by Coimbra et al. (2022). Briefly, 5 µL of different concentrations of the EOs methanolic solutions were separately mixed with 195 µL of DPPH methanolic solutions in 96-well microtiter plates. Gallic acid (Acros Organics, Geel, Belgium) and Trolox (Acros Organics, Geel, Belgium) were used as positive controls, while methanol was considered as negative control. All tests were performed in triplicate and the absorbances were measured at 515 nm. The antioxidant activity was expressed through the Antioxidant Activity Index (AAI), calculated as follows: AAI = (final concentration of DPPH in the control sample at 60 min)/IC_{50} (Scherer and Godoy, 2009).

4.2.3.2 β-Carotene bleaching assay

The antioxidant activity evaluated by *β*-carotene bleaching assay was described by Coimbra et al. (2022). Briefly, methanolic dilutions of EOs were prepared, butylated hydroxytoluene (BHT, purity 99 %, Acros Organics, Geel, Belgium) and methanol were used as positive and negative control, respectively. A volume of 943.4 µL of the emulsion was added to 56.6 µL of each EO methanolic or control solutions to be tested. All tests were performed in triplicate and the absorbances were measured at 470 nm, against a blank containing an emulsion without β-carotene. After the determination of the absorbance values, the percentage of inhibition of β-carotene oxidation was calculated using the equation: % Inhibition = ((Abs sample$^{t=1h}$ – Abs control$^{t=1h}$)/(Abs control$^{t=oh}$ – Abs control$^{t=1h}$))×100.

4.2.4 Antimicrobial activity

4.2.4.1 Microorganisms and culture media

In this study, the antimicrobial activity of EOs was assessed towards four Gram-positive (*Staphylococcus aureus* ATCC 25923 and MRSA 10/08, *Bacillus cereus* ATCC 11778 and *Listeria monocytogenes* LMG 16779) and five Gram-negative bacteria (*Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, *Pseudomonas aeruginosa* ATCC 27853

and *Salmonella* Typhimurium ATCC 13311), as well as, against two yeast species (*Candida albicans* ATCC 90028 and *Candida tropicalis* ATCC 750). The culture media used were Müeller-Hinton broth (MHB) and Müeller-Hinton agar (MHA), except for *L. monocytogenes*, for which Brain Heart Infusion (BHI) and Brain Heart Infusion agar (BHIA) were used. For yeasts, the media used were Roswell Park Memorial Institute 1640 (RPMI 1640) supplemented with 3-(N-morpholino)propanesulfonic acid (MOPS) and Sabouraud Dextrose Agar (SDA). All microorganisms used were stored in cryogenic tubes at -80 ˚C in a suitable medium with 20% glycerol for long-term storage. For short-term storage during testing, culture plates were stored at 4 ˚C. These cultures were subcultured in suitable solid medium and subsequently incubated at 37 ˚C for 24 h for bacteria or 48 h for yeast.

4.2.4.2 Disc diffusion method and vapor-phase antimicrobial activity determination

The susceptibility of bacteria and yeasts to the EOs was performed using the disc diffusion method, according to Luís et al. (2017) and the effect of the EOs volatile compounds was evaluated as described by Coimbra et al. (2022). The inoculum used in the assay was prepared by direct suspension of isolated colonies in isotonic saline solution (0.85% (w/v) NaCl) adjusting the turbidity to 0.5 McFarland and the agar plates were uniformly inoculate with a swab. Filter paper discs (6 mm) impregnated with 10 µL of each EO or tetracycline discs (20 µg/disc) were placed on the surface of the inoculated plates. Regarding the EOs volatile compounds, the discs impregnated with 10 µL of the EOs were placed in the lid of the Petri dishes. The plates were incubated at 37 ˚C for 24 h for bacteria and 48 h for yeasts. After the incubation period, the inhibition halos were measured in mm. All tests were performed as three independent assays, and the results are presented as mean ± standard deviation.

4.2.4.3 Determination of the minimum inhibitory concentration (MIC)

The susceptibility of the bacteria and yeasts to the EOs was evaluated through the broth microdilution method as described by Coimbra et al. (2020b), with modifications. Briefly, EOs were serial diluted in media using Tween 80 as solvent (maximum concentration of 1% (v/v), not inhibiting growth) in 96 well plates. The inoculum used was adjusted to 0.5 McFarland and then diluted in medium to obtain a final concentration of 5×10^5 CFU/mL in each well for bacteria and $0.5 - 2.5 \times 10^3$ CFU/mL for yeasts. Growth, solvent, and sterility controls were included. The microplates were then incubated at 37 ˚C for 24 h for bacteria and 48 h for yeasts. 30 µL of a 0.01% solution of resazurin were added to the wells followed by incubation for 2 h (bacteria) or 3 h (yeasts) at 37 ˚C, no colour change (blue

resazurin colour remained unchanged) indicated inhibition of the test microorganism. At least duplicates of three independent determinations were performed, presenting the results as modal values.

4.2.5 Evaluation of EOs biocompatibility

To evaluate the cytotoxicity of the EOs, normal human dermal fibroblasts (NHDF) cells purchased from PromoCell were initially seeded at a density of 2×10^4 cells/well in 96-well flat-bottom culture plates, containing DMEM-F12 supplemented with 10% FBS. Adherent cells were grown at 37 °C, in an incubator with a humidified atmosphere containing 5% CO_2 for 24 h. Then, the culture medium was removed, and cells were incubated with different concentrations of EOs, to a final concentration ranging 0.061 to 4 µL/mL, for 24 h. The cell metabolic activity was monitored through a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The medium was removed and 50 µL of MTT (5 mg/mL PBS) was added to each sample (n = 5), followed by incubation for 4 h at 37 °C in a 5% CO_2 atmosphere. After, cells were treated with 200 µL of DMSO for 30 min. A microplate reader (Biorad xMark microplate spectrophotometer, Waltham, MA, USA) was used to read the absorbance at 570 nm of the samples from each well. Cells cultured without EOs were used as a negative control, whereas cells cultured with EtOH (96%) were used as a positive control.

4.3. Results and discussion

Essential oils may be presented as promising natural products due to its bioactive properties, which are mainly determined by its concentration and chemical composition (Rao et al., 2019). Considering EOs potential, in this work, we selected to study six EOs taking into account the distribution of the plants in Portugal and the therapeutic usage and bioactive properties of other species from the same genera, associated with a reduced knowledge regarding its bioactivity. Therefore, we started by determining the composition of the six essential oils (Table 4.1).

Table 4.1. Chemical composition of essential oils evaluated by GC-MS.

Compounds	KI	Reference KIs (Adams, 2007)	Area (%)					
			FV	HS	MP	PP	RG	TM
α-Pinene *	936	939	1.17	14.15		**29.13**		4.42
Camphene *	950	954				0.55		3.58
Sabinene *	973	975						3.54
β-Pinene *	978	979	1.21			15.52		4.17
α-Phellandrene *	1004	1002	1.47					

Table 4.1. Chemical composition of essential oils evaluated by GC-MS (continuation).

Compounds	KI	Reference KIs (Adams, 2007)	Area (%)					
			FV	HS	MP	PP	RG	TM
δ-3-Carene *	1011	1011				2.16		
p-Cymene *	1030	1024	1.69					
Limonene *	1030	1029	9.50	5.54		4.59	0.64	
1,8-Cineole *	1032	1031						**58.31**
β-*Trans*-ocimene	1048	1050	0.85			0.53		0.86
γ-Terpinene *	1060	1059						0.48
Cis-sabinene hydrate	1067	1070						0.85
Fenchone *	1087	1086	30.90					
α-Terpinolene *	1088	1088				0.53		
2-Nonanone	1092	1090					2.82	
β-Linalool *	1099	1096		1.13				1.04
Camphor *	1143	1146	0.87					5.03
Menthone *	1151	1152			0.56			
Isomenthone	1159	1162		1.07	30.27			
Borneol *	1166	1169						4.00
Isopulegone	1177	NF		0.77	1.10			
4-Terpineol *	1177	1177						1.02
$C_{10}H_{18}O$	1189	NF		1.33				
2-Decanone	1190	1190					2.67	
α-Terpineol	1190	1188				0.71		3.85
Estragole *	1196	1195	15.35					
Nerol *	1229	1229		1.93				
Pulegone *	1234	1237			**56.71**			
Anisaldehyde	1252	1250	1.12					
Bornyl acetate *	1284	1288						0.50
Anethole *	1285	1284	**33.54**					
2-Undecanone	1293	1294					**86.18**	
2-Undecanol	1311	NF					1.37	
8-Hydroxy-4-*p*-menthen-3-one	1315	NF			1.53			
α-Cubebene	1351	1348				0.59		
Neryl acetate *	1363	1361		**22.06**				
α-Copaene	1376	1376				0.98		
DIEPI-α-cedrene-(I)	1382	NF		1.34				
2-Dodecanone	1384	NF					1.11	
Italicene	1401	1405		3.92				
Junipene	1407	1402				1.66		
Cis-α-bergamotene	1415	1412		1.07				
β-Caryophyllene *	1420	1419		1.05	0.50	9.87		0.85
Thujopsene *	1432	1431					0.56	
Trans-α-bergamotene	1435	1434		2.16				
Aromadendrene *	1441	1441		1.15				
Neryl propanoate	1452	1454		3.66				
α-Humulene	1453	1454			0.64	1.72		

Table 4.1. Chemical composition of essential oils evaluated by GC-MS (continuation).

Compounds	KI	Reference KIs (Adams, 2007)	Area (%)					
			FV	HS	MP	PP	RG	TM
Alloaromadendrene	1460	1560		0.51				
γ-Muurolene	1476	1475				2.24		
γ-Curcumene	1480	1482		5.16				
Germacrene D *	1481	1487				5.45		
Ar-Curcumene	1482	1483		4.86				
β-Selinene	1486	1490		1.37				
Phenethyl isovalerate	1491	1491				0.66		
β-Muurolene	1494	NF				0.78		
2-Tridecanone	1499	1496					0.56	
α-Muurolene	1498	1500				0.88		
β-Bisabolene	1508	1505		3.49				
Cis-γ-Bisabolene	1512	1514		0.53				
γ-Cadinene	1513	1513				1.13		
δ-Cadinene	1523	1523				3.38		
Trans-Nerodiol	1561	1563		0.57				
Geranyl butanoate	1563	1564		0.49				
Caryophyllene oxide	1581	1583				0.68		
Guaiol *	1597	1600		0.72				
Humulene epoxide II	1606	1608		1.37				
α-Acorenol	1630	1633		0.59				
β-Eudesmol	1650	1650		0.60				
Abietatriene	2054	2056				0.56		
Abietadiene	2080	2087				0.68		

* Compared to the corresponding standard compounds; FV – *F. vulgare*; HS – *H. stoechas*; KI – Kovats index; MP – *M. pulegium*; NF- not found; PP – *P. pinaster*; RG – *R. graveolens*; TM – *T. mastichina*. The values in bold represent the major component of each EO.

Twelve compounds accounting for 98.14% of the total composition were identified in the EO of *F. officinale* (also known as *F. vulgare*), and presented as major compounds anethole, fenchone and estragole, which accorded with others studies (Ashokkumar et al., 2021; Chen et al., 2020a; Vieira et al., 2017). For the *H. stoechas* EO, twenty-seven compounds were found, representing 82.59% of the total composition, with neryl acetate (22.06%) and α-pinene (14.15%) as major components. Comparatively with *Helichrysum italicum,* one of the most known species of the genus *Helichrysum,* these two major components are common to the EO of both species. This EO also has other common compounds with the EO of *H. stoechas*, like bisabolene, β-caryophyllene, limonene, γ-curcumene and β-linalool (Oliva et al., 2020; Talić et al., 2019). Eight compounds representing 92.32% of the total composition of the *M. pulegium* EO were identified, with pulegone (56.71%) as the major compound, as presented in other studies (Baali et al., 2019; Luís and Domingues, 2021; Piras et al., 2019; Vieira et al., 2017). The analysis of the EO of *P. pinaster* showed the major

compounds as α-pinene (29.13%) and β-pinene (15.52%), in accordance with other studies, namely when the EO was obtained from different parts of the plant, such as wood, cones and needles (Gonçalves et al., 2020; Tümen et al., 2018). The EO of *R. graveolens* presented a mixture of ketones, being the major one the 2-undecanone (86.18%) followed by 2-nonanone (2.82%) and 2-decanone (2.67%). These results agree with the data reviewed by Coimbra et al. (2020a), which shows that the different species of the genus *Ruta* have some differences in composition, but the dominant class of compounds are the ketones (Coimbra, et al. 2020a). The *T. mastichina* EO possess similar composition to other reports, being the major compound the 1,8-cineole (58.31%) (Arantes et al., 2019; Ballester-Costa et al., 2013; Miguel et al., 2004).

An antioxidant can play a different role or have a different performance depending on the reactive species present or target substrate. Thus, different approaches should be used for antioxidant activity analysis (Ribeiro-Santos et al., 2018). In this study, the DPPH method evaluated the free radical scavenging activity and the β-carotene bleaching assay measured the capacity to inhibit the lipid peroxidation mediated by free radicals (Luís et al., 2019).

Regarding the antioxidant properties the EOs exhibited very strong or no antioxidant activity (Table 4.2), considering the DPPH results and Scherer and Godoy classification (Scherer and Godoy, 2009).

Table 4.2. Antioxidant properties of essential oils and standards measured by the DPPH method (results expressed as mean ± standard deviation) and β-carotene bleaching assay (results expressed as median).

	DPPH method			**β-carotene bleaching assay**
Samples	IC_{50} (%)	AAI	Antioxidant activity classification	IC_{50} (%)
F. vulgare	NA	NA	No activity	1.07
H. stoechas	10.96 ± 1.17	4.81 ± 1.22	Very strong	0.72
M. pulegium	18.14 ± 3.49	2.57 ± 0.57	Very strong	2.33
P. pinaster	NA	NA	No activity	0.90
R. graveolens	NA	NA	No activity	1.02
T. mastichina	NA	NA	No activity	1.92
Gallic acid	2.14 ± 0.39	22.16 ± 3.53	Very strong	-
Trolox	3.26 ± 1.21	15.02 ± 0.64	Very strong	-
BHT	-	-	-	0.1

The EO IC_{50}s are presented as % (v/v) and standards as % (w/v); AAI – Antioxidant activity index; NA – No activity; BHT – Butylated Hydroxytoluene.

Amongst the studied EOs, the ones from *H. stoechas* (IC_{50} of 10.96 ± 1.17%) and *M. pulegium* (IC_{50} of 18.14 ± 3.49%), considering the classification above mentioned, demonstrated very strong antioxidant activity by sequestration of DPPH free radicals, with the *H. stoechas* EO showing the strongest activity. However, when analysing the EOs of *F.*

vulgare, P. pinaster, R. graveolens and *T. mastichina*, no antioxidant activity was observed by the DPPH method.

The results of the β-carotene bleaching assay show that the EOs of *F. vulgare*, *P. pinaster, R. graveolens* and *T. mastichina* inhibited lipid peroxidation (Table 4.2), despite not presenting antioxidant activity by sequestration of free radicals. Foods with high lipid content are more susceptible to oxidation and peroxidation leading to quality deterioration, affecting its nutritional value, odour, flavour, taste, and texture (Falowo et al., 2014; Ribeiro-Santos et al., 2018). The ability of these EOs to inhibit lipid peroxidation may be an advantage for use in food preservation.

In sum, EOs of *H. stoechas* and *M. pulegium* show antioxidant activity through at least two different mechanisms, the sequestration of free radicals and inhibition of lipid peroxidation.

In this work, the scavenging activity of the *P. pinaster* EO was demonstrated as weak, such as previously reported by Ruas et al. (2022); however, some studies have shown a good activity of the EO of the *P. pinaster* (Tümen et al., 2018). Regarding the *R. graveolens* and *T. mastichina*, the weak antioxidant activity of these EOs was also previously described (Arantes et al., 2017; Delgado et al., 2014; Jianu et al., 2021).

EOs from other *Helichrysum* species, such as *H. italicum* and *H. microphyllum* have also demonstrated DPPH radical inhibition at different levels (Ornano et al., 2015; Talić et al., 2019). Despite sharing some compounds, the variation in the composition may be responsible for these differences in antioxidant activity.

Previous reports evaluating *M. pulegium* EO antioxidant activity revealed a weaker antioxidant activity, with AAI values ranging from 0.005 to 0.01 (Benahmed et al., 2019; Teixeira et al., 2012), contrasting with the strong antioxidant activity through the DPPH assay observed in our work (AAI of 2.57) and the results obtained by Luís and Domingues (2021). Baali et al. (2019) evaluated the inhibition of lipid peroxidation and the EO of *M. pulegium* showed a weak activity (Antioxidant Activity (AA)=13.24 ± 0.61%) when compared with BHT (AA=85.66 ± 1.55%) (Baali et al., 2019). Likewise, our results also reveal that *M. pulegium* EO show weak antioxidant activity through lipid peroxidation.

An initial screening of antimicrobial activity of the six EOs was performed through the disc diffusion methodology (Table 4.3). According to the obtained results, a general higher antimicrobial activity was found for the *M. pulegium* EOs. The *Candida* species were the most susceptible with inhibition halos between 30.94 ± 5.34 and 36.90 ± 7.22 mm. When considering the analysis of the volatile compounds' antimicrobial activity of the EOs, only volatiles compounds from *M. pulegium* EO demonstrated inhibitory activity against *C. albicans* (19.28 ± 3.45 mm).

Table 4.3. Diameters of inhibition zone (mm) for disc diffusion method, presented as mean ± standard deviation.

Species	EOs						TET
	FV	HS	MP	PP	RG	TM	
S. aureus ATCC 25923	8.91 ± 0.39	8.73 ± 0.28	15.43 ± 1.71	7.28 ± 1.20	9.09 ± 0.35	8.98 ± 0.65	27.85 ± 0.53
S. aureus MRSA 10/08	8.13 ± 0.46	8.60 ± 0.69	19.21 ± 2.00	6.40 ± 0.70	9.93 ± 0.44	8.07 ± 0.74	28.42 ± 1.20
B. cereus ATCC 11778	9.44 ± 0.70	11.60 ± 1.76	18.5 ± 2.13	9.27 ± 0.93	11.21 ± 1.80	9.19 ± 0.43	30.61 ± 0.92
L. monocytogenes LMG 16779	9.62 ± 1.29	11.04 ± 0.55	13.24 ± 0.78	10.12 ± 0.61	9.65 ± 0.32	8.37 ± 0.45	33.07 ± 0.27
E. coli ATCC 25922	11.64 ± 1.10	6.00 ± 0.00	23.43 ± 1.41	12.01 ± 0.96	6.00 ± 0.00	13.93 ± 1.83	26.06 ± 0.51
K. pneumoniae ATCC 13883	18.52 ± 3.12	6.00 ± 0.00	16.31 ± 0.94	9.14 ± 1.66	7.21 ± 1.11	15.64 ± 1.83	25.43 ± 0.73
P. aeruginosa ATCC 27853	6.00 ± 0.00	6.00 ± 0.00	9.12 ± 1.62	6.00 ± 0.00	6.95 ± 1.65	6.89 ± 0.68	16.01 ± 0.96
S. Typhimurium ATCC 13311	9.19 ± 0.41	6.00 ± 0.00	19.14 ± 3.42	6.46 ± 0.80	6.00 ± 0.00	12.10 ± 2.22	21.82 ± 1.11
C. albicans ATCC 90028	11.16 ± 1.70	9.81 ± 0.39	30.94 ± 5.34	6.00 ± 0.00	11.82 ± 1.07	12.33 ± 1.45	-
C. tropicalis ATCC 750	9.29 ± 0.33	6.00 ± 0.00	36.90 ± 7.22	6.00 ± 0.00	11.15 ± 1.02	9.56 ± 0.85	-

FV – *F. vulgare;* HS – *H. stoechas*; MP – *M. pulegium*; PP – *P. pinaster*; RG – *R. graveolens;* TM – *T. mastichina*; TET – Tetracycline.

Concerning the evaluation of the antimicrobial activity through the determination of the MIC (Table 4.4), it was demonstrated that the EOs of *M. pulegium* and *T. mastichina* showed the highest antibacterial activity against the strains tested with MIC values between 0.25 and 8 µL/mL, apart from *P. aeruginosa* (32 µL/mL).

Table 4.4. Minimum inhibitory concentration (µL/mL) of EOs and standards (µg/mL) in bacterial and yeast species presented as modal values.

	EOs						TET	AMP B
Species	FV	HS	MP	PP	RG	TM		
S. aureus ATCC 25923	32	128	0.25	2	128	8	0.125	-
S. aureus MRSA 10/08	64	128	0.5	2	256	8	0.125	-
B. cereus ATCC 11778	8	16	0.5	64	128	2	0.015	-
L. monocytogenes LMG 16779	64	128	4	128	> 256	8	0.25	-
E. coli ATCC 25922	8	256	1	4	> 256	4	0.25	-
K. pneumoniae ATCC 13883	32	128	2	8	128	8	8	-
P. aeruginosa ATCC 27853	32	> 256	32	16	128	8	4	-
S. Typhimurium ATCC 13311	16	> 256	2	16	> 256	4	0.5	-
C. albicans ATCC 90028	16	64	1	4	32	4	-	0.5
C. tropicalis ATCC 750	1	16	0.06	0.125	2	0.5	-	1

FV – *F. vulgare;* HS – *H. stoechas*; MP – *M. pulegium*; PP – *P. pinaster*; RG – *R. graveolens;* TM – *T. mastichina*; TET – Tetracycline; AMP B - Amphotericin B.

These two EOs also demonstrated antifungal activity, with MIC values between 0.06 and 4 µL/mL against the tested yeasts. On the other hand, the *H. stoechas* and *R. graveolens* EOs showed lower activity with MIC values between 16 and >256 µL/mL, except for *R. graveolens* against *C. tropicalis* with a MIC value of 2 µL/mL.

The obtained results for the EO of *M. pulegium* are in accordance with the results previously reported (Baali et al., 2019; Luís and Domingues, 2021; Vieira et al., 2017). The activity of the EO of *M. pulegium* may potentially be linked with its main component pulegone, which was capable of inhibiting the growth of multi-drug resistant *E. coli*, as described in the literature (Gong et al., 2021).

Regarding *T. mastichina* EO, Cutillas et al. (2018b) evaluated different isolated compounds against several microorganisms, namely their main components 1,8-cineole and linalool. The authors showed that 1,8-cineole had MIC values highest than 2.3 mg/mL against *E. coli, S. aureus* and *C. albicans*, whereas linalool presented lower MIC values, indicating that the EOs with lower values of linalool were less active against the tested microorganisms (Cutillas et al., 2018b). Considering the present study, the EO' activity may not be correlated to the concentration of linalool. Within several species tested, *P. aeruginosa* demonstrated to be the most resistant to *T. mastichina* EO, as shown in other studies (Cutillas et al.,

2018b; Vieira et al., 2017). Regarding the antifungal activity, Pina-Vaz et al. (2004) evaluated the antifungal activity of *Thymus* oils and their major compounds against several *Candida* strains. The *T. mastichina* results showed MIC values between 1.25 and 10 μL/mL and for 1,8-cineole the MIC values obtained were between 5 and 20 μL/mL. With this study, it can be seen that the antifungal activity of *T. mastichina* may be related to its major compound, but also the synergism between several compounds present in its composition can be also contribute for its activity against fungi (Pina-Vaz et al., 2004).

To evaluate the cytotoxic profile EOs on human cells, the current investigation was carried out by using cultured normal human dermal fibroblasts cell line (Figure 4.1). The results revealed EOs reduced the viability of these cells in a dose-dependent manner, with EOs cytotoxicity varying in ranges of IC_{50} of 0.26 ± 0.13 and 0.95 ± 0.62 μL/mL, suggesting that the use of EOs as antimicrobials should take into account this constraint.

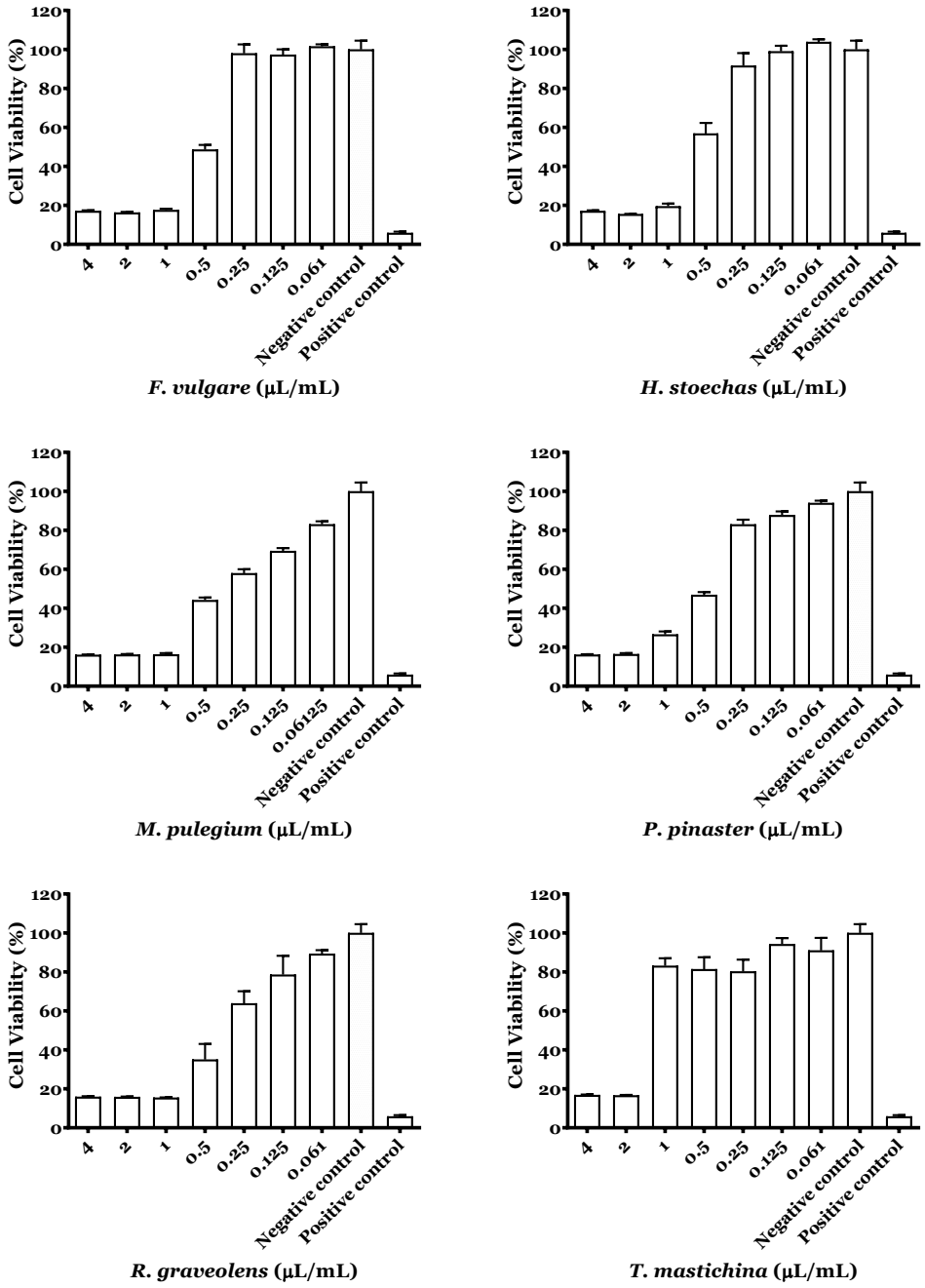

Figure 4.1. Essential oils biocompatibility on cultured normal human dermal fibroblasts cell line measured by MTT assay after 24 h of treatment. The negative control were untreated cells and cells cultured with EtOH (96%) were used as a positive control. Results are expressed as mean ± standard deviation of at least three independent experiments.

The cytotoxicity of *R. graveolens* EO (IC_{50} of 0.26 ± 0.13 µL/mL) is in agreement to previous works, in which EOs extract from these extracts and its isolated compounds showed to be cytotoxic against several cell lines (Coimbra, et al. 2020a). The EO of *P. pinaster* also proved to be cytotoxic (IC_{50} of 0.38 ± 0.15 µL/mL), similar to *Juniperus communis* EO from needles, with concentrations above 0.32 µL/mL showing cytotoxicity in HaCaT keratinocytes (Cabral et al., 2012). Both EOs have α-pinene as one of the main compounds and this compound may be one of those responsible for the cytotoxicity of this EO (Machado

et al., 2021). The highest cytotoxic effect was observed with *R. graveolens* EO and the less cytotoxic was the *T. mastichina* EO. The decreased viability of these essential oils may be justified by the EOs capacity to impair the cell membrane integrity of prokaryotic and eukaryotic cells (Perricone et al., 2015).

Based on these findings, it is important to notice that the concentration of these EOs must be chosen carefully, taking into account the envisioned application.

4.4. Conclusion

Considering the results, it can be concluded that the studied EOs have promising bioactive properties, highlighting the EOs of *M. pulegium* and *H. stoechas* that showed to have the best free radicals scavenging activity, and *H. stoechas* and *P. pinaster* for their ability to inhibit the lipid peroxidation. The *M. pulegium*, *P. pinaster* and *Thymus mastichina* EOs revealed the highest antimicrobial activity against different species of bacteria and *Candida* strains, while the EO of *R. graveolens* was effective against *C. tropicalis,* evidencing its potential to be applied in diverse fields.

4.5. Supplementary material from Chapter 4

This chapter contains additional results that were not published. The results are preceded by a short description of the methodology applied, followed by the results and a brief conclusion.

4.5.1 Screening of antimicrobial activity of the *Thymus zygis* EO

The susceptibility of bacteria and yeasts to the *T. zygis* EO was performed as described in the Materials and Methods section of Chapter 4. Briefly, it was analysed using the disc diffusion method (Luís et al., 2017) and the broth microdilution method based on Coimbra et al. (2020b), furthermore the effect of the EO volatile compounds was evaluated according to Coimbra et al. (2022).

Table 4.S1. Diameters of inhibition zone (mm) for disc diffusion method and volatiles compounds, presented as mean ± standard deviation, and minimum inhibitory concentration (µL/mL), presented as modal values, of *T. zygis* EO and standards (µg/mL) against bacterial and yeast species.

	Inhibition zone (mm)			**MIC**		
Species	EO (10 µL/disc)	TET (20 µg/disc)	Volatile compounds (10 µL/disc)	EO	TET	AMP B
S. aureus ATCC 25923	33.45 ± 4.57	27.85 ± 0.53	30.64 ± 1.05	1	0.125	-
S. aureus MRSA 10/08	35.81 ± 5.60	28.42 ± 1.20	30.60 ± 3.62	2	0.125	-
B. cereus ATCC 11778	36.70 ± 1.29	30.61 ± 0.92	34.71 ± 3.62	1	0.015	-
L. monocytogenes LMG 16779	37.66 ± 6.91	33.07 ± 0.27	32.87 ± 5.45	1	0.25	-
E. coli ATCC 25922	35.00 ± 2.44	26.06 ± 0.51	33.13 ± 3.88	0.5	0.25	-
K. pneumoniae ATCC 13883	33.03 ± 1.71	25.43 ± 0.73	29.13 ± 2.25	0.5	8	-
P. aeruginosa ATCC 27853	10.11 ± 1.25	16.01 ± 0.96	6.00 ± 0.00	16	4	-
S. Typhimurium ATCC 13311	33.83 ± 3.36	21.82 ± 1.11	27.87 ± 3.74	0.5	0.5	-
C. albicans ATCC 90028	69.11 ± 0.67	-	65.18 ± 8.78	2	-	0.5
C. tropicalis ATCC 750	64.35 ± 4.44	-	56.68 ± 2.57	2	-	1

AMP B - Amphotericin B; MIC - Minimum inhibitory concentration; TET – Tetracycline.

The results reveal antibacterial and antifungal activity of the *T. zygis* EO, with the results obtained with the disc diffusion method and by the determination of the MIC values showing higher activity of the *T. zygis* EO than the other EOs tested in Chapter 4. The good antimicrobial activity of *T. zygis* EO has been demonstrated in previous works, being that some differences can be found due, for example, to the constitution of the EOs, location, chemotypes, amongst others (Ballester-Costa et al., 2013; Cutillas et al., 2018a; Dorman and Deans, 2004; Pina-Vaz et al., 2004; Rota et al., 2008).

The volatile compounds also showed good inhibitory activity on the growth of different species of bacteria and yeasts, while almost completely inhibiting the growth of the tested

yeasts. These results for bacteria agrees with the results obtained by Ghabraie et al. (2016) in which the volatile compounds of the *T. zygis* EO present strong antibacterial activity against different species of Gram-positive and Gram-negative bacteria, except against *P. aeruginosa* (Ghabraie et al., 2016).

These results led us to conclude that the EO of *T. zygis* was the EO with higher antimicrobial activity within the EOs studied in this thesis, thus being further explored in the following chapters.

Chapter 5

Thymus zygis essential oil: Phytochemical characterization, bioactivity evaluation and synergistic effect with antibiotics against *Staphylococcus aureus*

This chapter corresponds to a published manuscript with the following reference:

Coimbra, A., Miguel, S., Ribeiro, M., Coutinho, P., Silva, L., Duarte, A.P., Ferreira, S., 2022. *Thymus zygis* essential oil: Phytochemical characterization, bioactivity evaluation and synergistic effect with antibiotics against *Staphylococcus aureus*. Antibiotics. 11, 146. https://doi.org/10.3390/antibiotics11020146

Abstract

Staphylococcus aureus is a nosocomial bacterium causing different infectious diseases, ranging from skin and soft-tissue infections to more serious and life-threatening infections such as sepsis, meningitis and endocarditis, which may be exacerbated by antibiotic resistance. Plant products may be seen as an alternative as antibacterial agents, namely, against *S. aureus*. Thus, the aim of this work was to characterize the chemical composition and evaluate the bioactive properties of the *T. zygis* essential oil (EO), with a focus on antimicrobial activity against *S. aureus*. Gas chromatography coupled with mass spectrometry was used to assess the chemical composition of the *T. zygis* EO, and the antioxidant activity was evaluated using the DPPH method and β-carotene-bleaching assay. The antimicrobial activity against *S. aureus* strains, the interaction with different antibiotics and the attenuation of this bacterium's virulence were evaluated. The *T. zygis* EO showed antioxidant activity acting through two different mechanisms and antibacterial activity against *S. aureus*, with antibiofilm and antihaemolytic properties. This EO also demonstrated synergistic or additive interactions in combination with ampicillin, ciprofloxacin or vancomycin against *S. aureus* strains and, in some cases, changed the antibiotic-resistance phenotype from resistant to susceptible. Therefore, the present work demonstrates the good bioactive properties of the EO of *T. zygis,* mainly the antimicrobial activity against *S. aureus*, revealing its potential to be used as an antibacterial agent.

Keywords: *Thymus zygis*; essential oil; antioxidant activity; antimicrobial agent; *Staphylococcus aureus*; interaction with antibiotics

5.1. Introduction

Antibiotics are used as the primary weapon against infections; while, at first, antibiotics were highly effective, their inappropriate use and high selective pressure have led to the emergence and spread of antibiotic-resistant bacteria (Wu et al., 2019). In fact, antibiotic resistance has increased dramatically in recent decades and is now considered one of the greatest global health threats (Langeveld et al., 2014; Owen and Laird, 2018).

Staphylococcus aureus is a Gram-positive facultative anaerobic human pathogen of both nosocomial and community-acquired infections worldwide (Lee et al., 2017, 2014). *S. aureus* is a commensal bacterium located on the skin and mucous membranes, but also a virulent bacterial pathogen associated with high morbidity and mortality (Otto, 2014; Singh and Phukan, 2019; Vandenesch et al., 2012; Zhang et al., 2013). This opportunistic pathogen can cause numerous acute and chronic infections (Lee et al., 2017; Quave and Horswill,

2014), such as moderately severe skin infections, fatal pneumonia, sepsis, meningitis, endocarditis, or toxic-shock syndrome (Cheung et al., 2021; Korem et al., 2007; Otto, 2014). The higher rates of colonization, augmented use of surgical implants, immunosuppressive conditions, and escalation of antibiotic resistance have increased the prevalence of these infections (Quave and Horswill, 2014). Due to frequently occurring antibiotic resistance in *S. aureus* isolates, *S. aureus* infections are particularly problematic, and methicillin-resistant *S. aureus* (MRSA) is of utmost importance clinically. The World Health Organization states that people with MRSA infections are 64% more likely to die than people with drug-sensitive infections, and so it is on the list of microorganisms for which further investigation is critical (WHO, 2021).

When bacteria become resistant to first-line medicines, alternative therapies may be used (Langeveld et al., 2014). The development of novel antibiotics remains a dominant approach for the treatment of bacterial-associated infections; however, this discovery is challenging (Wu et al., 2019). Thus, it is important to explore alternative strategies and molecules to fight antibiotic-resistant *S. aureus* (Wu et al., 2019). One possible solution is to combine antibiotics with other nonantibiotic drugs or to combine antibiotics with adjuvants or antimicrobials selected from the reservoir of bioactive compounds in nature (Ju et al., 2020; Langeveld et al., 2014).

Plant products have been used in folk medicine throughout human history and are the primary source of healthcare for much of the world's population (Owen and Laird, 2018). Hereupon, the need for novel antibacterial therapies has led to an increase in research into natural products as antibacterial agents (Owen and Laird, 2018). Plants naturally produce a wide diversity of secondary metabolites, such as essential oils (EOs), that serve as defence compounds protecting against pathogens; therefore, they are important sources for the discovery of natural bioactive products (Wu et al., 2019).

Essential oils are complex blends of secondary metabolites, mainly terpenes and terpenoids (Vázquez-Ucha et al., 2020), extracted by steam distillation, hydrodistillation or solvent extraction (Raut and Karuppayil, 2014; Trifan et al., 2020), which are usually stored in resin ducts, oil ducts, glands or trichomes of the plants (Raut and Karuppayil, 2014). EOs are natural products obtained from aromatic plant materials, with a broad spectrum of valuable biological properties and recognized uses in various areas (pharmaceutical, food, cosmetic and textile industries) (Ribeiro-Santos et al., 2018; Trifan et al., 2020). They have been known to present antibacterial activity for centuries and so have been investigated for this purpose (Owen and Laird, 2018).

Thymus zygis, also known as red thyme, is predominantly found in the Mediterranean region, Asia, Southern Europe and North Africa and has been used for a long time as a spice

or drug (Cutillas et al., 2018a; Gonçalves et al., 2010). Its EO is known for its bioactive properties, such as antibacterial (Lagha et al., 2019), antifungal (Debonne et al., 2019; Yang and Clausen, 2007) antiviral (Santoyo et al., 2014), antigiardial (Machado et al., 2010), insecticidal (Park et al., 2016; Sangha et al., 2017) and other properties.

Thus, considering the relevance of *S. aureus* resistance to antibiotics and the bioactive effects of *T. zygis*, this work aimed to evaluate the chemical composition of the *T. zygis* EO and to provide a better understanding of its antioxidant properties and antimicrobial activity against *S. aureus* strains, as well as cytotoxicity. The effect of the *T. zygis* EO on the virulence attenuation of *S. aureus* and also its interaction with antibiotics were evaluated.

5.2. Materials and Methods

5.2.1 Essential oil and bacterial strains

The commercial *Thymus zygis* essential oil was acquired from the company Pharmaplant (Algarve, Portugal) and was obtained by steam distillation from the aerial parts. The essential oil was protected from light and stored at 4 ˚C until further use.

The reference strain *S. aureus* ATCC 25923 and two clinical isolates *S. aureus* SA 03/10 and *S. aureus* MRSA 12/08 were used as test microorganisms.

5.2.2 GC-MS analysis of the *T. zygis* essential oil

The *T. zygis* EO was analysed on an Agilent Technologies 7890A GC-System apparatus equipped with a fused silica DB-5 capillary column (Agilent J&W Column, part number: 122–5032) with a 30 m × 0.25 mm inner diameter and 0.25 μm film thickness coupled with a mass spectrometer (MS) (Agilent Technologies 5975C, Inert XL MSD) Triple-Axis detector. The operating conditions for the mass spectrometer were set as follows: ion source temperature, 250 ˚C; ionization voltage, 70 eV; interface temperature, 280 ˚C. As the carrier gas, helium was used at a flow rate of 1 mL/min. The initial oven temperature was 40 ˚C, with a hold time of 5 min, and it was increased to 250 ˚C at a rate of 5 ˚C/min. A 1 μL volume of *T. zygis* EO at a concentration of 10% (*v*/*v*) in dichloromethane was injected. The NIST Mass Spectral Software and Agilent GC/MSD ChemStation Software were used to calculate the relative concentration and perform identification.

5.2.3 Antioxidant activity

The antioxidant activity of the *T. zygis* EO was evaluated using two methodologies, the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) and the β-carotene-bleaching assays, according to Coimbra et al. (2020) with some adaptations. In the first one, 5 μL of each concentration

of *T. zygis* EO methanolic solution was separately mixed with 195 µL of DPPH methanolic solution in 96-well microtiter plates. Methanol was used as the negative control, and Trolox (Acros Organics, Geel, Belgium) and gallic acid (Acros Organics, Geel, Belgium), as standards. The absorbances were measured at 515 nm, and the antioxidant activity was expressed through the antioxidant activity index (AAI), calculated according to:

$$AAI = (\text{final concentration of DPPH in the control sample}/IC_{50}. \quad (1)$$

In the second methodology, 56.6 µL of a *T. zygis* EO methanolic dilution was added to 943.4 µL of the emulsion. The emulsion was prepared with 500 µL of β-carotene solution at 20 mg/mL in chloroform, 40 µL of linoleic acid, 400 µL of Tween 40 and 1 mL of chloroform. Then, the chloroform was evaporated under vacuum at 45 °C, and 100 mL of oxygenated distilled water was added to form an emulsion. Butylated hydroxytoluene (BHT, purity 99%, Acros Organics, Geel, Belgium) was used as a standard, and methanol, as a negative control. The absorbances were read at 470 nm, against a blank containing an emulsion without β-carotene, and the percentage of inhibition of β-carotene oxidation was calculated using the equation:

$$\%\ \text{Inhibition} = ((\text{Abs sample}^{t=1h} - \text{Abs control}^{t=1h})/(\text{Abs control}^{t=0h} - \text{Abs control}^{t=1h}))\times 100. \quad (2)$$

All tests were performed in triplicate.

5.2.4 Antimicrobial activity

5.2.4.1 Disc-diffusion method and vapour-phase antimicrobial activity determination

The disc-diffusion method was performed to evaluate the susceptibility of the *S. aureus* strains to the *T. zygis* EO as described by Luís et al. (2017). Tetracycline at 20 µg/disc was used as a positive control. The susceptibility of the *S. aureus* strains to the volatile compounds of the *T. zygis* EO was evaluated as described by Duarte et al. (2016). These methodologies were performed with tryptic soy agar (TSA) medium. The inhibition halos were measured in millimetres, and the results are presented as means ± standard deviations. At least three independent assays were performed.

5.2.4.2 Determination of the minimum inhibitory concentration (MIC)

The susceptibility of the *S. aureus* strains to the *T. zygis* EO was evaluated through the broth microdilution method according to Coimbra et al. (2020) with modifications. Briefly, in 96-well plates, the essential oil was serially diluted with tryptic soy broth (TSB, RPD microbiology, Spain). Dimethyl sulfoxide (DMSO) was used as the solvent for the improvement of the solubility, with a maximum concentration of 2% (*v*/*v*) (no growth

inhibition). The inoculum, with a concentration of 0.5 McFarland, was diluted in medium, and 50 µL was added to the wells to obtain a final volume of 100 µL and a concentration of 5×10^5 colony-forming units (CFU)/mL per well. The MIC was determined as the lowest concentration of *T. zygis* EO without visible growth. At least three independent determinations with duplicates were performed, and the results are presented as modal values.

5.2.4.3 Time–kill curves

The time–kill curve assay was performed based on Ferreira and Domingues (2016) with minor modifications. Briefly, *S. aureus* strains grown overnight were used to prepare a cellular suspension to give a final cell concentration of 10^6 CFU/mL, and it was exposed to several concentrations of *T. zygis* EO (from 0.125× to 2× MIC). A solvent control with DMSO (1% (*v*/*v*)) and growth control were also performed. The viable counts were determined by the drop-plate method at 0, 2, 4, 6 and 8 h of incubation from the tubes incubated at 37 °C. The independent experiments were performed at least thrice.

5.2.4.4 Antibiofilm activity of *T. zygis* EO

Biofilm formation

The inhibition of biofilm formation was based on the previously described method of Stepanović et al. (2004) with modifications. Briefly, *S. aureus* strains were grown overnight at 37 °C, at 250 rpm, in TSB. Afterwards, the turbidity of the suspension was adjusted to an $OD_{600\ nm}$ ~1.5 and diluted to achieve a final concentration in the wells of 1×10^7 CFU/mL. Serial two-fold dilutions of *T. zygis* EO (0.25 to 2× MIC) were prepared in TSB, supplemented with 0.5% glucose, in 96-well flat-bottom polystyrene microtiter plates, and 100 µL of each bacterial suspension was added to the wells to obtain a final volume of 200 µL. The plates were incubated at 37 °C for 24 h. For the positive control, the bacterial suspension with medium was used, whereas for the negative control, only the culture medium was used. A solvent control in the presence of DMSO (0.125 to 1%) was also performed. After incubation, the contents of the plates were poured off, and each well was washed twice with 200 µL of distilled water to remove the loosely attached cells. The remaining attached bacteria were fixed with methanol (200 µL) for 20 min; after methanol removal, the plates were air dried. Staining was achieved with 0.1% (*w*/*v*) crystal violet (200 µL) for 10 min, the dye was removed, and the wells were washed thrice with 400 µL of distilled water. The crystal violet bound was dissolved with 33% (*v*/*v*) glacial acetic acid per well (200 µL), and the absorbance at 570 nm was determined using a microplate reader. At least five replicates of three independent experiments were conducted.

Biofilm dispersion

The effect of the *T. zygis* EO on preformed biofilms was evaluated based on Duarte et al. (2015) with some adaptations. Briefly, biofilms were prepared as mentioned above by inoculating 100 µL of the bacterial suspension into the wells of 96-well flat-bottom polystyrene microtiter plates containing 100 µL of TSB supplemented with 0.5% glucose. Following incubation at 37 °C for 24 h, the medium was removed and 100 µL of each *T. zygis* EO or DMSO concentration was added to the biofilm in the wells. The plates were further incubated at 37 °C for 24 h. For the positive control, 100 µL of bacterial suspension was added, whereas for the negative control, only the culture medium (200 µL) was used. After incubation, the biofilm biomass was evaluated by the crystal-violet staining method as described above. At least five replicates of three independent experiments were conducted.

5.2.4.5 Inhibition of quorum sensing

The anti-quorum-sensing activity of the *T. zygis* EO was assessed with the biosensor strain *Chromobacterium violaceum* ATCC 12472 and performed based on Asensio et al. (2020) with some modifications. A bacterial suspension of *C. violaceum* ATCC 12472 was obtained from an overnight culture at 30 °C and 250 rpm in Luria–Bertani (LB) broth and then diluted in fresh LB broth to achieve $OD_{600\ nm}$ 0.02. *T. zygis* EO and resveratrol (positive control) were serially 2-fold diluted with LB (final concentrations of 0.0015 to 0.013% and 0.063 to 1.0%, respectively), and 500 µL of each solution was applied to 48-well flat-bottom polystyrene microtiter plates. DMSO with a final concentration of 0.125% was used as the solvent control. Then, 500 µL of the bacterial suspension was added to the wells, and the plate was incubated at 30 °C without shaking for 48 h. After incubation, 750 µL from each well was transferred to a centrifuge tube and centrifuged at 5000× *g* for 3 min. The supernatants were discarded, and the pellets were vigorously vortexed with 750 µL of DMSO to dissolve the violacein. The samples were centrifuged again at 8000× *g* for 5 min to remove the *C. violaceum* cells and to evaluate the violacein production. A 200 µL volume of violacein-containing supernatant was added into a 96-well microplate in triplicate, and the optical density at 585 nm was measured using a plate reader. The growth inhibition of *C. violaceum* was evaluated by suspending the removed cells in 750 µL of distilled water, 200 µL of the suspension was applied into a 96-well microplate in triplicate, and the absorbance was measured at 600 nm. The violacein inhibition (%) was calculated using the equation

$$100 - ((OD_{sample}/OD_{growth\ control}) \times 100). \quad (3)$$

5.2.4.6 Scanning electron microscopy (SEM)

The effect of *T. zygis* EO on biofilm formation by the strain *S. aureus* MRSA 12/08 was observed through SEM according to Luís et al. (2014) with slight modifications. Biofilm formation was performed as described above but, in this case, in 24-well plates containing a polystyrene coupon with dimensions of 1 cm × 1 cm. Initially, the coupons were washed and submerged in a 70% ethanol solution overnight, followed by exposure to UV radiation for 30 min on both sides. A 500 µL volume of *T. zygis* EO (1× MIC), DMSO (0.5% (*v*/*v*), solvent control) or TSB supplemented with 0.5% glucose (growth control) was added to the plate, and 500 µL of bacterial suspension was added. After 24 h of incubation at 37 °C, the wells were washed twice with an isotonic saline solution (0.85% (*w*/*v*) NaCl) and fixed with 500 µL of 2% glutaraldehyde and 4% formaldehyde solution in PBS for 3 h at room temperature. The coupons were then carefully washed with PBS, dehydrated in a graded ethanol series (25, 50, 70, 90 and twice with 100%) and dried in a desiccator overnight. Lastly, the coupons were mounted on a stub, sputter-coated with gold and examined with a scanning electron microscope (Hitachi S-3400 N).

5.2.4.7 Checkerboard assay

The checkerboard method was used to test the combined effect of the *T. zygis* EO and antibiotics, according to Silva et al. (2011) with some adaptations. The inoculum was prepared as described in Section 5.2.4.2 and the suspension was then diluted 1:67 in TSB to ensure a final cell concentration of 5×10^5 CFU/mL in each well. Two microplates were prepared, one where *T. zygis* EO was successively diluted with TSB, vertically, with a final volume of 50 µL, and another plate, where successive dilutions of the antibiotics (ampicillin, ciprofloxacin or vancomycin) were carried out with TSB, in the horizontal direction. Subsequently, with a multichannel pipette, 50 µL from the plate with the antibiotic was transferred to the plate with the *T. zygis* EO, with the addition of 50 µL of inoculum to obtain a final volume of 150 µL per well. The concentrations of *T. zygis* EO and the antibiotics were selected based on the MIC values previously determined. The plate was incubated at 37 °C for 24 h.

The results for the combined effects of the *T. zygis* EO and antibiotics were calculated and are expressed in terms of the fractional inhibitory concentration index (FICI), equal to the sum of the fractional inhibitory concentration (FIC) of the *T. zygis* EO and FICs of the antibiotics. The FIC was defined as the MIC of the EO and antibiotic in combination divided by the MIC of the EO and antibiotic used alone. If FICI ≤ 0.5, it was considered to have a synergistic effect; for 0.5 < FICI ≤ 1, there was an additive effect; 1 < FICI < 4 showed an indifferent effect; and with FICI ≥ 4, the effect was antagonistic (Roudashti et al., 2017).

5.2.4.8 Effect of the *T. zygis* EO on the haemolytic capacity of *S. aureus*

The haemolytic activity of the *S. aureus* strains was evaluated as described by Lee et al. (2017) with adaptations. Briefly, the *S. aureus* strains were grown overnight at 37 °C, at 250 rpm, for 16 h, and used to prepare a cellular suspension at a final concentration of 10^6 CFU/mL. Tubes were prepared by adding *T. zygis* EO (0.06 to 0.5× MIC) in TSB and the cellular suspension in a final volume of 3 mL. A solvent control with DMSO (0.25% (*v*/*v*)) and a growth control were performed, and all the tubes were incubated at 37 °C for 20 h. After the incubation, 100 µL from each tube was transferred to a U-bottom 96-well plate, and 100 µL of 2% (*v*/*v*) human erythrocytes were added. A negative control (PBS without bacteria) and positive control for total haemolysis (1% (*v*/*v*) Triton-X 100) were also included. The erythrocytes were collected from one healthy volunteer into a blood collection tube with ethylenediamine tetraacetic acid (EDTA) and washed thrice with PBS, and a stock solution was prepared in the same buffer. The plate was incubated at 37 °C for 1 h and, after the incubation, was centrifuged at 1000× *g* for 5 min. A 100 µL volume of each supernatant was transferred to a 96-well flat-bottom microtiter plate, and the absorbance at 492 nm was measured. At least four replicates of three independent experiments were conducted.

5.2.5 Evaluation of *T. zygis* EO biocompatibility

The cytotoxicity of the *T. zygis* EO was evaluated using normal human dermal fibroblasts (NHDF cells isolated from the dermis of adult skin and acquired from PromoCell GmbH (Heidelberg, Germany)) that were initially seeded in 96-well flat-bottom culture plates with 2×10^4 cells/well and containing DMEM-F12 supplemented with 10% FBS. Adherent cells were grown in an incubator with a humidified atmosphere containing 5% CO_2 at 37 °C for a day. Then, the culture medium was removed, and the cells were incubated with several concentrations of *T. zygis* EO (0.0030 and 0.4%) for 24 h. Cells cultured with EtOH (96%) were used as a positive control, and those without materials were used as a negative control. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to monitor the cell metabolic activity. For that, the medium was removed, and a PBS solution of 5 mg/mL of MTT (50 µL in each well) was added to each sample (n = 5). The plate was incubated in a 5% CO_2 atmosphere for 4 h at 37 °C. To dissolve the pigmented formazan formed, 200 µL of DMSO was added to the cells for 30 min. Afterwards, a microplate reader (Biorad xMark microplate spectrophotometer, Waltham, MA, USA) was used to read the absorbance at 570 nm.

5.3. Results

5.3.1 *T. zygis* EO chemical composition

The analysis of the chemical composition of the *T. zygis* EO through gas chromatography coupled with mass spectrometry (GC-MS) showed eighteen compounds, accounting for 94% of the total composition of the EO. The main components were identified as thymol (43.17%), carvacrol (13.00%) and *p*-cymene (10.58%) (Table 5.1).

Table 5.1. Chemical composition of *T. zygis* essential oil according to GC-MS.

Compounds	Retention Time	Kovats Index	%
α-Thujene	10.75	929	0.73
α-Pinene	10.98	936	1.01
Camphene	11.50	950	1.19
β-Myrcene	13.14	989	1.29
α-Terpinene	13.98	1017	1.38
p-Cymene	14.31	1024	10.58
Limonene	14.42	1030	0.56
γ-Terpinene	15.50	1060	8.04
Trans-sabinene hydrate	15.71	1098	1.14
β-Linalool	16.85	1099	3.77
Camphor	18.16	1143	1.10
Trans-pinocarveol	18.31	1140	0.89
Borneol	18.90	1166	3.79
4-Terpineol	19.21	1177	0.46
Thymol	22.78	**1290**	**43.17**
Carvacrol	22.99	1300	13.00
β-Caryophyllene	26.03	1420	1.43
Caryophyllene oxide	30.01	1581	0.59

The value in bold represents the major component of *T. zygis* EO.

5.3.2 *T. zygis* EO antioxidant activity

A very strong antioxidant activity was exhibited by the *T. zygis* EO according to the DPPH method and based on Scherer and Godoy classification (Scherer and Godoy, 2009), with IC_{50} values of 2.00 ± 0.15% (Table 5.2).

Table 5.2. Results for antioxidant activity of *T. zygis* EO and standards measured using the DPPH method (mean ± standard deviation) and β-carotene-bleaching assay (results expressed as medians).

	DPPH Method			β-Carotene-Bleaching Assay
Samples	IC_{50} (%)	AAI	Antioxidant Activity Classification	IC_{50} (%)
T. zygis	2.00 ± 0.15	12.87 ± 3.65	Very strong	0.27
Gallic acid	2.14 ± 0.39	22.16 ± 3.53	Very strong	-
Trolox	3.26 ± 1.21	15.02 ± 0.64	Very strong	-
BHT	-	-	-	0.10

T. zygis EO IC_{50}s are presented as % (v/v) and standards as % (w/v); AAI—Antioxidant activity index; BHT—Butylated hydroxytoluene.

The antioxidant activity of the *T. zygis* EO proved to have an identical effect to the Trolox standard with similar AAI values. Regarding the antioxidant activity according to a β-carotene-bleaching assay, the *T. zygis* EO showed antioxidant activity through the inhibition of lipid peroxidation (Table 5.2). For this reason, it can be said that the *T. zygis* EO has antioxidant activity through at least two different mechanisms, the inhibition of lipid peroxidation and sequestration of free radicals.

5.3.3 *T. zygis* EO antibacterial activity

The antimicrobial activity of the *T. zygis* EO was evaluated by using different methodologies and considering different parameters. Thus, first, it was screened through the disc-diffusion methodology, considering the EO and its volatile compounds (Table 5.3).

Table 5.3. Diameters of inhibition zones for disc-diffusion method and volatile compounds of *T. zygis* EO.

	Inhibition Zone (mm)			MIC (%)	
Species	*T. zygis* (10 µL/Disc)	Tetracycline (20 µg/Disc)	Volatile Compounds (10 µL/Disc)	*T. zygis*	Tetracycline
S. aureus ATCC 25923	35.10 ± 4.57	31.17 ± 2.73	27.54 ± 4.10	0.05	0.013
S. aureus SA 03/10	20.67 ± 1.59	8.24 ± 0.49	16.26 ± 5.15	0.05	6.4
S. aureus MRSA 12/08	30.93 ± 4.64	8.42 ± 0.75	16.45 ± 3.63	0.1	6.4

MIC—minimum inhibitory concentration of *T. zygis* EO (%, v/v) and tetracycline (%, w/v). Values for inhibition zone are presented as means ± standard deviations, and MIC values of *T. zygis* EO and tetracycline are presented as modal values.

According to the obtained results, higher antimicrobial activity was found against *S. aureus* ATCC 25923, with an inhibition halo of 35.10 ± 4.57 mm. The *S. aureus* SA 03/10 strain was the most resistant to the *T. zygis* EO, with an inhibition halo of 20.67 ± 1.59 mm. Concerning the evaluation of the volatile compounds of the *T. zygis* EO (Table 5.3), the *T. zygis* EO's compounds released from the disc during incubation demonstrated inhibitory

activity against all three *S. aureus* strains, showing inhibition halos between 16.26 ± 5.15 and 27.54 ± 4.10 mm. Regarding the study of the antimicrobial activity through the MIC determination (Table 5.3), the essential oil of *T. zygis* presented the same MIC value of 0.05% for the strains *S. aureus* ATCC 25922 and SA 03/10 and 0.1% against MRSA 12/08.

The antimicrobial activity can also be observed by looking at the time–kill curves showing that the *T. zygis* EO had a bactericidal effect at 1× and 2×MIC for all the strains of *S. aureus* (Figure 5.1). Furthermore, after 4 h of incubation, significant reductions in the logarithmic bacterial counts were observed for MRSA 12/08 with 0.5× MIC, 0.25× MIC and 0.125× MIC of *T. zygis* EO ($p < 0.0001$), while only the subinhibitory concentration of 0.5× MIC led to a significant reduction in *S. aureus* SA 03/10 ($p < 0.0001$).

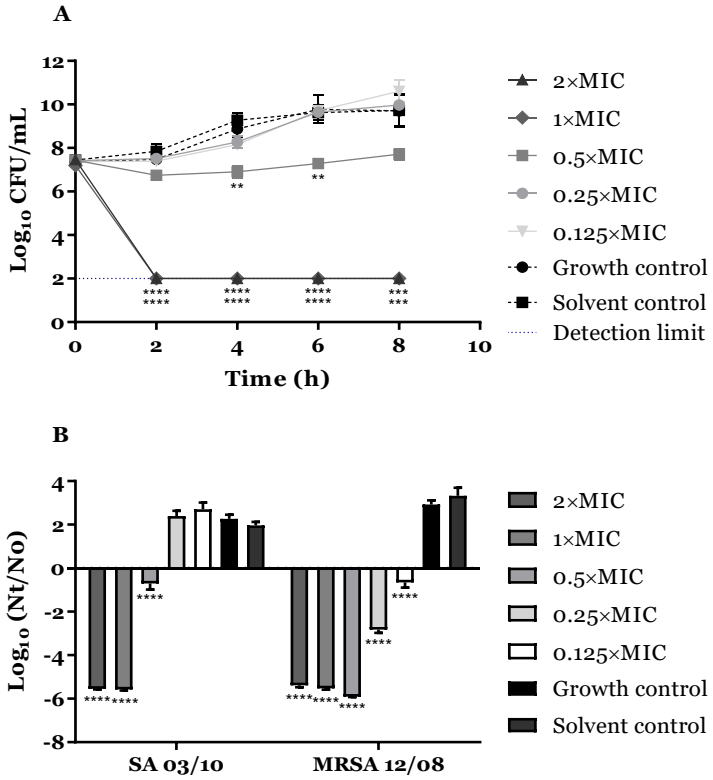

Figure 5.1. Time–kill curves for *Staphylococcus aureus* ATCC 25923 (A) and Log_{10} (N_t/N_0) of *Staphylococcus aureus* SA 03/10 and MRSA 12/08 strains at 4 h (B) incubated with *T. zygis* EO from 0.125× MIC to 2× MIC at 37 °C. Pointed line corresponds to the detection limit of the method. ** ($p < 0.01$); *** ($p < 0.001$); **** ($p < 0.0001$).

The combined use of non-antibiotic compounds (known as antibiotic adjuvants) and antibiotics can be a strategy to enhance the activity of antibiotics and thus increase the susceptibility of resistant strains of bacteria (Owen and Laird, 2018; Vázquez-Ucha et al., 2020). According to the results presented in Figure 5.2, the *T. zygis* EO showed interactions with the antibiotics ampicillin, ciprofloxacin and vancomycin, demonstrating a synergistic (FICI ≤ 0.5) or additive (0.5 < FICI ≤ 1) effect, according to the classification of (Roudashti et al., 2017). With this association, a synergic interaction occurred for the MRSA 12/08

strain with ampicillin or ciprofloxacin and the *T. zygis* EO, while the other combinations presented an additive interaction. Furthermore, the *T. zygis* EO resensitised *S. aureus* SA 03/10 to the antibiotics, ampicillin, ciprofloxacin and vancomycin, and *S. aureus* MRSA 12/08, to ampicillin or ciprofloxacin.

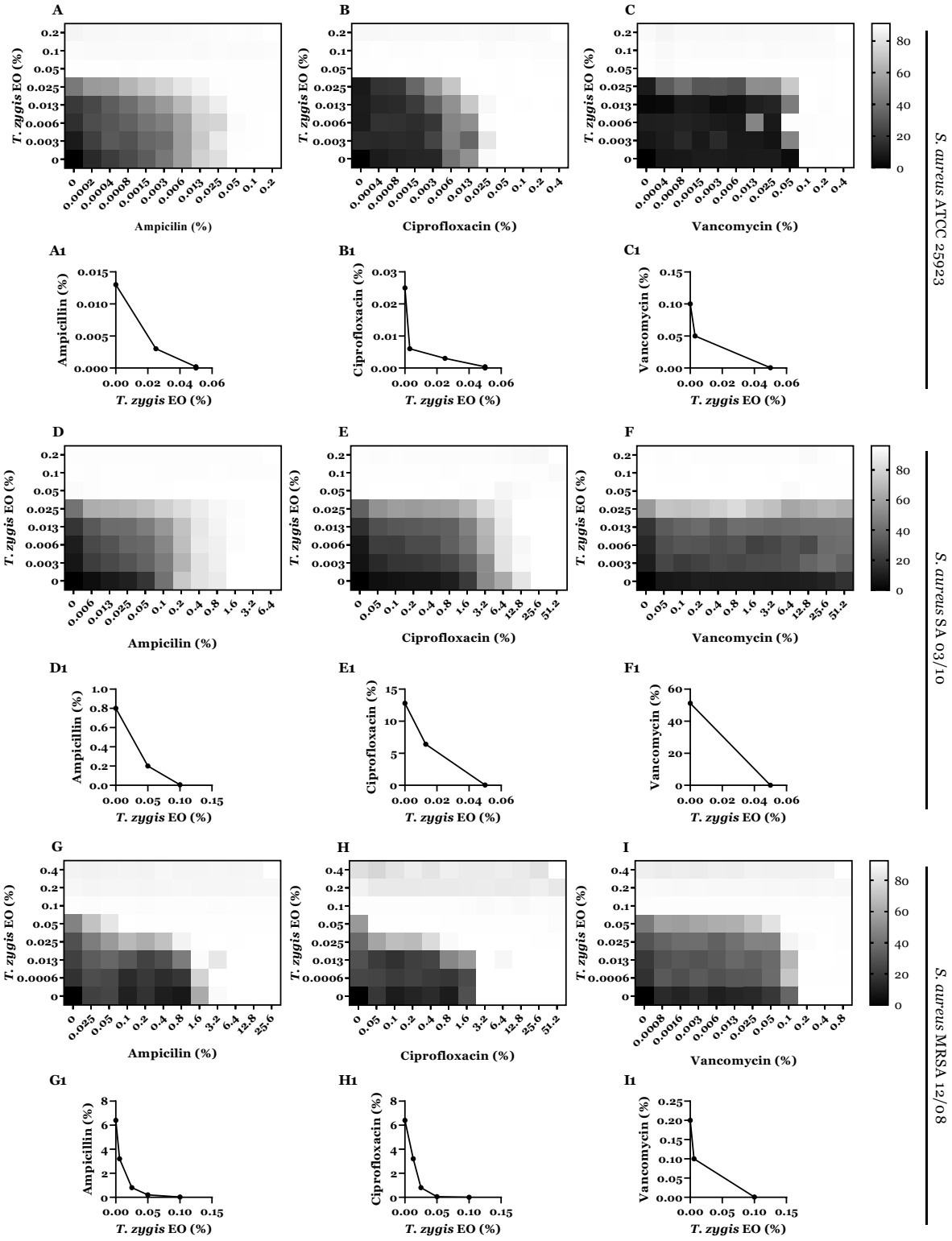

Figure 5.2. Checkerboards of *T. zygis* EO and (A,D,G) ampicillin, (B,E,H) ciprofloxacin and (C,F,I) vancomycin for growth inhibition of *S. aureus* ATCC 25923 (FICI = 0.74–1; 0.54–1; 0.56–1), *S. aureus* SA 03/10 (FICI =

0.75–1; 0.75–1; 1) and *S. aureus* MRSA 12/08 (FICI = 0.31; 0.27–0.38; 0.56–1). The graphs (A1–I1) are the corresponding isobolograms. In the checkerboard graphics, white indicates 0% growth and black indicates 100% growth in relative terms. Points on isobolograms represent combinations of *T. zygis* EO and antibiotics (relative to their MICs alone) that exhibited > 90% growth inhibition.

5.3.4 *T. zygis* EO anti-virulence activity

The essential oil of *T. zygis* was shown to affect virulence factors of *S. aureus*, such as by the inhibition of biofilm formation, the elimination of biofilms formed or affecting their haemolytic ability. The *T. zygis* EO was shown to be able to inhibit the formation of biofilms by the strains of *S. aureus* and also to partially eliminate preformed biofilms even at subinhibitory concentrations (Figure 5.3 and 5.4). The effect of the essential oil of *T. zygis* in inhibiting biofilm formation was more pronounced than that in eliminating preformed biofilms, with the exception of the *S. aureus* SA 03/10 strain.

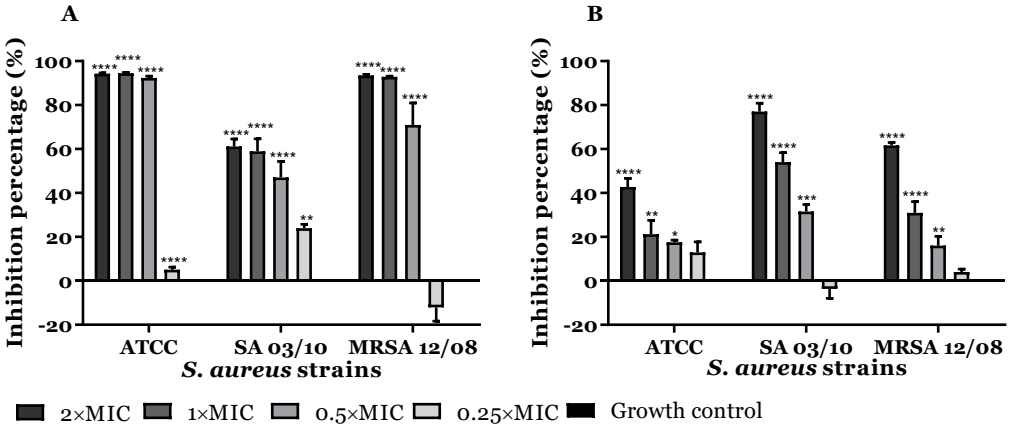

Figure 5.3. Effects of different concentrations of *T. zygis* EO on the formation of biofilms (A) and on elimination of pre-established biofilms (B) of *S. aureus* strains. Biofilm formation was estimated by the crystal-violet assay, and results are expressed as % of biofilm biomass inhibition regarding the correspondent solvent control (DMSO). * ($p < 0.05$); ** ($p < 0.01$); *** ($p < 0.001$); **** ($p < 0.0001$).

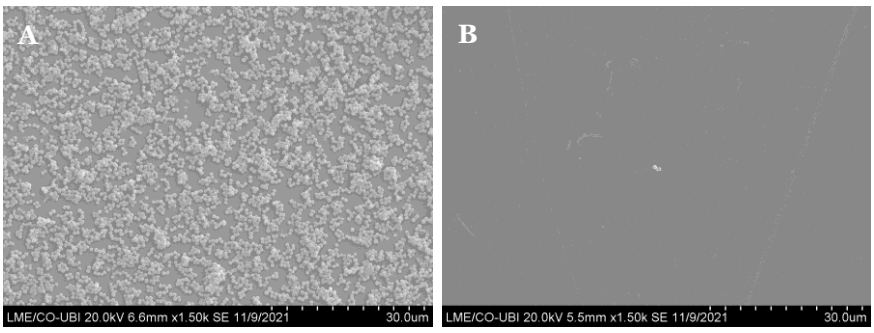

Figure 5.4. SEM micrographs showing the effect of the *T. zygis* EO on the biofilm formation: (A) untreated *S. aureus* MRSA 12/08; (B) *S. aureus* biofilm formed in the presence of *T. zygis* EO at 1× MIC. Micrographs are presented at 1500× magnification.

The interference of the *T. zygis* EO with the haemolytic ability of the strains was also evaluated. Of the three strains of *S. aureus* under study, only the SA 03/10 strain demonstrated haemolytic capacity. The pre-exposure of *S. aureus* to subinhibitory *T. zygis*

EO concentrations was shown to significantly reduce the haemolytic activity of *S. aureus* SA 03/10 compared to the respective controls (Figure 5.5) in a dose-dependent way.

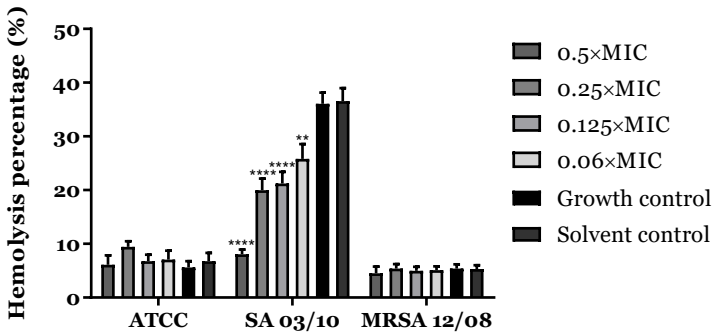

Figure 5.5. Effects of different concentrations of *T. zygis* EO on haemolytic capacity of *S. aureus* strains. ** ($p < 0.01$); **** ($p < 0.0001$).

As quorum sensing is a mechanism that allows bacteria to control the regulation and the secretion of virulence factors, we further tested its potential inhibition by the EO (Algburi et al., 2017; Trifan et al., 2020). Using *C. violaceum* as a biosensor strain to evaluate the potential of the *T. zygis* EO as a quorum-sensing inhibitor, it was observed that the concentration of 0.006% led to a significant reduction in the violet pigment production ($p < 0.0001$) without affecting the growth of *C. violaceum* (Figure 5.6). The concentration of 0.006% inhibited almost 100% of the violacein production, and the concentration of 0.003% inhibited approximately 50% of the pigment. It can also be observed that the *T. zygis* EO worked in a dose-dependent manner, and when the concentration of the *T. zygis* EO was decreased, the violacein inhibition also decreased. Resveratrol was used as a positive control, and there was a statistically significant inhibition of violacein production at all the concentrations under study.

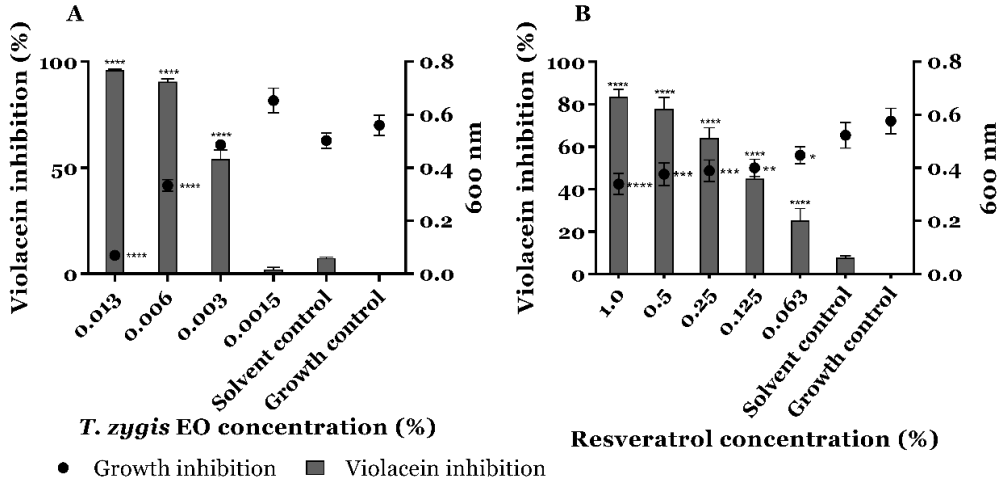

Figure 5.6. Quorum-sensing inhibition by *T. zygis* EO (A) and resveratrol (B) against *Chromobacterium violaceum*. Percentage of violacein inhibition (%) by different concentrations of EO or resveratrol and evaluation of microbial viability ($OD_{600\ nm}$) after 48 h of incubation. * ($p < 0.05$); ** ($p < 0.01$); *** ($p < 0.001$); **** ($p < 0.0001$).

To understand the biocompatibility of the *T. zygis* EO for human cells, the effect of this essential oil was studied using a normal human dermal-fibroblast cell line for the evaluation of cytotoxicity (Figure 5.7). The incubation with the *T. zygis* EO reduced the viability of these cells in a dose-dependent manner. The results obtained in an MTT assay showed that the viability of NHDF was more than 70% when they were seeded in contact with the *T. zygis* EO at concentrations between 0.0125 and 0.0030%, for 24 h. Therefore, considering the ISO 10993:5–2009, it is possible to consider that such concentrations are non-cytotoxic when compared to the negative-control group (untreated cells).

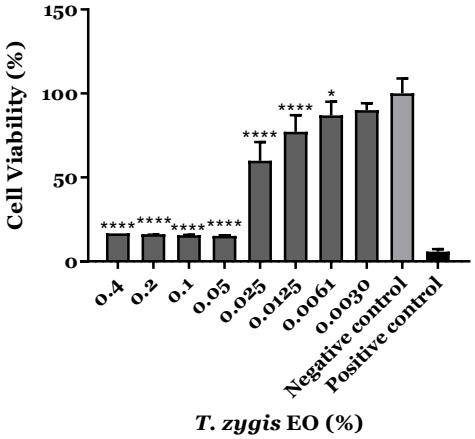

Figure 5.7. *T. zygis* EO biocompatibility for normal human dermal-fibroblast cell line measured by MTT assay after 24 h of treatment. The negative control was performed using untreated cells, and cells cultured with EtOH (96%) were used as a positive control. Results are expressed as means ± standard deviations of at least three independent experiments. * ($p < 0.05$); **** ($p < 0.0001$).

5.4. Discussion

EOs have been used for centuries in perfumery, cosmetics and medicine and as part of spices and herbs in foods, and they are associated with a broad range of bioactive properties such as antibacterial and antioxidant activities (Perricone et al., 2015). The potential use of essential oils for developing promising antimicrobial agents with potential against *S. aureus* has been widely studied (Idrees et al., 2021; Vieira et al., 2017). In the literature, it is described that *T. zygis* EO has antibacterial and antifungal activity against several microorganisms (Ballester-Costa et al., 2013; Dorman and Deans, 2004; Pina-Vaz et al., 2004) and several other bioactive properties (Lagha et al., 2019; Sánchez-Hidalgo et al., 2011; Vázquez-Ucha et al., 2020); however, its interaction with antibiotics and several forms of antimicrobial activity required more in-depth studies.

The bioactive properties of EOs are correlated with their compositions. Therefore, this determination is important and may allow relating the composition with the biological activities (Ribeiro-Santos et al., 2018; Rota et al., 2008). The composition of the *T. zygis* EO used in this work is similar to that presented in the literature, where thymol is presented as the major compound (Andrés et al., 2018; Ballester-Costa et al., 2013; Marinković et al.,

2020; Pina-Vaz et al., 2004) and the cymene and carvacrol are found with considerable percentages in relation to other compounds (Machado et al., 2010; Marinković et al., 2020; Pina-Vaz et al., 2004; Solarte et al., 2018).

Antioxidants are important because they can compete with free radicals and avoid the propagation of oxidation reactions (Ribeiro-Santos et al., 2018). An increase in free-radical production and decline in the activities of antioxidant-enzyme systems can damage membranes, lipids and lipoproteins and can induce DNA mutations (Carrasco et al., 2015; Youdim et al., 2002), and the implications of lipid peroxidation can lead to a diverse number of pathological disorders (Carrasco et al., 2015; Jordán et al., 2009; Youdim et al., 2002). The results in this work show the ability of *T. zygis* EOs to scavenge free radicals, as well as the results for the inhibition of lipid peroxidation (Youdim et al., 2002), and are corroborated by other authors (Ballester-Costa et al., 2017; Cutillas et al., 2018a). Different samples of *T. zygis* EOs were evaluated by Carrasco et al. (2015), and it was shown that *T. zygis* EO with a high proportion of thymol led to better antioxidant activity, using different methodologies. As can be seen in the review of Escobar et al. (2020), there are several studies showing the antioxidant activity of the isolated compound thymol. Considering that thymol is the major compound of the *T. zygis* EO under study, the high antioxidant activity may be mainly due to this compound.

The broad spectrum of antibacterial activity of many EOs suggests a wide range of applications as antibacterial agents (Owen and Laird, 2018). The *T. zygis* EO and its volatile compounds showed good antimicrobial activity against *S. aureus*, as previously described (Ghabraie et al., 2016). The authors related the high amount of monoterpenes in the vapour of the EO with the presented activity, as it is easier for these compounds to attack the bacterium compared to the liquid phase (Ghabraie et al., 2016). In accordance with our results, it was described that *T. zygis* EO also shows good inhibition of the growth of MRSA isolates (Abdallah et al., 2020). The amount of thymol was also correlated with a better antimicrobial activity of *T. zygis* EO against *S. aureus*, pointing to its role in the activity of the EO (Cutillas et al., 2018a), which may correlate with the activity observed in this work. The antimicrobial activity of the *T. zygis* EO was further validated by time–kill curves, demonstrating its bactericidal action even at subinhibitory concentrations, similarly to what has been described for other essential oils (Bilia et al., 2014; Brochot et al., 2017; Wang et al., 2020) and for *A. baumannii* and *K. pneumoniae* strains with *T. zygis* EO (Vázquez-Ucha et al., 2020).

Drug discovery has looked to natural products for the purpose of combating infections caused by multiresistant bacteria (Owen and Laird, 2018). The combination of multitarget antivirulence compounds, such as EOs, and antibiotics can help to restore the effectiveness

of antibiotics, as can be seen in the review of Owen and Laird (2018), and is a promising approach for combating antibiotic-resistant *S. aureus* (Owen and Laird, 2018; Vázquez-Ucha et al., 2020; Wu et al., 2019). In fact, *T. zygis* EO shows promising results in this area. The majority of the combinations of the *T. zygis* EO and antibiotics investigated mainly showed additive interactions; however, several of these additive combinations restored antibiotic sensitivity according to Clinical and Laboratory Standards Institute breakpoints (CLSI, 2021). The combination of the *T. zygis* EO and ampicillin, ciprofloxacin or vancomycin changed the resistance phenotype from resistant to sensitive in SA 03/10 strain (Figure 5.2). Regarding the two synergistic effects obtained, namely, that between the *T. zygis* EO and the antibiotic ampicillin or ciprofloxacin against the MRSA 12/08 strain, the presence of the EO also changed the resistance phenotype from resistant to sensitive in this strain. Moreover, the isobolograms show additive or synergistic effects between the combinations of *T. zygis* EO and ampicillin, ciprofloxacin or vancomycin (Figure 5.2). These results correlate with the synergistic interaction between thymol and ampicillin previously described (Palaniappan and Holley, 2010). In fact, as reviewed by Langeveld et al. (2014), several studies showed interactions between thymol and different classes of antibiotics among different microorganisms. This interaction may be associated with the mechanism of action of thymol. Wang et al. (2017) showed that thymol disrupted *S. aureus* cell membrane integrity, which may decrease cell viability and also increase the ability of other drugs to permeate the membrane (Wang et al., 2017).

The data obtained here showed that the combinations of the *T. zygis* EO and the antibiotics ampicillin, ciprofloxacin and vancomycin were able to decrease the MICs of antibiotics substantially and restore sensitivity to them, showing the EO's potential in combating antibiotic-resistant *S. aureus* strains. As far as we know, there are no studies about the interaction of *T. zygis* EOs with antibiotics.

Antibiotic-resistant *S. aureus* poses a severe threat to human health, and antivirulence therapy is a potential antibacterial strategy for combating *S. aureus*-associated infections (Wu et al., 2019). In fact, the *T. zygis* EO presents activity against some of the virulence factors of *S. aureus*. Among these factors, biofilms are associated with indwelling-medical-device-associated infections, endocarditis, osteomyelitis, conjunctivitis and other diseases (Cheung et al., 2021). Furthermore, biofilms can be a form of resistance to antimicrobials, host defence systems and external stresses (Algburi et al., 2017; Idrees et al., 2021; Lee et al., 2017). The *T. zygis* EO decreased *S. aureus* virulence through the inhibition of biofilm formation and even the elimination of previously formed biofilms, even at subinhibitory concentrations. The antibiofilm-formation effect was further validated by SEM analysis. This antibiofilm efficacy of *T. zygis* EO was already described in the literature (Abdallah et al., 2020; Marinković et al., 2020).

S. aureus is a major human pathogen that produces diverse virulence factors, such as α-haemolysin (Hla; also known as α-toxin) (Lee et al., 2014; Otto, 2014; Singh and Phukan, 2019), one of the main cytotoxic agents secreted by *S. aureus*, which has been implicated in the pathogeneses of sepsis, pneumonia and severe skin infections (Lee et al., 2014; Singh and Phukan, 2019). The EO from *T. zygis* in the present work was shown to reduce the haemolytic capacity of *S. aureus*, comparable to other EOs that presented a similar effect (Qiu et al., 2012; Shi et al., 2016). To the best of our knowledge, this is the first report showing the efficacy of *T. zygis* EO in reducing the haemolytic capacity of *S. aureus*.

One of the antivirulence strategies aims to interfere with cell–cell communication or quorum sensing. Thus, the discovery of quorum-sensing inhibitor candidates has been presented as a step in the path toward the integration of the antivirulence strategy into the management and treatment of *S. aureus* infections (Quave and Horswill, 2014). The essential oil of *T. zygis* demonstrated the ability to inhibit the formation of violacein in *C. violaceum,* indicating its potential as an inhibitor of quorum sensing. Thus, the results show that, in addition to *T. zygis* having antimicrobial activity against planktonic cells, it also reduces virulence factors such as quorum sensing and biofilm formation.

The chemical composition and the biological activities of EOs are important to know, but determining their utilization limits, including their safety, is also important (Ribeiro-Santos et al., 2018).

Similarly to our work, where a strong decrease in MTT reduction was observed for 0.25 μL/mL, the cytotoxicity of the EO of *T. zygis* against different cell lines was previously shown. In a fetal mouse-skin dendritic cell line (FSDC), no cytotoxic effect was observed at concentrations ranging from 0.08 to 0.16 μL/mL, and only for higher concentrations of the *T. zygis* EO (0.32 μL/mL) was a strong decrease in MTT reduction for the FSDC cell line noted (Gonçalves et al., 2010). Moreover, the EO of *T. zygis* did not cause a significant alteration in the viability of macrophages (RAW 264.7) and bovine aortic endothelial cells when compared to controls (Machado et al., 2010). Nonetheless, when the *T. zygis* EO was tested on normal fetal lung fibroblasts (MRC-5 cell line), a significant decrease in cell viability was observed, comparable to the that for the positive control Triple antibiotic paste (metronidazole, ciprofloxacin and minocycline, at the ratio 1:1:1) (Marinković et al., 2020). In these studies, the essential oils of *T. zygis* had differences in their composition compared to the one studied in the present work, which must be considered when the biomedical application of these essential oils is envisioned.

Nonetheless, at the concentrations of *T. zygis* EO for which the cytotoxicity of the EO is low, interesting biological activities were also obtained. In the range of concentrations showing a low level of cytotoxicity, the *T. zygis* EO was shown (i) to significantly reduce the

logarithmic bacterial counts of *S. aureus*, (ii) to potentiate the effects of the studied antibiotics, (iii) to inhibit the haemolytic capacity in the SA 03/10 strain or inhibit biofilm formation, and (iv) to potentiate the elimination of preformed biofilms in the *S. aureus* strains, as well as inhibiting quorum sensing in *C. violaceum*.

5.5. Conclusions

To summarize, this work shows that *T. zygis* EO presents good antioxidant and antimicrobial properties. *T. zygis* EO presents activity against resistant *S. aureus* strains with bactericidal activity, while showing antibiofilm and antihaemolytic activities against *S. aureus*. The *T. zygis* EO's prospects for improving the effect of antimicrobial agents was highlighted, since the combination of the *T. zygis* EO with the antibiotics ampicillin, ciprofloxacin and vancomycin potentiated the effects of these antibiotics against the *S. aureus* strains. These results show the possible use of *T. zygis* EO as an alternative antibacterial agent for the control of *S. aureus*.

Chapter 6

Antimicrobial activity of *Thymus zygis* essential oil against *Listeria monocytogenes* and its application as food preservative

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Abstract

Thymus zygis is an aromatic plant used in folk medicine. This work aimed to evaluate the anti-*Listeria monocytogenes* activity of *T. zygis* essential oil (EO), whose thymol is its major compound. Furthermore, the attenuation of this bacterium's virulence, namely by the inhibition of biofilm formation, motility and invasion of human cells, and the possible application of the EO in food were evaluated. The *T. zygis* EO showed antibacterial activity against *L. monocytogenes* with a minimum inhibitory concentration (MIC) of 0.05%, while showing a bactericidal effect. The EO significantly reduced the biofilm formation (inhibition from 16.85 to 89.86%) and motility (halos between 6.66 and 10.98 mm, compared to controls 13.12 to 17.22), and not inducing cross-resistance to antibiotics, such as ampicillin, cefotaxime, erythromycin, gentamicin, tetracycline, and vancomycin. *L. monocytogenes* counts (initial inoculum of ~10^6 CFU/mL) were lowered by the use of 2× MIC of *T. zygis* EO in the chicken juice (1.53 log CFU/mL) and lettuce model (to below the detection limit) after two days of storage. The use of EO (0.2% (v/v)) for sanitizing fresh vegetables, reduce *L. monocytogenes* and natural microbiota for values below the detection limit of the method for iceberg lettuce after an immersion of 5 min. For the spinach, *L. monocytogenes* was reduced in 4.35 log CFU/mL and the natural microbiota was diminished in a range of 4.47 to 5.94 log CFU/mL, when compared with the washing with water. Overall, the *T. zygis* EO has demonstrated a promising antimicrobial activity and these findings point to the potential of EO as a natural food preservative or sanitizer for controlling *L. monocytogenes* in food products.

Keywords: *Thymus zygis*; *Listeria monocytogenes*; antimicrobial activity; sanitizer; vegetables; milk; chicken

6.1. Introduction

Among the foodborne pathogens, *L. monocytogenes* is an opportunistic pathogen and a major causative agent of foodborne illness worldwide. This bacterium is associated with high rates of hospitalization and mortality and can be isolated from a variety of environments (Balali et al., 2020; Lemon et al., 2010; Lucera et al., 2012).

L. monocytogenes is a Gram-positive pathogenic rod-shaped bacterium and facultative anaerobe (Kannan et al., 2020). This species is subclassified into 13 serotypes, with all serotypes being able to cause a severe invasive disease called listeriosis. However, the serotypes 1/2a, 1/2b, and 4b are considered to be more widespread and associated with disease in humans (Lomonaco et al., 2015; Matle et al., 2019; Ranjbar and Halaji, 2018).

This pathogen causes various syndromes in humans, ranging from mild to severe, with a host-dependent susceptibility (Duze et al., 2021). In healthy individuals, the symptoms usually are mild, such as self-limiting gastroenteritis accompanied by symptoms, such as vomiting, nausea, diarrhea, and fever (Duze et al., 2021). Considering pregnant women, fetuses or neonates, the elderly, and people with compromised immune systems, the risk is high for severe infections of the bloodstream and/or the central nervous system that leads to septicaemia, miscarriage, and stillbirth (Kannan et al., 2020; Lomonaco et al., 2015; Matle et al., 2019).

The ability of *L. monocytogenes* to survive and grow in harsh environmental conditions, such as dry environments, high temperatures, refrigeration temperature (4 °C), low oxygen conditions, low pH, and high salt content, as well as sublethal concentrations of biocides, allows to this bacterium be widely distributed in different environments and matrices, such as water, soil, and various food products, including fish products, meat, dairy products, vegetables, and ready-to-eat food (Duze et al., 2021; Matereke and Okoh, 2020; NicAogáin and O'Byrne, 2016; Wiktorczyk-Kapischke et al., 2021). The tolerance to desiccation also allows the bacteria to survive on a surface for extended periods of time with little access to water and nutrients, which can be associated with *L. monocytogenes* ability to persist in food production and subsequently cross-contaminate food products (Hingston et al., 2017). In the same way, the capacity of *L. monocytogenes* to form a persistent biofilm, also limits its removal by standard sanitation protocols favouring its presence in food processing establishments and retail (Matereke and Okoh, 2020; Yousefi et al., 2020). The major transmission route to humans is through the consumption of contaminated food (Matereke and Okoh, 2020).

The production of high-quality and safe food products is one of the most important issues in the food industry (Balali et al., 2020; Yousefi et al., 2020). Diverse synthetic preservatives have been utilized in the food industry, but consumers have shown increasing concern about the use of these compounds due to the possible negative associated effects (Froiio et al., 2019). This has potentiated the research and the development of potential natural additives and antimicrobial compounds for use in the food industry (Balali et al., 2020; Yousefi et al., 2020). The plants are seen as an accepted source of natural antimicrobial agents, whose compounds may control and/or prevent the growth of pathogenic microorganisms, as well as, natural spoilage processes (Martínez-Graciá et al., 2015). Among the natural additives, EOs from aromatic plants can be considered as potential natural alternatives to synthetic preservatives to improve the safety and the shelf life of food products (Yousefi et al., 2020). EOs are lipophilic and volatile liquids, obtained from plant organs such as roots, wood, stems, buds, flowers, and seeds (Perricone et al., 2015), for which antimicrobial properties against *L. monocytogenes* have been reported (Perricone et al., 2015; Yousefi et al., 2020).

Thymus zygis (*T. zygis*) is an aromatic plant belonging to the *Lamiaceae* family, which is widely distributed in the Iberian Peninsula, and has been used since a long time as a condiment (Abdallah et al., 2020; Gonçalves et al., 2010). The EO of this plant, obtained from its aerial parts, has been highlighted for its antimicrobial activity against bacteria and fungi (Afonso et al., 2018; Ballester-Costa et al., 2013; Gonçalves et al., 2010; Pérez-Sánchez et al., 2007). Thymol is one of the major constituents of *T. zygis* EOs (Coimbra et al., 2022; Ghabraie et al., 2016; Van Haute et al., 2016) and presents diverse bioactive properties, such as antimicrobial activity (Hernández et al., 2015). The major components of EOs are known by their relevance for biological activity, but the minor components also play a significant role since they can increase the impact of major components, through synergistic and additive effects. Therefore, it is important to study the EO as it can have more activity than the isolated compounds (Perricone et al., 2015).

The adaptive response of *L. monocytogenes* to the stress factors in the food processing environment (Wiktorczyk-Kapischke et al., 2021) and also the increase of the antibiotic resistance among *L. monocytogenes* strains isolated from food products, evidence the problems associated with this bacterium, which have been potentiating the research for natural options to fight these problems (Olaimat et al., 2018). Thus, considering the relevance of *L. monocytogenes* as a foodborne pathogen and the beneficial effects of EOs, particularly the EO of *T. zygis*, in this work, (i) the antimicrobial activity of *T. zygis* EO against *L. monocytogenes,* (ii) the effect on virulence attenuation, (iii) issues associated with a safe application of the EO as the development of tolerance, and (iv) its possible application as a natural food preservative or in the decontamination of vegetables were studied. As far as we know, it is the first time that *T. zygis* EO has been evaluated against the *L. monocytogenes* virulence traits and tolerance to stress factors and also tested on vegetables in order to use this oil as a natural disinfectant.

6.2. Materials and Methods

6.2.1 EO and bacterial strains

The commercial EO obtained by steam distillation from *T. zygis* (aerial parts) was achieved at the company *Pharmaplant* (Algarve, Portugal). The EO was stored at 4 °C and protected from light until further use.

L. monocytogenes LMG 13305 (serotype 4b), *L. monocytogenes* LMG 16779 (serotype 1/2a), and *L. monocytogenes* LMG 16780 (serotype 1/2b), obtained from the BCCM/LMG collection (Belgium), were used as test microorganisms.

6.2.2 Disc diffusion method and vapor-phase antimicrobial activity determination

The susceptibility of each *L. monocytogenes* strain to the *T. zygis* EO was performed using the disc diffusion method (Luís et al., 2017) and the effect of the *T. zygis* EO volatile compounds against *L. monocytogenes* strains was evaluated as described by Duarte et al. (2016). The inoculum used was prepared by adjusting a cellular suspension to 0.5 McFarland and then the plates were uniformly inoculated with this suspension. Ten µg/disc of ampicillin was used as a positive control. After the incubation period, the inhibition halos were measured in mm. All tests were performed in three independent assays and the results were presented as mean ± standard deviation.

6.2.3 Determination of the minimum inhibitory concentration (MIC)

The susceptibility of *L. monocytogenes* strains to the *T. zygis* EO was evaluated through the broth microdilution method accordingly to Coimbra et al. (2022). Briefly, the EO was serially emulsified in tryptic soy broth (TSB, RPD microbiology, Spain) using dimethyl sulfoxide (DMSO) as solvent, following by double dilutions given eight test concentrations, ranging from 0.003 to 0.4% (v/v) (maximum final concentration of 2% (v/v) of DMSO). The inoculum was prepared by adjusting a cellular suspension to 0.5 McFarland, and then diluted in medium and added to the wells to obtain a final concentration of 5×10^5 colony-forming units (CFU)/mL in each well. After 24 h of incubation at 37 °C, the MIC was determined by the lowest concentration of *T. zygis* EO without visible growth. At least duplicates of three independent determinations were performed, and the results were presented as modal values.

6.2.4 Time-kill curves

The time-kill curves assay was performed based on Ferreira and Domingues (2016) with some adaptations. Briefly, *L. monocytogenes* obtained from an overnight culture were exposed to 0.125×, 0.25×, 0.5×, 1×, and 2× MIC of *T. zygis* EO, using a final cell concentration of 10^6 CFU/mL. Solvent (1% (v/v) of DMSO) and growth controls were performed. The tubes were incubated at 37 °C and viable counts were determined by the drop plate method at 0, 2, 4, 6, and 8 h of incubation. At least three independent experiments were conducted.

6.2.5 Motility assay

The effect of *T. zygis* EO on *L. monocytogenes* motility was evaluated as described by Marini

et al. (2018), with some modifications. From overnight cultures, *L. monocytogenes* strains were incubated in a TSB medium, for 18 hours at 37˚C. After incubation, 5 µL of the bacterial suspension was pipetted onto soft TSB plates with 0.3% (w/v) agar supplemented with 0.125× MIC and 0.25× MIC of *T. zygis* EO. TSB plates with 0.3% (w/v) agar were used as control. The plates were incubated at 30˚C and the diameter of the motility was measured after 24, 48, and 72 h. At least three independent experiments were performed.

6.2.6 Inhibition of biofilm formation

The inhibition of biofilm formation was based on the previously described method (Stepanović et al., 2004) with modifications. Briefly, *L. monocytogenes* strains were grown overnight in TSB at 37 ˚C, 250 rpm, and the culture adjusted to an OD_{600nm} ~0.1. Serial two-fold dilutions of *T. zygis* EO (0.25×, 0.5×, 1×, and 2× MIC) were prepared in TSB in 96-well flat-bottom polystyrene microtiter plates (100 µL per well). Then, 100 µL of each bacterial suspension was added to the wells. The plates were incubated for 24 h at 37 ˚C. For control, strains were incubated in the absence of *T. zygis* EO or presence of DMSO (0.125, 0.25, 0.5, and 1%), whereas for negative control only the culture medium was used. After incubation, the content of the plates was poured off and the loosely attached cells were removed from each well by washing twice with 200 µL of distilled water. The remaining attached bacteria were fixed with 200 µL of methanol for 20 min and after the removal of methanol, the plates were air-dried. The plates were stained with 200 µL of 0.1% (w/v) crystal violet for 10 min. Then, the dye was removed, and the wells were washed thrice with distilled water. The crystal violet bound to the adherent cells was dissolved with 200 µL of 33% (v/v) glacial acetic acid per well and the absorbance at 570 nm was determined using a microplate reader. At least five replicates of three independent experiments were conducted.

6.2.7 Effect of a pre-exposure to *T. zygis* EO on the tolerance to adverse conditions

6.2.7.1 Pre-exposure to *T. zygis* EO

The pre-exposure of *L. monocytogenes* LMG 13305 was performed by one-step exposure to a subinhibitory concentration of *T. zygis* EO. Initially, a colony from a culture in tryptic soy agar (TSA) for 24 h was suspended in TSB, and cultured at 37 ˚C, 250 rpm, for 16 h. Following, tubes were prepared by adding the cellular suspension to reach a final concentration of 10^6 CFU/mL and a *T. zygis* EO concentration of 0.125× MIC in TSB, in a final volume of 3 mL. A solvent control with DMSO at the maximum concentration used in this assay (0.125% (v/v)) and a growth control were performed. All tubes were incubated at 37 ˚C for 18 hours. After incubation, cells were collected by centrifugation at 8000 ×g, 4 ˚C,

for 5 min. The supernatant of each sample was removed, and the cells were washed with TSB and suspended in medium to obtain a concentration of 10^8 CFU/mL, which was used to test tolerance to adverse conditions, development of cross-resistance to antibiotics, and effect in invasion capacity to Caco-2 cell line.

6.2.7.2 Evaluation of the tolerance to adverse conditions

The influence of pre-exposure to *T. zygis* EO on the tolerance to several stress conditions was tested. Desiccation tolerance assay was conducted as described by Chen et al. (2020b) with adaptations. The previously prepared suspensions were diluted to a final concentration of 10^7 CFU/mL, and 100 µL were then transferred to 6-well culture plates. Then, the plates were incubated at 40 °C for 4 h, dried in a flow chamber, and placed at 25 °C in a desiccator for 7 days. The content of plate wells was resuspended in 1 mL of TSB on days 0 and 7, and CFU was determined by the drop plate method.

The effect of heat stress was also evaluated, following Oliveira et al. (2017) with some modifications. The previous cellular suspensions (30 µL) were added to 2970 µL of TSB heated to 55 °C, followed by incubation at the same temperature in a thermostatic water bath for 1 h.

The effect on osmotic stress tolerance was evaluated by adapting the method proposed by Walecka et al. (2011). The cellular suspension (30 µL) was transferred to 2970 µL of TSB supplemented with NaCl to a final concentration of 12% and incubated at 37 °C for 24 h.

The influence on the tolerance of *L. monocytogenes* to acidic pH was evaluated, according to Oliveira et al. (2017) with minor modifications. Exactly 30 µL of the previously prepared suspension was added to 2970 µL of TSB medium acidified with hydrochloric acid to pH 2.4. After 0, 2, 4, and 8 h of incubation, samples were removed and serially diluted in phosphate buffer saline (PBS 1×, pH 7.2). For all the assays, the count of CFU was performed by the drop plate method. At least duplicates of three independent experiments were conducted.

6.2.7.3 Development of cross-resistance

The potential development of cross-resistance was evaluated as described in sub-Section 6.2.3 and following the assay carried out by Oliveira et al. (2017) with adaptations. The MIC determination of several antibiotics from different classes (ampicillin, cefotaxime, erythromycin, gentamicin, tetracycline, and vancomycin) was performed, using as inoculum the cells obtained from a pre-exposure with *T. zygis* EO or the controls. At least

duplicates of three independent determinations were performed and the results were presented as modal values.

6.2.7.4 Caco-2 invasion assay

The effect of the exposure to *T. zygis* EO in the invasion of the human colon carcinoma Caco-2 cell line by *L. monocytogenes* LMG 13305 was evaluated as described by Su et al. (2019) with modifications. Caco-2 cells were routinely cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich) supplemented with 10% (v/v) fetal bovine serum, 1% non-essential amino acids, 100 μg/mL of streptomycin and 100 μg/mL penicillin at 37 ˚C in an atmosphere containing 5% CO_2. 500 μL of cells were seeded in 24-well plates and incubated for 48 h at 37 ˚C and 5% CO_2 to obtain a final concentration of approximately 2.6 × 10^5 cells/well.

Infection was performed by suspending bacterial cells from 6.2.7.1. in DMEM medium and adding 500 μL of the bacterial suspensions to Caco-2 cells washed twice with phosphate-buffered saline (PBS, pH 7.2) in a multiplicity of infection of 100. Bacteria and cell line were incubated for 1 h at 37 ˚C in 5% CO_2. Then, monolayers were washed thrice with PBS and incubated for 90 min with 500 μL of DMEM containing 150 μg/mL of gentamicin. After cells were washed thrice with PBS and lysed in 500 μL Triton X-100 1% (v/v) for 5 min. The lysates were serially diluted, and bacterial counts were performed by the drop plate method in TSA. At least triplicates in three independent experiments were conducted.

6.2.8 Antimicrobial effect of *T. zygis* EO against *L. monocytogenes* in liquid food models

The effect of *T. zygis* EO against *L. monocytogenes* LMG 13305 was evaluated in four food matrices: chicken juice, lettuce leaf model, ultra-high-temperature (UHT)-treated skim, and whole milk. The liquid food models were prepared as described previously (Ferreira and Domingues, 2016) and inoculated with *L. monocytogenes* LMG 10335, as described for time-kill assays. Three concentrations of *T. zygis* EO were used (0.5× MIC, 1× MIC, and 2× MIC). The different food matrices samples were incubated at 4 ˚C for 14 days and samples were taken on days 0, 2, 4, 7, 10, and 14. For controls, non-inoculated food models, a solvent control with DMSO, and the growth control were used. At least duplicates of two independent experiments were conducted.

6.2.9 Antimicrobial effect of *T. zygis* EO against *L. monocytogenes* in fresh vegetables

The effect of *T. zygis* EO on the survival of *L. monocytogenes* LMG 13305 in fresh vegetables was determined as described by De Sousa et al. (2012) and Moore-Neibel et al. (2013) with some modifications. Portions of iceberg lettuce or spinach leaves were cut into small pieces and submerged in a water suspension of *L. monocytogenes* with a proportion of 10 g of vegetables leaf to 100 mL of inoculum with a final concentration of 10^7 CFU/mL. The samples were softly rotated with a sterile spatula for 5 min to ensure uniform inoculation and air-dried for 1 h in a bio-safety cabinet. Then, 10 g of lettuce or spinach samples were submerged in 100 mL of the *T. zygis* EO solution (0.2% (v/v)) or distilled water for 5 min. Afterward, in aseptic conditions, 10 g sample of each vegetable was transferred into a sterile stomacher bag containing 90 mL of buffered peptone water and homogenized in the stomacher at high speed for 2 min. Subsequently, a decimal dilution series (10^{-1} to 10^{-4}) was made in buffered peptone water and bacterial enumeration was performed by spreading 100 µL of the appropriate sample dilution on Polymyxin acriflavine lithium chloride ceftazidime aesculin mannitol (PALCAM) agar. The plates were incubated at 37 ˚C for 48 h. At least duplicates of two independent experiments were conducted.

6.2.10 Survival of naturally occurring microorganisms in fresh vegetables

The effect of *T. zygis* EO on the survival of microorganisms that are naturally present in fresh vegetables was determined accordingly by De Sousa et al. (2012) and Moore-Neibel et al. (2013) with adaptations. Portions of 10 g iceberg lettuce or spinach leaves were cut into small pieces and directly submerged in 100 mL of the *T. zygis* EO solution (0.2% (v/v)) or distilled water for 5 min. Non-submerged samples were used as control. The samples of each vegetable were then treated as previously described. The enumeration of the natural flora was performed by pour-plating 1 mL of the appropriate sample dilutions on different media: the Yeast Extract Glucose Chloramphenicol Agar (YCGA) for molds and yeasts (25 ˚C, 3-5 days); the Violet Red Bile Glucose Agar (VRBGA) for *Enterobacteriaceae* (37 ˚C, 48 h); and the Plate Count Agar (PCA) were used to determine aerobic psychrotrophic bacteria (4 ˚C, 7-10 days) and total aerobic mesophilic bacteria (30 ˚C, 72 h). At least duplicates of two independent experiments were conducted.

6.2.11 Statistical analysis

The statistical analysis of the results was performed using the one-way ANOVA and Tukey's test or Dunnett test (time kill curve and liquid food models) using the GraphPad Prism

v8.02 software, with a 95% confidence interval, considering the values of $p < 0.05$ as statistically significant.

6.3. Results and discussion

6.3.1 Antimicrobial activity of *T. zygis* EO against *L. monocytogenes*

T. zygis EO has been described as possessing antibacterial activity, with higher potential against Gram-positive bacteria (Cutillas et al., 2018a; Lagha et al., 2019). In fact, the *T. zygis* EO exhibited a high antibacterial activity with an inhibition zone between 41.55 ± 2.63 mm and 55.04 ± 3.64 mm, and a MIC of 0.05 % (v/v) for the three *L. monocytogenes* strains studied. Besides, the volatile compounds of the *T. zygis* EO also demonstrated high antimicrobial activity with diameter inhibition zones from 36.13 ± 3.41 to 50.43 ± 2.59 mm (Table 6.1).

Table 6.1. Diameters of inhibition zone (mm) for disc diffusion method and volatiles compounds of *T. zygis* EO, presented as mean ± standard deviation and minimum inhibitory concentration (MIC) of *T. zygis* EO (%) and ampicillin (%) presented as modal values.

Strains	Inhibition zone (mm)			MIC (%)	
	T. zygis (10 µL/disc)	Ampicillin (10 µg/disc)	Volatile compounds (10 µL/disc)	*T. zygis*	Ampicillin
L. monocytogenes LMG 13305	41.55 ± 2.63[a]	31.46 ± 1.81	36.13 ± 3.41[a]	0.05	0.05
L. monocytogenes LMG 16779	55.04 ± 3.64[b]	39.83 ± 2.78	50.43 ± 2.59[b]	0.05	0.013
L. monocytogenes LMG 16780	47.34 ± 2.89[a,b]	30.87 ± 1.64	42.96 ± 2.95[a,c]	0.05	0.05

Different letters indicate statistical differences between values of the same column ($p < 0.05$).

Also, the antibacterial activity can be observed by the time-kill curves showing that *T. zygis* EO has a bactericidal effect at 1× and 2× MIC for all strains of *L. monocytogenes* (Figure 6.1). Further, after 8 h of incubation, significant reductions in the logarithmic bacterial counts were observed for *L. monocytogenes* LMG 16779 and LMG 16780 with 0.5× MIC, 0.25× MIC, and 0.125× MIC of *T. zygis* EO ($p < 0.01$), while only the sub-inhibitory concentration of 0.5× MIC had a significant reduction in *L. monocytogenes* LMG 13305 ($p < 0.001$). Thereby, it can be shown that the EO from *T. zygis* has antimicrobial activity against *L. monocytogenes*, with a bactericidal mode of action. Despite the resistance of *L. monocytogenes* strains to antimicrobial agents, the EOs have been shown to be effective in combating it with a bactericidal effect (Kakhki et al., 2020; Melo et al., 2013; Silva et al., 2019).

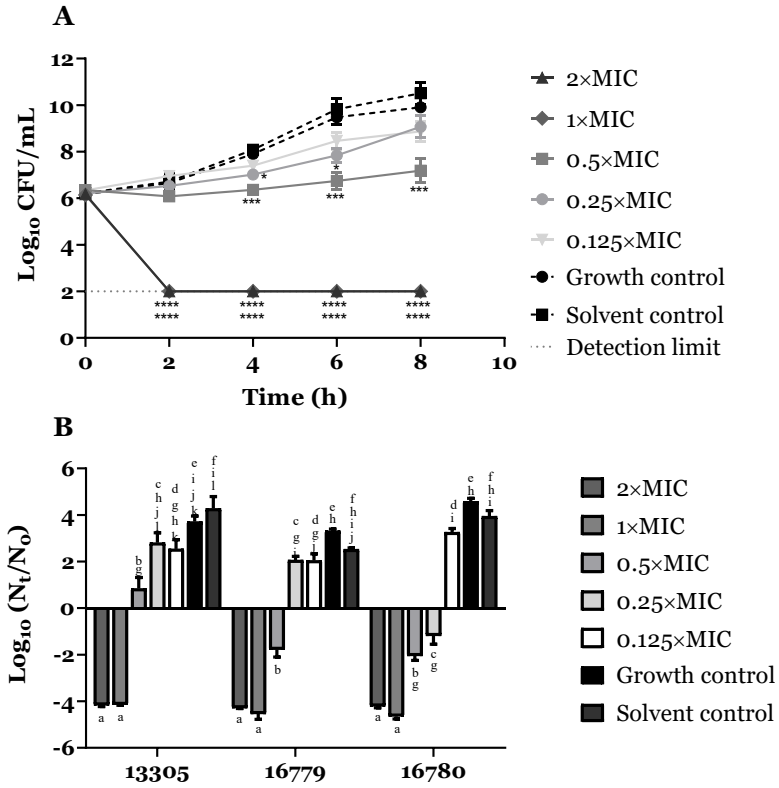

Figure 6.1. Time-kill curves of *L. monocytogenes* LMG 13305 (A) and Log_{10} (N_t/N_0) of LMG 13305, LMG 16779 and LMG 16780 strains at 8 h (B) incubated with *T. zygis* EO concentrations from 0.125× MIC to 2× MIC for 8 h at 37 °C. * ($p < 0.05$); *** ($p < 0.001$); **** ($p < 0.0001$). Different letters indicate statistical difference between samples of each *L. monocytogenes* strain ($p < 0.05$).

The antimicrobial activity of essential oils against bacteria, such as *L. monocytogenes*, could be associated to the presence of diverse compounds in their chemical composition, namely *L. monocytogenes* proved to be more susceptible to EO with a greater amount of thymol in its composition (Teixeira et al., 2013). In fact, the *T. zygis* EO used in the present work has thymol as its major compound (Coimbra et al., 2022). Thymol is a compound known by its antimicrobial activity (Kachur and Suntres, 2020; Palaniappan and Holley, 2010), by acting at several sites of action, correlated with cell membrane disruption, inhibition of motility, inhibition of membrane-bound ATPases, and inhibition of efflux pumps (Reviewed by Kachur and Suntres, 2020; Salehi et al., 2018). Wherefore, thymol may be one of those responsible for the antimicrobial activity of the *T. zygis* EO described in this work.

6.3.2 Effect of *T. zygis* EO in *L. monocytogenes* motility and biofilm formation

Motility was assessed by phenotypic approaches by the soft agar motility assay, showing a significant reduction in motility diameter for *L. monocytogenes* strains (Figure 6.2), in a concentration-dependent way.

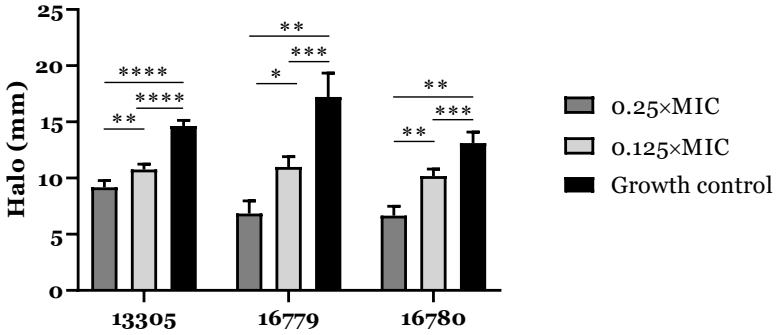

Figure 6.2. Inhibitory effect of *T. zygis* EO on the motility of *L. monocytogenes* LMG 13305, *L. monocytogenes* LMG 16779, and *L. monocytogenes* LMG 16780 measured on TSB + 0.3% agar supplemented with subinhibitory concentrations of EO. The plates were incubated at 30 °C for 24 h. Motility medium without EO was maintained as a negative control. * ($p < 0.05$); ** ($p < 0.01$); *** ($p < 0.001$); **** ($p < 0.0001$).

The use of 0.25× MIC of EO led to a reduction in the motility diameter from 14.63 ± 0.50 mm, 17.22 ± 2.12 mm and 13.12 ± 0.97 mm to 9.18 ± 0.60 mm, 6.85 ± 1.13 mm and 6.66 ± 0.84 mm for the strains *L. monocytogenes* LMG 13305, LMG 16779 and LMG 16780, respectively. When using a concentration of 0.125× MIC, a significant decrease of the motility was also observed. Thyme EO (unspecified specie) was previously described as preventing the motility by the flagellum of *L. monocytogenes*, by inhibiting flagellar synthesis, but also disturbing motility related to chemotaxis and adaptation (Sarengaowa et al., 2019). Inhibition of *L. monocytogenes* motility by other EO has also been reported with *Cannabis sativa* EO (Marini et al., 2018) and *Plectranthus barbatus* EO (Chatterjee and Vittal, 2021), thus showing that EOs can be used for the attenuation of certain virulence factors, such as the motility.

Biofilms allow *L. monocytogenes* to survive, persist and disseminate in the food production environment while increasing tolerance to washing and sterilization in the food production chain (Kannan et al., 2020). Therefore, the effect of *T. zygis* EO in the formation of biofilms by *L. monocytogenes* strains was evaluated, showing that biofilm formation was inhibited even at subinhibitory concentrations (Figure 6.3).

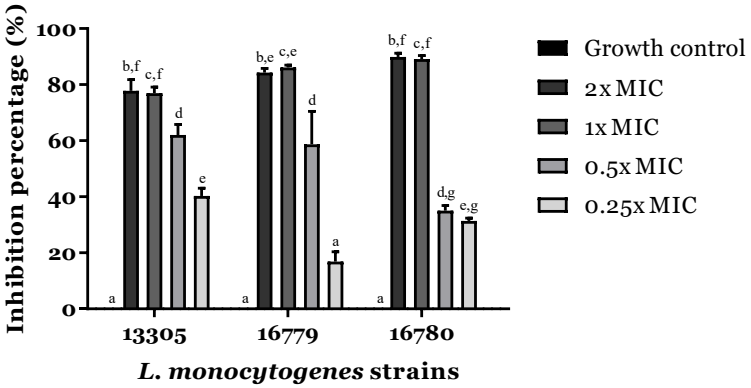

Figure 6.3. Effect of different concentrations of *T. zygis* EO on total biofilm biomass of *L. monocytogenes* strains. Biofilm formation was estimated by crystal violet assay and results are expressed as % biofilm biomass inhibition regarding the correspondent solvent control (DMSO) and compared with the growth control. Different letters indicate statistical difference between samples of each *L. monocytogenes* strain ($p < 0.05$).

The *T. zygis* EO showed biofilm inhibition values between 35 and 62.02% with the concentration of 0.5× MIC and between 16.85 and 40.27% for the concentration of 0.25× MIC, showing the potential of biofilm inhibition by the EO even at sub-inhibitory concentrations. This activity may be related with the potential of the EO of *T. zygis* to act as a quorum sensing inhibitor, as previously demonstrated through inhibition of the violacein pigment of the biosensor *Chromobacterium violaceum* (Coimbra et al., 2022).

EOs have shown good results in inhibiting the formation of *L. monocytogenes* biofilms (Chatterjee and Vittal, 2021; Gao et al., 2020; Zhang et al., 2020), with for example the *Plectranthus barbatus* EO showing antibiofilm activity against *L. monocytogenes*, which was associated with the promising quorum quenching activity (Chatterjee and Vittal, 2021). The antibiofilm efficacy of *T. zygis* EO was previously demonstrated against biofilms formed by single species and multispecies of oral biofilms (Marinković et al., 2020), *E. coli* clinical isolates associated with urinary tract infection (Lagha et al., 2019) and methicillin-resistant *Staphylococcus aureus* clinical isolates (Abdallah et al., 2020; Coimbra et al., 2022). However, to the best of our knowledge, there are no studies about the underlying mechanism of action of the *T. zygis* EO. Nonetheless, when considering methicillin-resistant *S. aureus* and *Pseudomonas aeruginosa,* it has been shown that thymol can (i) decrease the enzyme activity of the biofilm, reducing the hydrolytic activity of pathogenic bacteria (Walczak et al., 2021), (ii) inhibit the formation of biofilm and also remove the mature biofilms by inhibiting the production of polysaccharide intracellular adhesin and the release of extracellular DNA, and (iii) inhibit the transcript levels of key biofilm-regulated genes (Yuan et al., 2020). Possibly, these mechanisms of action of thymol may also influence the antibiofilm activity of *T. zygis* EO since it is its major compound, however, more studies must be carried out to know exactly how this EO acts.

6.3.3 Tolerance and virulence of *L. monocytogenes* after exposure to *T. zygis* EO

There are some reports about the development of cross and/or direct protection by *L. monocytogenes* when exposed to these compounds at subinhibitory concentrations, which may question its use (Berdejo et al., 2021; De Souza, 2016). To assess whether a single exposure to a subinhibitory concentration of *T. zygis* EO may induce cellular protection in adverse conditions, cells of *L. monocytogenes* LMG 13305 obtained from previous exposure to *T. zygis* EO for 18 h at 0.125× MIC were then evaluated regarding its ability to survive to stressful conditions. Thus, the cells were exposed to desiccation for 7 days, to 12% of NaCl, a low pH of 2.4, and a high temperature of 55 ˚C (Figure 6.4).

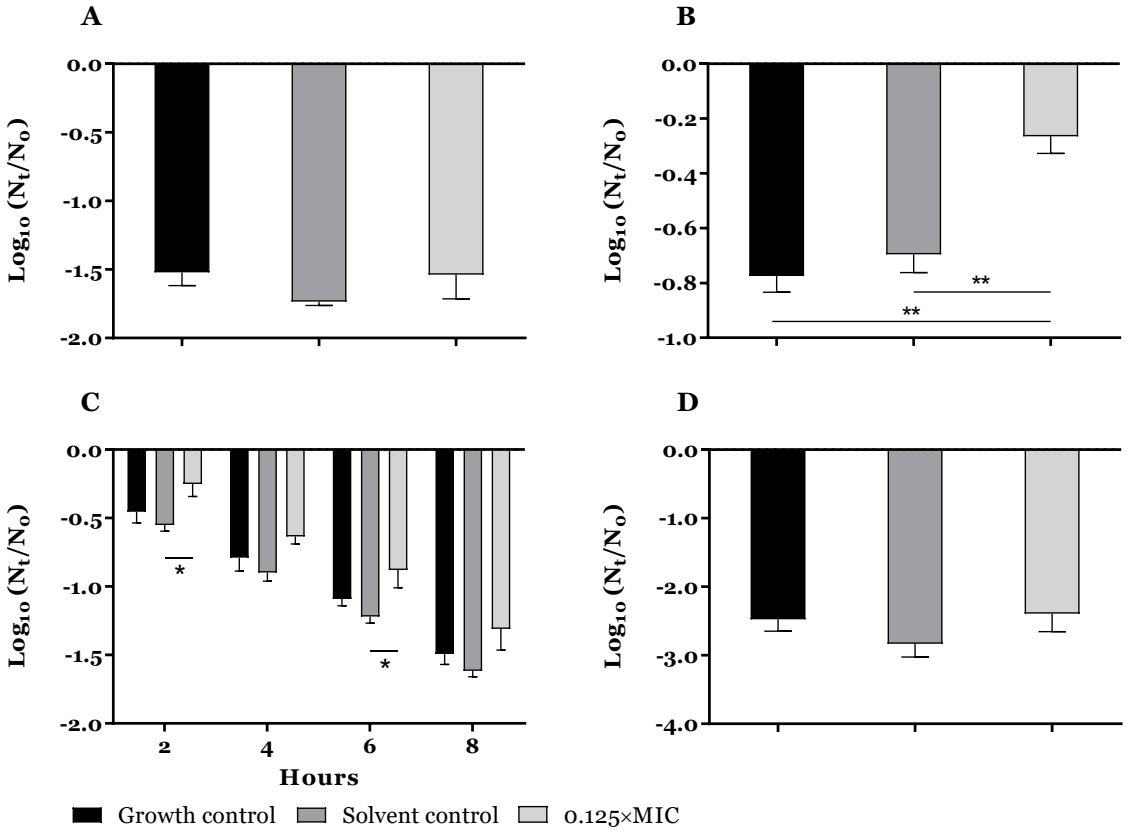

Figure 6.4. Effect of (A) desiccation after 7 days; (B) osmotic stress to NaCl 12%; (C) pH 2.4 and (D) temperature at 55 ˚C in *L. monocytogenes* LMG 13305 survivals after the incubation with *T. zygis* EO. * ($p < 0.05$); ** ($p < 0.01$).

Considering the assay of osmotic stress, a significant increase in survival of *L. monocytogenes* was observed for cells pre-exposed to *T. zygis* EO (Log_{10} (N_t/N_o) = –0.265) when compared to the control (Log_{10} (N_t/N_o) = –0.774) (Figure 6.4B). In turn, when cells were subject to acid stress (pH 2.4), heat shock (55 ˚C, 1 h), or desiccation (7 days), no significant difference was observed (exposed *L. monocytogenes* versus non-exposed) (Figure 6.4A, C, D).

As far as we know, there are no studies about the effect of *T. zygis* EO on cross-protection of *L. monocytogenes* to stress conditions. Considering studies about the exposure of *L. monocytogenes* to subinhibitory concentrations of other EOs and their effect under stressful conditions, *Rosmarinus officinalis* L. EO and the related compound 1,8-cineole did not induce protection in *L. monocytogenes* against lactic acid, osmotic stress with NaCl, or high temperature (Neto et al., 2012). In another study, the exposure of *L. monocytogenes* cells overnight to sublethal concentrations of *Origanum vulgare* or carvacrol did also not induce cross-protection against high temperature (45 ˚C), lactic acid (pH 5.2) or salt (NaCl at 10 g/100 mL) (Luz et al., 2012).

The development of cross-resistance to antibiotics by the exposure of *L. monocytogenes* LMG 13305 to subinhibitory concentrations of *T. zygis* EO was further evaluated, with the

results showing that the exposure did not induce cross-resistance to none of the tested antibiotics from different classes, when compared with cells that were not exposed to subinhibitory concentration of *T. zygis* EO (Table 6.2).

Table 6.2. Minimum inhibitory concentration (μg/mL) of antibiotics against *L. monocytogenes* LMG 13305 after incubation with *T. zygis* EO presented as modal values.

Antibiotics	MIC (μg/mL)		
	Growth control	Solvent control	Pre-exposure to 0.125×MIC
Ampicillin	1	1	1
Cefotaxime	8	8	8
Erythromycin	0.125	0.125	0.125
Gentamicin	4	4	4
Tetracycline	0.5	0.5	0.5
Vancomycin	0.5	0.5	0.5

Considering, the results obtained more studies are needed considering a longer exposure. In past, the development of resistance to EOs has been discarded due to its complexity; however, some authors have recently described the potential adaptation of bacteria to EOs, such as to *Citrus sinensis* EO by *S. aureus* (Berdejo et al., 2020) or to *Thymus capitata* EO by *L. monocytogenes* (Berdejo et al., 2021). In the latter study, the evolved strains also presented cross-resistance to some antibiotics (Berdejo et al., 2021). Previous studies have shown that EOs may slow the growth rate and interfere with the membrane permeability and the cell membrane integrity of bacteria, thus increasing the lag phase that can lead to an decrease of susceptibility to antimicrobial agents (Berdejo et al., 2021; Bouyahya et al., 2019; Braschi et al., 2018).

In general, *L. monocytogenes* infect the human host by oral route and the ingestion of contaminated food products allows this pathogen to cross physiological barriers, such as the intestinal, the blood-brain, and the fetoplacental barriers (Camejo et al., 2011). *L. monocytogenes* may penetrate the epithelial cell layer, disseminate to the bloodstream, and reach organs such as the spleen and liver, in the absence of an adequate response by the immune system (Camejo et al., 2011). To understand if the pre-exposure to *T. zygis* EO may influence this invasion ability, it was tested in the Caco-2 cell line. The invasion efficiency of Caco-2 cells by *L. monocytogenes* was similar when bacteria were pre-exposed to *T. zygis* EO or not (Figure 6.5).

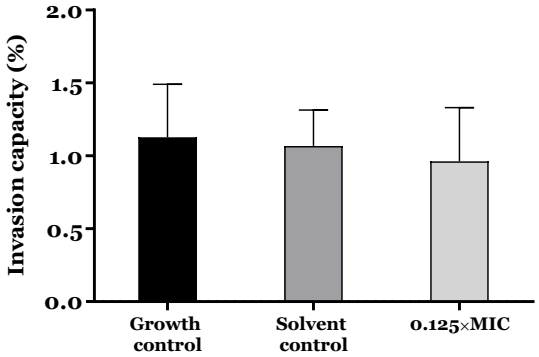

Figure 6.5. Caco-2 cell invasion by *L. monocytogenes* LMG 13305 non- and pre-exposed to a sub-inhibitory concentration of *T. zygis* EO. Data are expressed as a percentage of invasion.

Despite the lack of effects of *T. zygis* EO in the ability of *L. monocytogenes* to invade Caco-2 cells, this feature is recognized in other natural products. Marini et al. (2018) showed that the invasion efficiency of Caco-2 cells by *L. monocytogenes* strains was strongly reduced when bacteria were grown in presence of the *Cannabis sativa* EO. Further, subinhibitory concentrations of thymoquinone effectively inhibited the ability of *L. monocytogenes* to adhere and invade Caco-2 cells (Miao et al., 2019).

6.3.4 Anti-listerial effect of *T. zygis* EO in food models

A major concern for the food processing industry is food contamination and preservation, which is associated with the consumers' demand for the reduction of the use of synthetic compounds have led to the increased interest in natural compounds as food preservatives or sanitizers (Yousefi et al., 2020). Thus, EOs may be presented as interesting compounds; nonetheless, it is known that food components can interfere with the efficacy of EOs. For understanding the anti-listerial activity of the *T. zygis* EO in presence of food components, we used four food matrices: chicken juice, lettuce leaf model, ultra-high-temperature (UHT)-treated skim, and whole milk, to represent food products where *L. monocytogenes* may appear as a foodborne pathogen (Figure 6.6). *T. zygis* EO inhibited the *L. monocytogenes* LMG 13305 growth in the chicken juice and lettuce model medium (Figure 6.6A and 6.6B, respectively). A significant reduction in counts was observed for the two highest EO concentrations tested from days 4 to 14 for chicken juice (Figure 6.6A) and days 2 to 14 for lettuce model medium (Figure 6.6B) ($p < 0.05$). The concentration of 0.5× MIC had a significant reduction only for the lettuce model medium for days 4 to 14 (Figure 6.6B).

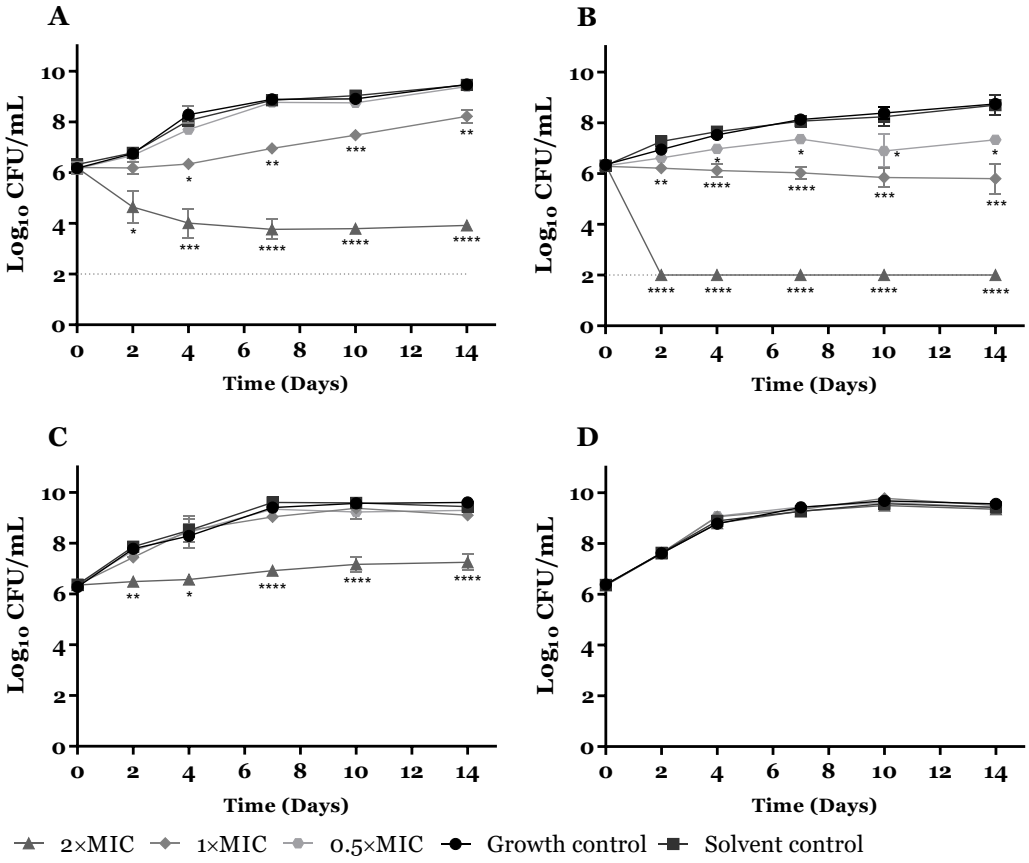

Figure 6.6. *T. zygis* EO activity against *L. monocytogenes* LMG 13305 in liquid food models (A) chicken juice, (B) lettuce leaf model medium, (C) skim UHT milk, and (D) whole UHT milk, at 4 ˚C for 14 days. * ($p < 0.05$); ** ($p < 0.01$); *** ($p < 0.001$); **** ($p < 0.0001$).

The different lipid content of the two milk samples influenced the effect of *T. zygis* EO against *L. monocytogenes* LMG 13305 (Figure 6.6C and 6.6D), with no significant reduction being observed when using whole milk as a food model. Regarding skim milk, the logarithmic bacterial counts presented a significant reduction from day 2 with the 2× MIC concentration of *T. zygis* EO ($p < 0.01$). In another work, in which the effect of one of the major compounds of *T. zygis* EO, thymol, was studied, the results showed antimicrobial effectiveness against *L. monocytogenes* in both food models (reduced-fat milk and apple cider), with the activity of thymol being also affected by pH and food matrix (Shah et al., 2012). Machado et al. (2014), evaluated the antimicrobial efficacy of an EO and its interaction with different food ingredients against *L. monocytogenes*, and the results showed that proteins and lipids had a negative impact on the EO effectiveness and the carbohydrates have a positive effect (Machado et al., 2014). Taking these results into account, it is clear that the composition of food products can negatively affect the antimicrobial activity of EOs even presenting promising bioactivity *in vitro* (reviewed by Perricone et al. 2015). Altogether, the anti-listerial activity of *T. zygis* EO in food was relevant in lettuce model medium, chicken juice, and skim milk.

6.3.5 *T. zygis* EO as a leafy vegetable sanitizer

Taking the results obtained in the lettuce leaf model and the use of short exposure, considering the effects of pre-exposure on tolerance to adverse conditions, we proceeded with the evaluation of the effect of *T. zygis* EO at 0.2% (v/v) in the sanitizing of vegetable leaves contaminated with *L. monocytogenes*. In fact, washing iceberg lettuce and spinach leaves with *T. zygis* EO aqueous solution significantly reduced the amount of inoculated *L. monocytogenes* with higher efficacy for iceberg lettuce than spinach leaves (Figure 6.7A). In fact, the washing with *T. zygis* EO aqueous solution eliminated *L. monocytogenes* for values below the detection limit of the method for iceberg lettuce, while reduced in 4.35 log CFU/mL for spinach leaves compared with the washing with water. When washing the leaf samples with water, only have a significant effect on iceberg lettuce, but the effect obtained by washing with EO was much more evident.

Other studies also used natural products to wash fresh leafy products as an alternative to commercial disinfectants. *Morinda citrifolia* fruit extract was evaluated to assess its applicability for the washing of romaine lettuce, spinach, and kale and the results showed a good antibacterial activity against *L. monocytogenes* inoculated in the samples (Kang and Song, 2019). A grape stem extract was applied as a disinfectant in lettuce and spinach, reducing significantly the numbers of the inoculated pathogenic bacteria *L. monocytogenes* (Vázquez-Armenta et al., 2017). The application of the individual or combined *Cymbopogon citratus* and *Allium cepa* EOs caused a reduction in *L. monocytogenes* bacterial growth when applied on spinach and romaine lettuce leaves (Ortega-Ramirez et al., 2016).

Additionally, the effect of *T. zygis* EO on the natural microbiota of the vegetable was also evaluated (Figure 6.7). The lettuce and spinach samples dipped in the solutions containing 0.2% (v/v) of *T. zygis* showed a statistically significant reduction in the amount of molds and yeasts (Figure 6.7B), *Enterobacteriaceae* (Figure 6.7C), total aerobic mesophilic bacteria (Figure 6.7D), and aerobic psychrotrophic bacteria (Figure 6.7E) comparatively with each control groups. In general, the reduction for iceberg lettuce was to values below detection limit, and for spinach was from 4.47 to 5.94 log CFU/mL for total aerobic mesophilic bacteria and aerobic psychrotrophic bacteria, respectively, when compared with the washing with water control.

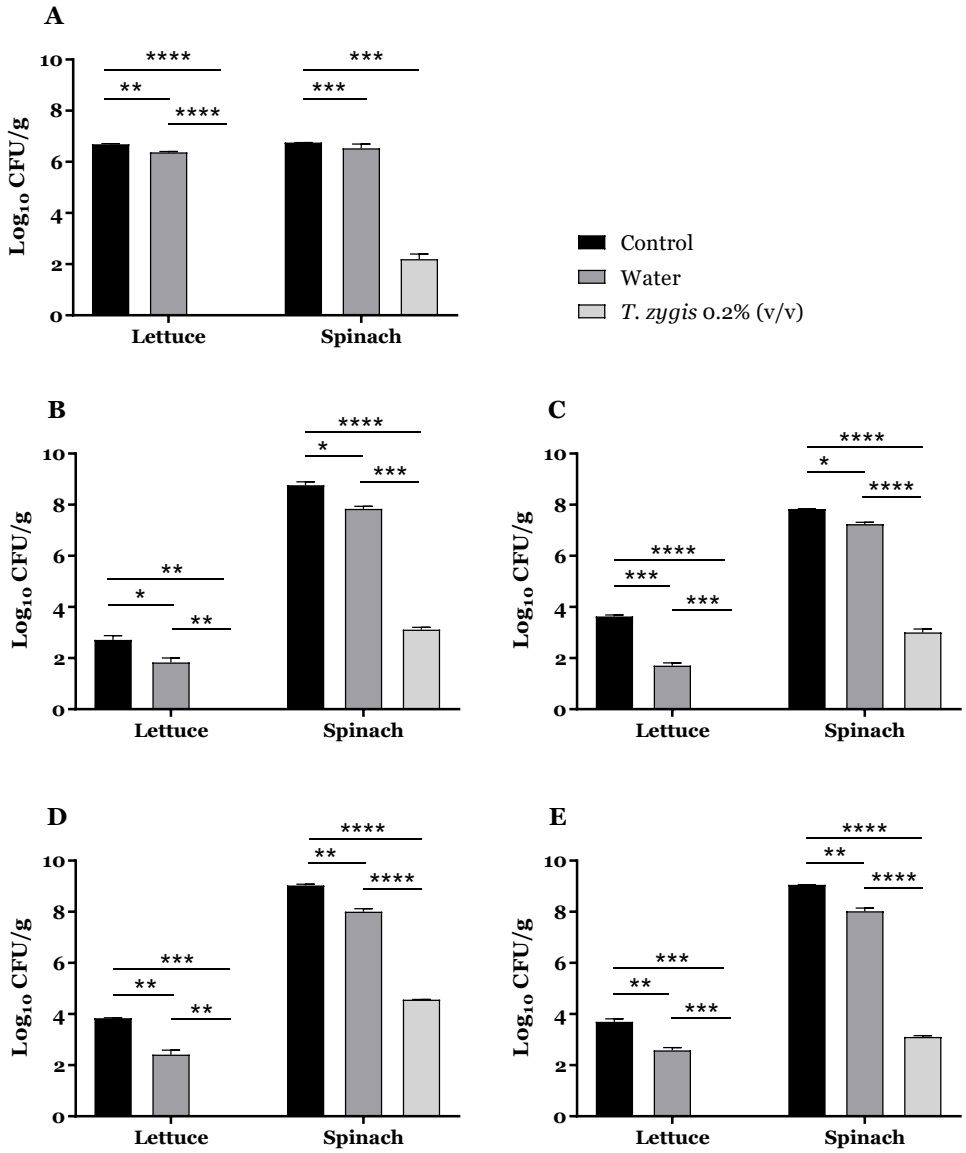

Figure 6.7. *T. zygis* EO activity against *L. monocytogenes* LMG 13305 in fresh vegetables (A) and *T. zygis* EO effect in survival of naturally occurring microorganisms in fresh vegetables, lettuce, and spinach. (B) Molds and yeasts in YGCA medium, (C) *Enterobacteriaceae* in VRBGA medium, (D) total aerobic mesophilic bacteria in PCA at 30 ˚C, and (E) aerobic psychrotrophic bacteria in PCA at 4 ˚C. * ($p < 0.05$); ** ($p < 0.01$); *** ($p < 0.001$); **** ($p < 0.0001$).

The effect of natural antimicrobial agents on the survival of naturally occurring microorganisms in fresh vegetables was also described in the literature for isolated compounds, and also other EOs used isolated or in combination. Treatment with carvacrol reduced microbial counts in fresh-cut kiwifruit and honeydew melon at chill temperatures (Roller and Seedhar, 2002), and the counts of mesophilic and psychrotrophic bacteria, *Enterobacteriaceae,* and molds and yeasts on fresh-cut iceberg lettuce, chard and rocket (De Sousa et al., 2012). De Azeredo et al. (2011) studied the application of EOs from *Origanum vulgare* L. and *Rosmarinus officinalis* L. in the inhibition of bacteria and autochthonous microflora in minimally processed vegetables. The results showed that the *O. vulgare* EO caused a significant reduction of the inoculated *L. monocytogenes* and the

microflora of lettuce, beet, and rocket, and no difference was found for the microbial counts in vegetables exposed to the *R. officinalis* EO. The authors hypothesized that the EO having the strongest antibacterial activity contains high amounts of carvacrol and in this case was the *O. vulgare* EO (De Azeredo et al., 2011). This compound could be one of the compounds responsible for the antimicrobial activity of *T. zygis* EO used in our work.

The effect of washing spinach leaves with oregano aqueous extract had also significant reductions in total mesophilic microbiota compared to water washing, but with a higher significant decrease in lettuce samples (Poimenidou et al., 2016), similarly to what happened with *T. zygis* EO. Burnett and Beuchat (2001) reviewed the information about the difficulties in decontamination of food and concluded that one factor that was responsible for the lack of efficacy of EOs as sanitizers in fresh-cut vegetables was the possibility of bacteria attaching or infiltrating into the protective structures of vegetables such as lenticels, broken trichomes, and bruises. This can impair the contact of EOs with target bacteria and reduce their antimicrobial activity (Burnett and Beuchat, 2001). Likewise, the results obtained between lettuce and spinach samples in the present work, in which EO most efficiently decontaminated lettuce, may be associated with the different morphology and microflora load of the samples.

Washing lettuce and spinach leaves with a 0.2% (v/v) solution of *T. zygis* EO effectively reduced the number of microorganisms present in these vegetables and maybe a natural alternative to the use of commercial sanitizers that may be harmful to the health of consumers.

6.4. Conclusion

The *T. zygis* EO had the ability to inhibit planktonic cells, and also presented antibiofilm activity against *L. monocytogenes*. This EO also decreases virulence factors, such as motility, and does not affect the invasive capacity of *L. monocytogenes*. The pre-exposure of sub-inhibitory concentrations of *T. zygis* EO only increased the strain's robustness when subjected to osmotic stress and, in relation to other stress factors, the EO did not have a significant effect on its tolerance or induce cross-resistance to antibiotics. *T. zygis* EO proved to inhibit *L. monocytogenes* growth in chicken juice and in the lettuce model medium. Further, the use of *T. zygis* EO showed to be more effective in reducing the natural microbiota and *L. monocytogenes* from fresh iceberg lettuce and spinach leaves than a simple wash with water.

Therefore, the present work provides a baseline to define a possible use of *T. zygis* EO as an alternative antibacterial agent for the control of *L. monocytogenes* in food products as a natural food preservative and sanitizer.

Chapter 7

Concluding remarks and Future perspectives

Concluding remarks and Future perspectives

Plants have been used in different fields, mainly in folk medicine to prevent and treat diseases and to promote good health. For a long time, plants have been a valuable resource in the supply of herbal cosmetics and food supplements and as alternative therapies to improve human health. The growing interest in traditional phytotherapeutic methods allows a further study of plants, searching for more information about their medicinal and bioactive properties and discovering new drugs and therapies for human welfare.

EOs from different plants have been studied, however, not all have been thoroughly examined, namely considering their different properties and possible applications. Thus, in order to deepen the knowledge about EOs, screening tests of the biological activity of seven commercial EOs from plants present in the Portuguese flora (*Foeniculum vulgare*, *Helichrysum stoechas*, *Mentha pulegium*, *Pinus pinaster*, *Ruta graveolens*, *Thymus mastichina*, and *Thymus zygis*) were carried out.

The GC-MS identified the presence of twelve compounds in the EO of *F. vulgare*, twenty-seven in *H. stoechas* EO, eight in *M. pulegium* EO, twenty-four in *P. pinaster* EO, eight in *R. graveolens* EO, sixteen in *T. mastichina* EO and eighteen in *T. zygis* EO. All the EOs revealed antioxidant activity acting through inhibition of lipid peroxidation, while only three EOs (*H. stoechas*, *M. pulegium,* and *T. zygis*) scavenged the free radicals of DPPH. That is, the last three EO have antioxidant activity through at least two different mechanisms of action. *M. pulegium, T. mastichina* and *T. zygis* EOs showed the strongest antimicrobial activity with the highest inhibition halos and the lowest MIC values against most of the microorganisms in the study. Considering the analysis of the EOs volatile compounds, only volatiles compounds from *M. pulegium* EO demonstrated inhibitory activity against *C. albicans* and the volatile compounds of the *T. zygis* EO inhibited the growth of all microorganisms under study, except *P. aeruginosa*. Also, the effect on the fibroblasts viability was directly proportional to the EOs concentration, with *R. graveolens* EO exhibiting the highest cytotoxic effect. Therefore, of the selected EOs, the *T. zygis* EO showed to be the most promising one with better antioxidant and antimicrobial activity followed by the *M. pulegium* and *T. mastichina* EOs. These results allowed a better understanding of the bioactive properties of some plants' EOs, thus serving as a baseline for a possible further investigation.

Taking into account the results obtained through the screening test of the EOs, it was decided to proceed with the study of the EO of *T. zygis* against *Staphylococcus aureus* and *Listeria monocytogenes*.

Staphylococcus aureus is one of the highly virulent and antibiotic-resistant bacterial pathogens belonging to the ESKAPE group that are the cause of life-threatening nosocomial or hospital-acquired infections. Considering the high resistance to antimicrobial agents used in the first line of treatment, the search for new alternatives or improvement to the existing ones needs extreme attention. Thus, in the next step of our work, the EO of *T. zygis* was studied against different strains of *S. aureus*. *T. zygis* EO demonstrated antibacterial activity against resistant *S. aureus* strains with bactericidal effect, while showing antibiofilm activity and the ability to reduce haemolysis of erythrocytes caused by *S. aureus*. Furthermore, this EO was also able to inhibit the quorum-sensing of the biosensor *C. violaceum*. That is, this EO was able to inhibit some of the virulence factors characteristic of the *S. aureus* strains. Likewise, the *T. zygis* EOs also improve the effect of antimicrobial agents, since the combination of this EO with the antibiotic's ampicillin, ciprofloxacin and vancomycin, potentiated their effect against the *S. aureus* strains under study and, in some cases, changing their phenotype from antibiotic-resistant to susceptible. These results show the possible use of *T. zygis* EO as an alternative antibacterial agent for the control of *S. aureus* or as a possible adjuvant of antibiotics used to combat these bacteria.

Considering the pathogenic potential of *Listeria monocytogenes* and its ability to survive under adverse conditions used in food processing and preservation, as well as the results obtained in the screening tests, the antilisterial activity of *T. zygis* EO was further studied. The results showed that the *T. zygis* EO had the ability to inhibit planktonic cells, but also presented antibiofilm activity and reduced the motility of *L. monocytogenes*, despite not affecting its invasive capacity, indicating once again that this EO interferes with the virulence factors, in this situation decreasing the virulence of *L. monocytogenes*. When *L. monocytogenes* was pre-exposed to sub-inhibitory concentrations of *T. zygis* EO, the strain's robustness was only increased when subjected to osmotic stress and, in relation to other stress factors such as high temperature, desiccation tolerance and low pH, the EO did not have a significant effect on its tolerance or even induced cross-resistance to antibiotics.

Based on the fact that consumers are increasingly looking for natural alternatives to preserve their food and the results obtained against *L. monocytogenes* strains, the *T. zygis* EO was studied to evaluate its potential as a food preservative. *T. zygis* EO proved to inhibit *L. monocytogenes* growth in chicken juice and in the iceberg lettuce model medium. Furthermore, the use of *T. zygis* EO has a vegetable sanitizer showed to be more effective in reducing the natural microbiota and *L. monocytogenes* from fresh iceberg lettuce and spinach leaves than a simple wash with water. The evaluation of the inhibitory activity of *T. zygis* against *L. monocytogenes* strains may contribute to further studies concerning the development of new control approaches for this pathogenic bacterium and the possible application of *T. zygis* EO as a food preservative and sanitizer.

Overall, in this thesis, we presented some biological activities of different EOs, contributing to the knowledge of these EOs, highlighting the antibacterial activity of the *T. zygis* EO against *S. aureus* and in the to control *L. monocytogenes* in food.

For further works, it could be interesting to evaluate the different isolated compounds of the *T. zygis* EO, or their combination, in order to understand which isolated compounds or combinations of compounds are responsible for the observed biological activities, which can be an asset when intending to use the EO. Also, additional studies should be carried out to understand the exact mechanisms of action of the *T. zygis* EO. Considering the observed effect in *L. monocytogenes* by pre-exposure to sub-inhibitory concentrations of *T. zygis* EO, more studies are in need considering a more prolonged exposure and the potential for tolerance development also to low temperatures, high pressure, UV-light, or pulsed electric fields, or other adverse conditions. For a possible application in food for consumption, it would be essential to perform sensory tests in order to understand whether or not the application of EO changes the organoleptic properties of the food. Considering that the direct application of EOs in foods may have limitations due its volatility and lipophilicity and, in some cases, can change the organoleptic properties of the products, it would be interesting to study different types and materials of delivery vehicles in order to overcome these disadvantages, for example, incorporation of EOs in edible coatings or films, in active packaging, or even encapsulation.

References

References

Aazza, S., El-Guendouz, S., Miguel, M.G., Dulce Antunes, M., Leonor Faleiro, M., Isabel Correia, A., Cristina Figueiredo, A., 2016. Antioxidant, anti-inflammatory and anti-hyperglycaemic activities of essential oils from *Thymbra capitata*, *Thymus albicans*, *Thymus caespititius*, *Thymus carnosus*, *Thymus lotocephalus* and *Thymus mastichina* from Portugal. Nat. Prod. Commun. 11, 1029–1038. https://doi.org/10.1177/1934578x1601100739

Abbas, A., Ikram, R., Adil, A., Bukhari, Q. ul A., Hasan, F., 2021. Impact of *Foeniculum vulgare* on hemoglobin, red blood cell indices and white blood cell count: A laboratory based randomized controlled study. J. Pharm. Res. Int. 33, 97–102. https://doi.org/10.9734/jpri/2021/v33i30a31620

Abbasi-Maleki, S., Maleki, S.G., 2021. Antidepressant-like effects of *Foeniculum vulgare* essential oil and potential involvement of dopaminergic and serotonergic systems on mice in the forced swim test. PharmaNutrition. 15, 100241. https://doi.org/10.1016/j.phanu.2020.100241

Abbou, F., Azzi, R., Ouffai, K., El Haci, I.A., Belyagoubi-Benhammou, N., Bensouici, C., Benamar, H., 2022. Phenolic profile, antioxidant and enzyme inhibitory properties of phenolic-rich fractions from the aerial parts of *Mentha pulegium* L. S. Afr. J. Bot. 146, 196–204. https://doi.org/10.1016/j.sajb.2021.10.024

Abdallah, F. Ben, Lagha, R., Gaber, A., 2020. Biofilm inhibition and eradication properties of medicinal plant essential oils against methicillin-resistant *Staphylococcus aureus* clinical isolates. Pharmaceuticals. 13, 1–15. https://doi.org/10.3390/ph13110369

Abdel-Baki, A.A.S., Aboelhadid, S.M., Sokmen, A., Al-Quraishy, S., Hassan, A.O., Kamel, A.A., 2021. Larvicidal and pupicidal activities of *Foeniculum vulgare* essential oil, *trans*-anethole and fenchone against house fly *Musca domestica* and their inhibitory effect on acetylcholinestrase. Entomol. Res. 51, 568–577. https://doi.org/10.1111/1748-5967.12550

Aboelhadid, S.M., Arafa, W.M., Abdel-Baki, A.A.S., Sokmen, A., Al-Quraishy, S., Hassan, A.O., Kamel, A.A., 2021. Acaricidal activity of *Foeniculum vulgare* against *Rhipicephalus annulatus* is mainly dependent on its constituent from *trans*-anethone. PLoS ONE. 16, e0260172. https://doi.org/10.1371/journal.pone.0260172

Abuga, I., Sulaiman, S.F., Abdul Wahab, R., Ooi, K.L., Abdull Rasad, M.S.B., 2022. Phytochemical constituents and antibacterial activities of 45 Malay traditional medicinal plants. J. Herb. Med. 32, 100496.

https://doi.org/10.1016/j.hermed.2021.100496

Adams, R.P., 2007. Identification of essential oil components by Gas Chromatography/Mass Spectroscopy, 4th Edition. Allured Publishing Corporation, Carol Stream, Illinois 60188-2787 USA.

Afonso, A.F., Pereira, O.R., Válega, M., Silva, A.M.S., Cardoso, S.M., 2018. Metabolites and biological activities of *Thymus zygis*, *Thymus pulegioides*, and *Thymus fragrantissimus* grown under organic cultivation. Molecules. 23, 1514. https://doi.org/10.3390/molecules23071514

Ahmad, A., Elisha, I.L., van Vuuren, S., Viljoen, A., 2021. Volatile phenolics: A comprehensive review of the anti-infective properties of an important class of essential oil constituents. Phytochemistry. 190, 112864. https://doi.org/10.1016/j.phytochem.2021.112864

Ahmed, A., Ayoub, K., Chaima, A.J., Hanaa, L., Abdelaziz, C., 2018. Effect of drying methods on yield, chemical composition and bioactivities of essential oil obtained from Moroccan *Mentha pulegium* L. Biocatal. Agric. Biotechnol. 16, 638–643. https://doi.org/10.1016/j.bcab.2018.10.016

Aimad, A., Sanae, R., Anas, F., Abdelfattah, E.M., Bourhia, M., Mohammad Salamatullah, A., Alzahrani, A., Alyahya, H.K., Albadr, N.A., Abdelkrim, A., El Barnossi, A., Noureddine, E., 2021. Chemical characterization and antioxidant, antimicrobial, and insecticidal properties of essential oil from *Mentha pulegium* L. Evidence-Based Complementary Altern. Med. 2021, 1108133. https://doi.org/10.1155/2021/1108133

Akaberi, M., Sahebkar, A., Azizi, N., Emami, S.A., 2019. Everlasting flowers: Phytochemistry and pharmacology of the genus *Helichrysum*. Ind. Crops Prod. 138, 111471. https://doi.org/10.1016/j.indcrop.2019.111471

Akbari, B., Baghaei-Yazdi, N., Bahmaie, M., Mahdavi Abhari, F., 2022. The role of plant-derived natural antioxidants in reduction of oxidative stress. BioFactors. 48, 611–633. https://doi.org/10.1002/biof.1831

Akhbari, M., Kord, R., Nodooshan, S.J., Hamedi, S., 2019. Analysis and evaluation of the antimicrobial and anticancer activities of the essential oil isolated from *Foeniculum vulgare* from Hamedan, Iran. Nat. Prod. Res. 33, 1629–1632. https://doi.org/10.1080/14786419.2017.1423310

Albano, S.M., Sofia Lima, A., Graça Miguel, M., Pedro, L.G., Barroso, J.G., Figueiredo, A.C., 2012. Antioxidant, anti-5-lipoxygenase and antiacetylcholinesterase activities of essential oils and decoction waters of some aromatic plants. Rec. Nat. Prod. 6, 35–48.

Albayrak, S., Aksoy, A., Sagdic, O., Hamzaoglu, E., 2010. Compositions, antioxidant and antimicrobial activities of *Helichrysum* (Asteraceae) species collected from Turkey. Food Chem. 119, 114–122. https://doi.org/10.1016/j.foodchem.2009.06.003

Alexandrovich, I., Rakovitskaya, O., Kolmo, E., Sidorova, T., Shushunov, S., 2003. The effect of fennel (*Foeniculum vulgare*) seed oil emulsion in infantile colic: A randomized, placebo-controlled study. Altern. Ther. Health Med. 9, 58–61.

Algburi, A., Comito, N., Kashtanov, D., Dicks, L.M.T., Chikindas, M.L., 2017. Control of biofilm formation: Antibiotics and beyond. Appl. Environ. Microbiol. 83, 1–15. https://doi.org/10.1128/AEM.02508-16

Alimi, D., Hajri, A., Jallouli, S., Sebai, H., 2022. Phytochemistry, anti-tick, repellency and anti-cholinesterase activities of *Cupressus sempervirens* L. and *Mentha pulegium* L. combinations against *Hyalomma scupense* (Acari: Ixodidae). Vet. Parasitol. 303, 109665. https://doi.org/10.1016/j.vetpar.2022.109665

Alonso-Castro, A.J., Maldonado-Miranda, J.J., Zarate-Martinez, A., Jacobo-Salcedo, M.D.R., Fernández-Galicia, C., Figueroa-Zuñiga, L.A., Rios-Reyes, N.A., De LeónRubio, M.A., Medellín-Castillo, N.A., Reyes-Munguia, A., Méndez-Martínez, R., Carranza-Alvarez, C., 2012. Medicinal plants used in the Huasteca Potosina, México. J. Ethnopharmacol. 143, 292–298. https://doi.org/10.1016/j.jep.2012.06.035

Álvarez, A., García García, B., Jordán, M.J., Martínez-Conesa, C., Hernández, M.D., 2012. The effect of diets supplemented with thyme essential oils and rosemary extract on the deterioration of farmed gilthead seabream (*Sparus aurata*) during storage on ice. Food Chem. 132, 1395–1405. https://doi.org/10.1016/j.foodchem.2011.11.127

Aminkhani, A., Sharifi, R., Ranjbar, E., Chalabian, F., Katouzian, F., 2019. Antimicrobial activities and chemical constituents of essential oil extracted from stem, leaf, and flower of *Thymus fedtschenkoi* from Khoy, Iran. J. Food Process. Preserv. 43, 1–8. https://doi.org/10.1111/jfpp.14149

Andrés, M.F., González-coloma, A., Muñoz, R., De la Peña, F., Julio, L.F., Burillo, J., 2018. Nematicidal potential of hydrolates from the semi industrial vapor-pressure extraction of Spanish aromatic plants. Environ. Sci. Pollut. Res. 25, 29834–29840. https://doi.org/10.1007/s11356-017-9429-z

Angane, M., Swift, S., Huang, K., Butts, C.A., Quek, S.Y., 2022. Essential oils and their major components: An updated review on antimicrobial activities, mechanism of action and their potential application in the food industry. Foods. 11, 464. https://doi.org/10.3390/foods11030464

Arantes, S., Piçarra, A., Candeias, F., Caldeira, A.T., Martins, M.R., Teixeira, D., 2017. Antioxidant activity and cholinesterase inhibition studies of four flavouring herbs from Alentejo. Nat. Prod. Res. 31, 2183–2187. https://doi.org/10.1080/14786419.2017.1278598

Arantes, S.M., Piçarra, A., Guerreiro, M., Salvador, C., Candeias, F., Caldeira, A.T., Martins, M.R., 2019. Toxicological and pharmacological properties of essential oils of *Calamintha nepeta*, *Origanum virens* and *Thymus mastichina* of Alentejo (Portugal). Food Chem. Toxicol. 133, 110747. https://doi.org/10.1016/j.fct.2019.110747

Araujo, A.R.T.S., Périno, S., Fernandez, X., Cunha, C., Rodrigues, M., Ribeiro, M.P., Jordao, L., Silva, L.A., Rodilla, J., Coutinho, P., Chemat, F., 2021. Solvent-free microwave extraction of *Thymus mastichina* essential oil: Influence on their chemical composition and on the antioxidant and antimicrobial activities. Pharmaceuticals. 14, 1–13. https://doi.org/10.3390/ph14080709

Asbahani, A. El, Miladi, K., Badri, W., Sala, M., Addi, E.H.A., Casabianca, H., Mousadik, A. El, Hartmann, D., Jilale, A., Renaud, F.N.R., Elaissari, A., 2015. Essential oils: From extraction to encapsulation. Int. J. Pharm. 483, 220–243. https://doi.org/10.1016/j.ijpharm.2014.12.069

Ascensao, L., Da Silva, J.A.T., Barroso, J.G., Figueiredo, A.C., Pedro, L.G., 2001. Glandular trichomes and essential oils of *Helichrysum stoechas*. Isr. J. Plant Sci. 49, 115–122. https://doi.org/10.1560/F85Y-YEN4-9VTF-B1AJ

Asensio, C.M., Quiroga, P.R., Al-Gburi, A., Huang, Q., Grosso, N.R., 2020. Rheological behavior, antimicrobial and quorum sensig inhibition study of an argentinean oregano essential oil nanoemulsion. Front. Nutr. 7, 1–12. https://doi.org/10.3389/fnut.2020.569913

Ashokkumar, K., Vellaikumar, S., Murugan, M., Dhanya, M.K., Karthikeyan, A., Ariharasutharsan, G., Arjun, P., Sivakumar, P., Aiswarya, S., 2021. GC/MS analysis of essential oil composition from selected seed spices. Natl. Acad. Sci. Lett. 44, 503–506. https://doi.org/10.1007/s40009-021-01066-7

Aumeeruddy, M.Z., Mahomoodally, M.F., 2021. Traditional herbal medicines used in obesity management: A systematic review of ethnomedicinal surveys. J. Herb. Med. 28, 100435. https://doi.org/10.1016/j.hermed.2021.100435

Ayaz, M., Ullah, Farhat, Sadiq, A., Ullah, Farman, Ovais, M., Ahmed, J., Devkota, H.P., 2019. Synergistic interactions of phytochemicals with antimicrobial agents: Potential strategy to counteract drug resistance. Chem.-Biol. Interact. 308, 294–303. https://doi.org/10.1016/j.cbi.2019.05.050

Azmin, S.N.H.M., Sulaiman, N.S., Mat Nor, M.S., Abdullah, P.S., Abdul Kari, Z., Pati, S., 2022. A review on recent advances on natural plant pigments in foods: Functions, extraction, importance and challenges. Appl. Biochem. Biotechnol. https://doi.org/10.1007/s12010-022-04050-z

Baali, F., Boumerfeg, S., Napoli, E., Boudjelal, A., Righi, N., Deghima, A., Baghiani, A., Ruberto, G., 2019. Chemical composition and biological activities of essential oils from two wild Algerian medicinal plants: *Mentha pulegium* L. and *Lavandula stoechas* L. J. Essent. Oil Bear. Plants. 22, 821–837. https://doi.org/10.1080/0972060X.2019.1642800

Babich, O., Dyshlyuk, L., Prosekov, A., Noskova, S., Ivina, O., Pavsky, V., Ivanova, S., Bulgakova, O., 2020. Study of the potential of the capsule shell based on natural polysaccharides in targeted delivery of the L-phenylalanine ammonia-lyase enzyme preparation. Pharmaceuticals. 13, 63. https://doi.org/10.3390/ph13040063

Badgujar, S.B., Patel, V. V., Bandivdekar, A.H., 2014. *Foeniculum vulgare* Mill: A review of its botany, phytochemistry, pharmacology, contemporary application, and toxicology. BioMed Res. Int. 2014, 1–32. https://doi.org/10.1155/2014/842674

Baindara, P., Mandal, S.M., 2022. Plant-derived antimicrobial peptides: Novel preservatives for the food industry. Foods. 11, 2415. https://doi.org/10.3390/foods11162415

Bakkali, F., Averbeck, S., Averbeck, D., Idaomar, M., 2008. Biological effects of essential oils - A review. Food Chem. Toxicol. 46, 446–475. https://doi.org/10.1016/j.fct.2007.09.106

Balali, G.I., Yar, D.D., Afua Dela, V.G., Adjei-Kusi, P., 2020. Microbial contamination, an increasing threat to the consumption of fresh fruits and vegetables in today's world. Int. J. Microbiol. 2020, 3029295. https://doi.org/10.1155/2020/3029295

Ballester-Costa, C., Sendra, E., Fernández-López, J., Pérez-Álvarez, J.A., Viuda-Martos, M., 2013. Chemical composition and *in vitro* antibacterial properties of essential oils of four *Thymus* species from organic growth. Ind. Crops Prod. 50, 304–311. https://doi.org/10.1016/j.indcrop.2013.07.052

Ballester-Costa, C., Sendra, E., Fernández-López, J., Pérez-Álvarez, J.A., Viuda-Martos, M., 2017. Assessment of antioxidant and antibacterial properties on meat homogenates of essential oils obtained from four *Thymus* species achieved from organic growth. Foods. 6, 1–11. https://doi.org/10.3390/foods6080059

Ballester-Costa, C., Sendra, E., Fernández-López, J., Viuda-Martos, M., 2016. Evaluation of

the antibacterial and antioxidant activities of chitosan edible films incorporated with organic essential oils obtained from four *Thymus* species. J. Food Sci. Tech. 53, 3374–3379. https://doi.org/10.1007/s13197-016-2312-y

Barbosa, P., Lima, A.S., Vieira, P., Dias, L.S., Tinoco, M.T., Barroso, J.G., Pedro, L.G., Figueiredo, A.C., Mota, M., 2010. Nematicidal activity of essential oils and volatiles derived from Portuguese aromatic flora against the pinewood nematode, *Bursaphelenchus xylophilus*. J. Nematol. 42, 8–16. https://doi.org/10.1094/PDIS-12-10-0902

Barros, L., Heleno, S.A., Carvalho, A.M., Ferreira, I.C.F.R., 2009. Systematic evaluation of the antioxidant potential of different parts of *Foeniculum vulgare* Mill. from Portugal. Food Chem. Toxicol. 47, 2458–2464. https://doi.org/10.1016/j.fct.2009.07.003

Barros, L., Oliveira, S., Carvalho, A.M., Ferreira, I.C.F.R., 2010. *In vitro* antioxidant properties and characterization in nutrients and phytochemicals of six medicinal plants from the Portuguese folk medicine. Ind. Crops Prod. 32, 572–579. https://doi.org/10.1016/j.indcrop.2010.07.012

Becit, M., Aydin, S., 2020. An *in vitro* study on the interactions of Pycnogenol® with Cisplatin in human cervical cancer cells. Turk. J. Pharm. Sci. 17, 1–6. https://doi.org/10.4274/tjps.galenos.2018.97759

Bektašević, M., Politeo, O., Carev, I., 2021. Comparative study of chemical composition, cholinesterase inhibition and antioxidant potential of *Mentha pulegium* L. essential oil. Chem. Biodivers. 18, 1–9. https://doi.org/10.1002/cbdv.202000935

Belcaro, G., Cesarone, M.R., Errichi, B.M., Ledda, A., Di Renzo, A., Stuard, S., Dugall, M., Pellegrini, L., Gizzi, G., Rohdewald, P., Ippolito, E., Ricci, A., Cacchio, M., Cipollone, G., Ruffini, I., Fano, F., Hosoi, M., 2006. Diabetic ulcers: Microcirculatory improvement and faster healing with Pycnogenol. Clin. Appl. Thromb./Hemostasis. 12, 318–323. https://doi.org/10.1177/1076029606290133

Benahmed, A., Harfi, B., Belkhiri, A., 2019. Biological activity of essential oils of *Mentha pulegium* from field-grown and acclimated *in vitro* plants. Curr. Sci. 116, 1897–1904. https://doi.org/10.18520/cs/v116/i11/1897-1904

Benlamdini, N., Elhafian, M., Rochdi, A., Zidane, L., 2014. Étude floristique et ethnobotanique de la flore médicinale du Haut Atlas oriental (Haute Moulouya). J. Appl. Biosci. 78, 6771. https://doi.org/10.4314/jab.v78i1.17

Bentes, J., Miguel, M.G., Monteiro, I., Costa, M., Figueiredo, A.C., Barroso, J.., Pedro, L.G., 2009. Antioxidant activities of the essential oils and extracts of Portuguese *Thymbra*

capitata and *Thymus mastichina*. Ital. J. Food Sci. 21, 183–195.

Berdejo, D., Pagán, E., Merino, N., García-Gonzalo, D., Pagán, R., 2021. Emerging mutant populations of *Listeria monocytogenes* EGD-e under selective pressure of *Thymbra capitata* essential oil question its use in food preservation. Food Res. Int. 145, 110403. https://doi.org/10.1016/j.foodres.2021.110403

Berdejo, D., Pagán, E., Merino, N., Pagán, R., García-Gonzalo, D., 2020. Incubation with a complex orange essential oil leads to evolved mutants with increased resistance and tolerance. Pharmaceuticals. 13, 1–17. https://doi.org/10.3390/ph13090239

Bhatti, S., Ali Shah, S.A., Ahmed, T., Zahid, S., 2018. Neuroprotective effects of *Foeniculum vulgare* seeds extract on lead-induced neurotoxicity in mice brain. Drug Chem. Toxicol. 41, 399–407. https://doi.org/10.1080/01480545.2018.1459669

Bilia, A.R., Guccione, C., Isacchi, B., Righeschi, C., Firenzuoli, F., Bergonzi, M.C., 2014. Essential oils loaded in nanosystems: A developing strategy for a successful therapeutic approach. Evid.-based Compl. Alt. Med. 2014, 1–14. https://doi.org/10.1155/2014/651593

Bjørklund, G., Rahaman, S., Shanaida, M., Lysiuk, R., Oliynyk, P., Lenchyk, L., Chirumbolo, S., Chasapis, C.T., Peana, M., 2022. Natural dietary compounds in the treatment of arsenic toxicity. Molecules. 27, 4871. https://doi.org/10.3390/molecules27154871

Bogdadi, H.A.A., Kokoska, L., Havlik, J., Kloucek, P., Rada, V., Vorisek, K., 2007. *In vitro* antimicrobial activity of some Libyan medicinal plant extracts. Pharm. Biol. 45, 386–391. https://doi.org/10.1080/13880200701215026

Bouabida, H., Dris, D., 2020. Effect of rue (*Ruta graveolens*) essential oil on mortality, development, biochemical and biomarkers of *Culiseta longiareolata*. S. Afr. J. Bot. 133, 139–143. https://doi.org/10.1016/j.sajb.2020.07.005

Bouyahya, A., Abrini, J., Dakka, N., Bakri, Y., 2019. Essential oils of *Origanum compactum* increase membrane permeability, disturb cell membrane integrity, and suppress quorum-sensing phenotype in bacteria. J. Pharm. Anal. 9, 301–311. https://doi.org/10.1016/j.jpha.2019.03.001

Bouzeraa, H., Bessila-Bouzeraa, M., Labed, N., 2019. Repellent and fumigant toxic potential of three essential oils against *Ephestia kuehniella*. Biosyst. Divers. 27, 349–353. https://doi.org/10.15421/011946

Braschi, G., Serrazanetti, D.I., Siroli, L., Patrignani, F., De Angelis, M., Lanciotti, R., 2018. Gene expression responses of *Listeria monocytogenes* Scott A exposed to sub-lethal concentrations of natural antimicrobials. Int. J.Food Microbiol. 286, 170–178.

https://doi.org/10.1016/j.ijfoodmicro.2018.07.026

Bremner, P., Rivera, D., Calzado, M.A., Obón, C., Inocencio, C., Beckwith, C., Fiebich, B.L., Muñoz, E., Heinrich, M., 2009. Assessing medicinal plants from South-Eastern Spain for potential anti-inflammatory effects targeting nuclear factor-Kappa B and other pro-inflammatory mediators. J. Ethnopharmacol. 124, 295–305. https://doi.org/10.1016/j.jep.2009.04.035

Brochot, A., Guilbot, A., Haddioui, L., Roques, C., 2017. Antibacterial, antifungal, and antiviral effects of three essential oil blends. MicrobiologyOpen. 6, 1–6. https://doi.org/10.1002/mbo3.459

Burgos, M.J.G., Pulido, R.P., Aguayo, M. del C.L., Gálvez, A., Lucas, R., 2014. The cyclic antibacterial peptide enterocin AS-48: Isolation, mode of action, and possible food applications. Int. J. Mol. Sci. 15, 22706–22727. https://doi.org/10.3390/ijms151222706

Burlacu, E., Tanase, C., 2021. Anticancer potential of natural bark products—A review. Plants. 10, 1895. https://doi.org/10.3390/plants10091895

Burnett, S.L., Beuchat, L.R., 2001. Human pathogens associated with raw produce and unpasteurized juices, and difficulties in decontamination. J.Ind. Microbiol. Biotechnol. 27, 104–110. https://doi.org/10.1038/sj.jim.7000199

Cabral, C., Francisco, V., Cavaleiro, C., Gonçalves, M.J., Cruz, M.T., Sales, F., Batista, M.T., Salgueiro, L., 2012. Essential oil of *Juniperus communis* subsp. *alpina* (Suter) Čelak needles: Chemical composition, antifungal activity and cytotoxicity. Phytother. Res. 26, 1352–1357. https://doi.org/10.1002/ptr.3730

Calo, J.R., Crandall, P.G., O'Bryan, C.A., Ricke, S.C., 2015. Essential oils as antimicrobials in food systems - A review. Food Control. 54, 111–119. https://doi.org/10.1016/j.foodcont.2014.12.040

Camejo, A., Carvalho, F., Reis, O., Leitão, E., Sousa, S., Cabanes, D., 2011. The arsenal of virulence factors deployed by *Listeria monocytogenes* to promote its cell infection cycle. Virulence. 2, 379–394. https://doi.org/10.4161/viru.2.5.17703

Canali, R., Comitato, R., Schonlau, F., Virgili, F., 2009. The anti-inflammatory pharmacology of Pycnogenol® in humans involves COX-2 and 5-LOX mRNA expression in leukocytes. Int. Immunopharmacol. 9, 1145–1149. https://doi.org/10.1016/j.intimp.2009.06.001

Capewell, P., Krumrie, S., Katzer, F., Alexander, C.L., Weir, W., 2021. Molecular epidemiology of *Giardia* infections in the genomic era. Trends Parasitol. 37, 142–153.

https://doi.org/10.1016/j.pt.2020.09.013

Caputo, L., Cornara, L., Raimondo, F.M., De Feo, V., Vanin, S., Denaro, M., Trombetta, D., Smeriglio, A., 2021. *Mentha pulegium* L.: A plant underestimated for its toxicity to be recovered from the perspective of the circular economy. Molecules. 26, 1–19. https://doi.org/10.3390/Molecules.26082154

Carini, M., Aldini, G., Furlanetto, S., Stefani, R., Facino, R.M., 2001. LC coupled to ion-trap MS for the rapid screening and detection of polyphenol antioxidants from *Helichrysum stoechas*. J. Pharm. Biomed. Anal. 24, 517–526. https://doi.org/10.1016/S0731-7085(00)00431-3

Carnevali, I., La Paglia, R., Pauletto, L., Raso, F., Testa, M., Mannucci, C., Sorbara, E.E., Calapai, G., 2021. Efficacy and safety of the syrup "KalobaTUSS®" as a treatment for cough in children: A randomized, double blind, placebo-controlled clinical trial. BMC Pediatr. 21, 1–9. https://doi.org/10.1186/s12887-020-02490-2

Carrasco, A., Tomas, V., Tudela, J., Miguel, M.G., 2015. Comparative study of GC-MS characterization, antioxidant activity and hyaluronidase inhibition of different species of *Lavandula* and *Thymus* essential oils. Flavour Fragrance J. 31, 57–69. https://doi.org/10.1002/ffj.3283

Carrascosa, C., Raheem, D., Ramos, F., Saraiva, A., Raposo, A., 2021. Microbial biofilms in the food industry—A comprehensive review. Int. J. Environ. Res. Public Health. 18, 1–31. https://doi.org/10.3390/ijerph18042014

Cesarone, M.R., Belcaro, G., Rohdewald, P., Pellegrini, L., Ledda, A., Vinciguerra, G., Ricci, A., Gizzi, G., Ippolito, E., Fano, F., Dugall, M., Acerbi, G., Cacchio, M., Di Renzo, A., Hosoi, M., Stuard, S., Corsi, M., 2006a. Comparison of Pycnogenol® and Daflon® in treating chronic venous insufficiency: A prospective, controlled study. Clin. Appl. Thromb./Hemostasis.12, 205–212. https://doi.org/10.1177/107602960601200209

Cesarone, M.R., Belcaro, G., Rohdewald, P., Pellegrini, L., Ledda, A., Vinciguerra, G., Ricci, A., Gizzi, G., Ippolito, E., Fano, F., Dugall, M., Acerbi, G., Cacchio, M., Di Renzo, A., Hosoi, M., Stuard, S., Corsi, M., 2006b. Rapid relief of signs/symptoms in chronic venous microangiopathy with Pycnogenol®: A prospective, controlled study. Angiology. 57, 569–576. https://doi.org/10.1177/0003319706291392

Chaftar, N., Girardot, M., Labanowski, J., Ghrairi, T., Hani, K., Frère, J., Imbert, C., 2016. Comparative evaluation of the antimicrobial activity of 19 essential oils. Adv. Exp. Med. Biol. 901, 1–15. https://doi.org/10.1007/5584_2015_5011

Chaftar, N., Girardot, M., Quellard, N., Labanowski, J., Ghrairi, T., Hani, K., Frère, J., Imbert, C., 2015. Activity of six essential oils extracted from Tunisian plants against *Legionella pneumophila*. Chem. Biodivers. 12, 1565–1574. https://doi.org/10.1002/cbdv.201400343

Chang, S., Abbaspour, H., Nafchi, A.M., Heydari, A., Mohammadhosseini, M., 2016. Iranian *Foeniculum vulgare* essential oil and alcoholic extracts: Chemical composition, antimicrobial, antioxidant and application in olive oil preservation. J. Essent. Oil-Bear. Plants. 19, 1920–1931. https://doi.org/10.1080/0972060X.2016.1238324

Chatterjee, B., Vittal, R.R., 2021. Quorum sensing modulatory and biofilm inhibitory activity of *Plectranthus barbatus* essential oil: A novel intervention strategy. Arch. Microbiol. 203, 1767–1778. https://doi.org/10.1007/s00203-020-02171-9

Chen, F., Guo, Y., Kang, J., Yang, X., Zhao, Z., Liu, S., Ma, Y., Gao, W., Luo, D., 2020a. Insight into the essential oil isolation from *Foeniculum vulgare* Mill. fruits using double-condensed microwave-assisted hydrodistillation and evaluation of its antioxidant, antifungal and cytotoxic activity. Ind. Crops Prod. 144, 112052. https://doi.org/10.1016/j.indcrop.2019.112052

Chen, Y., Wen, Q., Chen, S., Guo, D., Xu, Y., Liang, S., Xia, X., Yang, B., Shi, C., 2020b. Effect of thymoquinone on the resistance of *Cronobacter sakazakii* to environmental stresses and antibiotics. Food Control. 109, 106944. https://doi.org/10.1016/j.foodcont.2019.106944

Cheng, Y.T., Yang, C.C., Shyur, L.F., 2016. Phytomedicine—Modulating oxidative stress and the tumor microenvironment for cancer therapy. Pharmacol. Res. 114, 128–143. https://doi.org/10.1016/j.phrs.2016.10.022

Cheung, G.Y.C., Bae, J.S., Otto, M., 2021. Pathogenicity and virulence of *Staphylococcus aureus*. Virulence. 12, 547–569. https://doi.org/10.1080/21505594.2021.1878688

Chinou, I.B., Roussis, V., Perdetzoglou, D., Tzakou, O., Loukis, A., 1997. Chemical and antibacterial studies of two *Helichrysum* species of Greek origin. Planta Med. 63, 181–183. https://doi.org/10.1055/s-2006-957641

Cho, K.J., Yun, C.H., Packer, L., Chunga, A.S., 2001. Inhibition mechanisms of bioflavonoids extracted from the bark of *Pinus maritima* on the expression of proinflammatory cytokines. Ann. N. Y. Acad. Sci. 928, 141–156. https://doi.org/10.1111/j.1749-6632.2001.tb05644.x

Chouhan, S., Sharma, K., Guleria, S., 2017. Antimicrobial activity of some essential oils—Present status and future perspectives. Medicines. 4, 58.

https://doi.org/10.3390/medicines4030058

Ciganda, C., Laborde, A., 2003. Herbal infusions used for induced abortion. J. Toxicol. Clin. Toxicol. 41, 235–239. https://doi.org/10.1081/CLT-120021104

Clinical and Laboratory Standards Institute (CLSI), 2021. Performance Standards for Antimicrobial Susceptibility Testing; Approvated standard, CLSI Document M100-S31, 31st ed. Wayne, PA, USA.

Coimbra, A., Miguel, S., Ribeiro, M., Coutinho, P., Silva, L., Duarte, A.P., Ferreira, S., 2022. *Thymus zygis* essential oil: Phytochemical characterization, bioactivity evaluation and synergistic effect with antibiotics against *Staphylococcus aureus*. Antibiotics. 11, 146. https://doi.org/10.3390/ antibiotics11020146

Coimbra, A.T., Ferreira, S., Duarte, A.P., 2020a. Genus *Ruta*: A natural source of high value products with biological and pharmacological properties. J. Ethnopharmacol. 260, 113076. https://doi.org/10.1016/j.jep.2020.113076

Coimbra, A.T., Luís, Â.F.S., Batista, M.T., Ferreira, S.M.P., Duarte, A.P.C., 2020b. Phytochemical characterization, bioactivities evaluation and synergistic effect of *Arbutus unedo* and *Crataegus monogyna* extracts with amphotericin B. Curr. Microbiol. 77, 2143–2154. https://doi.org/10.1007/s00284-020-02125-w

Crescenzi, M.A., D'urso, G., Piacente, S., Montoro, P., 2021. LC-ESI/LTQOrbitrap/MS metabolomic analysis of fennel waste (*Foeniculum vulgare* Mill.) as a byproduct rich in bioactive compounds. Foods. 10, 1893. https://doi.org/10.3390/foods10081893

Ćurković-Perica, M., Hrenović, J., Kugler, N., Goić-Barišić, I., Tkalec, M., 2015. Antibacterial activity of *Pinus pinaster* bark extract and its components against multidrug-resistant clinical isolates of *Acinetobacter baumannii*. Croat. Chem. Acta. 88, 133–137. https://doi.org/10.5562/cca2548

Cutillas, A.B., Carrasco, A., Martinez-Gutierrez, R., Tomas, V., Tudela, J., 2018a. Thyme essential oils from Spain: Aromatic profile ascertained by GC–MS, and their antioxidant, anti-lipoxygenase and antimicrobial activities. J. Food Drug Anal. 26, 529–544. https://doi.org/10.1016/j.jfda.2017.05.004

Cutillas, A.B., Carrasco, A., Martinez-Gutierrez, R., Tomas, V., Tudela, J., 2018b. *Thymus mastichina* L. essential oils from Murcia (Spain): Composition and antioxidant, antienzymatic and antimicrobial bioactivities. PLoS ONE. 13, 1–19. https://doi.org/10.1371/journal.pone.0190790

Cwikla, C., Schmidt, K., Matthias, A., Bone, K.M., Lehmann, R., Tiralongo, E., 2010. Investigations into the antibacterial activities of phytotherapeutics against

Helicobacter pylori and *Campylobacter jejuni*. Phytother. Res. 24, 649–656. https://doi.org/10.1002/ptr.2933

Da Silva, B.D., Bernardes, P.C., Pinheiro, P.F., Fantuzzi, E., Roberto, C.D., 2021. Chemical composition, extraction sources and action mechanisms of essential oils: Natural preservative and limitations of use in meat products. Meat Sci. 176, 108463. https://doi.org/10.1016/j.meatsci.2021.108463

Da Silva, F.G.E., Francisco, F.R., João, J.C., Santiago, G.M.P., Bezerra, M.A.X., Barbosa, F.G., Mafezoli, J., Rocha, R.R., 2014. Seasonal variation, larvicidal and nematicidal activities of the leaf essential oil of *Ruta graveolens* L. J. Essent. Oil Res. 26, 204–209. https://doi.org/ 10.1080/10412905.2014.882276

Dahmani, K., Moghrani, H., Deghbar, N., Ouarek, S., Allaf, K., Arab, K., 2022. Algerian wild fennel essential oils: Chromatographic profile, acute toxicity, antioxidant, and antimicrobial activities. Chem. Pap. 76, 1639–1652. https://doi.org/10.1007/s11696-021-02008-9

Dandlen, S.A., Lima, A.S., Mendes, M.D., Miguel, M.G., Faleiro, M.L., Sousa, M.J., Pedro, L.G., Barroso, J.G., Figueiredo, A.C., 2011. Antimicrobial activity, cytotoxicity and intracellular growth inhibition of Portuguese *Thymus* essential oils. Braz. J. Pharmacogn. 21, 1012–1024. https://doi.org/10.1590/S0102-695X2011005000155

Davies, R., Wales, A., 2019. Antimicrobial resistance on farms: A review including biosecurity and the potential role of disinfectants in resistance selection. Compr. Rev. Food Sci. Food Saf. 18, 753–774. https://doi.org/10.1111/1541-4337.12438

Davoodvandi, A., Shabani Varkani, M., Clark, C.C.T., Jafarnejad, S., 2020. Quercetin as an anticancer agent: Focus on esophageal cancer. J. Food Biochem. 44, 1–10. https://doi.org/10.1111/jfbc.13374

De Aguiar, F.C., Solarte, A.L., Gómez-Gascón, L., Galan-Relaño, A., Luque, I., Tarradas, C., Rodriguez-Ortega, M.J., Huerta, B., 2021. Antimicrobial susceptibility of cinnamon and red and common thyme essential oils and their main constituent compounds against *Streptococcus suis*. Lett. Appl. Microbiol. 74, 1–10. https://doi.org/10.1111/lam.13582

De Azeredo, G.A., Stamford, T.L.M., Nunes, P.C., Gomes Neto, N.J., De Oliveira, M.E.G., De Souza, E.L., 2011. Combined application of essential oils from *Origanum vulgare* L. and *Rosmarinus officinalis* L. to inhibit bacteria and autochthonous microflora associated with minimally processed vegetables. Food Res. Int. 44, 1541–1548. https://doi.org/10.1016/j.foodres.2011.04.012

De Martino, L., Nazzaro, F., Mancini, E., De Feo, V., 2015. Essential oils from

Mediterranean aromatic plants, in: The Mediterranean Diet: An Evidence-Based Approach. Elsevier, pp. 649–661. https://doi.org/10.1016/B978-0-12-407849-9.00058-0

De Rezende, R.A.E., Soares, M.P., Sampaio, F.G., Cardoso, I.L., Ishikawa, M.M., Lima Dallago, B.S., Rantin, F.T., Teixeira Duarte, M.C., 2021. Phytobiotics blend as a dietary supplement for *Nile tilapia* health improvement. Fish Shellfish Immunol. 114, 293–300. https://doi.org/10.1016/j.fsi.2021.05.010

De Sousa, J.P., De Azerêdo, G.A., De Araújo Torres, R., Da Silva Vasconcelos, M.A., Da Conceição, M.L., De Souza, E.L., 2012. Synergies of carvacrol and 1,8-cineole to inhibit bacteria associated with minimally processed vegetables. Int. J. Food Microbiol. 154, 145–151. https://doi.org/10.1016/j.ijfoodmicro.2011.12.026

De Souza, E.L., 2016. The effects of sublethal doses of essential oils and their constituents on antimicrobial susceptibility and antibiotic resistance among food-related bacteria: A review. Trends Food Sci. Technol. 56, 1–12. https://doi.org/10.1016/j.tifs.2016.07.012

Debonne, E., De Leyn, I., Verwaeren, J., Moens, S., Devlieghere, F., Eeckhout, M., Van Bockstaele, F., 2018a. The influence of natural oils of blackcurrant, black cumin seed, thyme and wheat germ on dough and bread technological and microbiological quality. Lwt-Food Sci. Technol. 93, 212–219. https://doi.org/10.1016/j.lwt.2018.03.041

Debonne, E., Van Bockstaele, F., De Leyn, I., Devlieghere, F., Eeckhout, M., 2018b. Validation of *in vitro* antifungal activity of thyme essential oil on *Aspergillus niger* and *Penicillium paneum* through application in par-baked wheat and sourdough bread. Lwt-Food Sci. Technol. 87, 368–378. https://doi.org/10.1016/j.lwt.2017.09.007

Debonne, E., Vermeulen, A., Van Bockstaele, F., Soljic, I., Eeckhout, M., Devlieghere, F., 2019. Growth/no-growth models of *in vitro* growth of *Penicillium paneum* as a function of thyme essential oil, pH, aw, temperature. Food Microbiol. 83, 9–17. https://doi.org/10.1016/j.fm.2019.04.003

Delgado, T., Marinero, P., Asensio-S.-Manzanera, M.C., Asensio, C., Herrero, B., Pereira, J.A., Ramalhosa, E., 2014. Antioxidant activity of twenty wild Spanish *Thymus mastichina* L. populations and its relation with their chemical composition. LWT-Food Sci. Technol. 57, 412–418. https://doi.org/10.1016/j.lwt.2013.12.041

Delgado-Adámez, J., Garrido, M., Bote, M.E., Fuentes-Pérez, M.C., Espino, J., Martín-Vertedor, D., 2017. Chemical composition and bioactivity of essential oils from flower and fruit of *Thymbra capitata* and *Thymus* species. J. Food Sci. Technol. 54, 1857–1865. https://doi.org/10.1007/s13197-017-2617-5

Devillers, J., Devillers, H., 2019. Toxicity profiling and prioritization of plant-derived antimalarial agents. SAR QSAR Environ. Res. 30, 801–824. https://doi.org/10.1080/1062936X.2019.1665844

Dhumal, C.V., Sarkar, P., 2018. Composite edible films and coatings from food-grade biopolymers. J. Food Sci. Technol. 55, 4369–4383. https://doi.org/10.1007/s13197-018-3402-9

Diánez, F., Santos, M., Parra, C., Navarro, M.J., Blanco, R., Gea, F.J., 2018. Screening of antifungal activity of 12 essential oils against eight pathogenic fungi of vegetables and mushroom. Lett. Appl. Microbiol. 67, 400–410. https://doi.org/10.1111/lam.13053

Djahafi, A., Taïbi, K., Abderrahim, L.A., 2021. Aromatic and medicinal plants used in traditional medicine in the region of Tiaret, North West of Algeria. Mediterr. Botan. 42, e71465. https://doi.org/10.5209/mbot.71465

Dolea, D., Rizo, A., Fuentes, A., Barat, J.M., Fernández-Segovia, I., 2018. Effect of thyme and oregano essential oils on the shelf life of salmon and seaweed burgers. Food Sci. Technol. Int. 24, 394–403. https://doi.org/10.1177/1082013218759364

Domingues, P.M., Santos, L., 2019. Essential oil of pennyroyal (*Mentha pulegium*): Composition and applications as alternatives to pesticides—New tendencies. Ind. Crops Prod. 139, 111534. https://doi.org/10.1016/j.indcrop.2019.111534

Donadu, M.G., Peralta-Ruiz, Y., Usai, D., Maggio, F., Molina-Hernandez, J.B., Rizzo, D., Bussu, F., Rubino, S., Zanetti, S., Paparella, A., Chaves-Lopez, C., 2021. Colombian essential oil of *Ruta graveolens* against nosocomial antifungal resistant *Candida* strains. J. Fungi. 7, 383. https://doi.org/10.3390/jof7050383

Dorman, H.J.D., Deans, S.G., 2004. Chemical composition, antimicrobial and *in vitro* antioxidant properties of *Monarda citriodora* var. *citriodora*, *Myristica fragrans*, *Origanum vulgare* ssp. *hirtum*, *Pelargonium* sp. and *Thymus zygis* oils. J. Essent. Oil Res. 16, 145–150. https://doi.org/10.1080/10412905.2004.9698679

Duarte, A., Alves, A.C., Ferreira, S., Silva, F., Domingues, F.C., 2015. Resveratrol inclusion complexes: Antibacterial and anti-biofilm activity against *Campylobacter* spp. and *Arcobacter butzleri*. Food Res. Int. 77, 244–250. https://doi.org/10.1016/j.foodres.2015.05.047

Duarte, A., Luís, Â., Oleastro, M., Domingues, F.C., 2016. Antioxidant properties of coriander essential oil and linalool and their potential to control *Campylobacter* spp. Food Control. 61, 115–122. https://doi.org/10.1016/j.foodcont.2015.09.033

Durán-Lara, E.F., Valderrama, A., Marican, A., 2020. Natural organic compounds for

application in organic farming. Agriculture. 10, 41. https://doi.org/10.3390/agriculture10020041

Dussault, D., Vu, K.D., Lacroix, M., 2014. *In vitro* evaluation of antimicrobial activities of various commercial essential oils, oleoresin and pure compounds against food pathogens and application in ham. Meat Sci. 96, 514–520. https://doi.org/10.1016/j.meatsci.2013.08.015

Duze, S.T., Marimani, M., Patel, M., 2021. Tolerance of *Listeria monocytogenes* to biocides used in food processing environments. Food Microbiol. 97, 103758. https://doi.org/10.1016/j.fm.2021.103758

El Aanachi, S., Gali, L., Rammali, S., Bensouici, C., Aassila, H., Dari, K., 2021. *In vitro* study of the antioxidant, photoprotective, anti-tyrosinase, and anti-urease effects of methanolic extracts from leaves of six Moroccan Lamiaceae. J. Food Meas. Charact. 15, 1785–1795. https://doi.org/10.1007/s11694-020-00759-9

El Azzouzi, F., Zidane, L., 2015. La flore médicinale traditionnelle de la région de Béni-Mellal (Maroc). J. Appl. Bios. 91, 8493. https://doi.org/10.4314/jab.v91i1.8

El Hafian, M., Benlandini, N., Elyacoubi, H., Zidane, L., Rochdi, A., 2014. Étude floristique et ethnobotanique des plantes médicinales utilisées au niveau de la préfecture d'Agadir-Ida-Outanane (Maroc). J. Appl. Bios. 81, 7198. https://doi.org/10.4314/jab.v81i1.8

El Hassani, F.Z., 2020. Characterization, activities, and ethnobotanical uses of *Mentha* species in Morocco. Heliyon. 6, e05480. https://doi.org/10.1016/j.heliyon.2020.e05480

El Yaagoubi, M., Mechqoq, H., El Hamdaoui, A., Jrv Mukku, V., El Mousadik, A., Msanda, F., El Aouad, N., 2021. A review on Moroccan *Thymus* species: Traditional uses, essential oils chemical composition and biological effects. J. Ethnopharmacol. 278, 114205. https://doi.org/10.1016/j.jep.2021.114205

El-Soud, N.A., El-Laithy, N., El-Saeed, G., Wahby, M.S., Khalil, M., Morsy, F., Shaffie, N., 2011. Antidiabetic activities of *Foeniculum vulgare* Mill. essential oil in streptozotocin-induced diabetic rats. Maced. J. Med. Sci. 4, 139–146. https://doi.org/10.3889/MJMS.1857-5773.2011.0173

Escobar, A., Pérez, M., Romanelli, G., Blustein, G., 2020. Thymol bioactivity: A review focusing on practical applications. Arabian J. Chem. 13, 9243–9269. https://doi.org/10.1016/j.arabjc.2020.11.009

Esquivel-Chávez, F., Colín-Chávez, C., Virgen-Ortiz, J.J., Martínez-Téllez, M.Á., Avena-Bustillos, R. de J., Peña-Madrigal, G., Miranda-Ackerman, M.A., 2021. Control of

mango decay using antifungal sachets containing of thyme oil/modified starch/agave fructans microcapsules. Future Foods. 3, 100008. https://doi.org/10.1016/j.fufo.2020.100008

European Commission, 2008. Regulation (EC) No 1334/2008 on flavourings. Official Journal of the European Union, 2008 L 354/34, 34–50.

European Pharmacopoeia, 10th ed, 2019. European Directorate for the Quality of Medicines & HealthCare of the Council of Europe, Strasbourg Cedex, France. https://doi.org/10.1136/bmj.4.5790.815-b

Faleiro, M.L., Miguel, M.G., Ladeiro, F., Venâncio, F., Tavares, R., Brito, J.C., Figueiredo, A.C., Barroso, J.G., Pedro, L.G., 2003. Antimicrobial activity of essential oils isolated from Portuguese endemic species of *Thymus*. Lett. Appl. Microbiol. 36, 35–40. https://doi.org/10.1046/j.1472-765X.2003.01259.x

Falowo, A.B., Fayemi, P.O., Muchenje, V., 2014. Natural antioxidants against lipid-protein oxidative deterioration in meat and meat products: A review. Food Res. Int. 64, 171–181. https://doi.org/10.1016/j.foodres.2014.06.022

Faria, J.M.S., Barbosa, P., Bennett, R.N., Mota, M., Figueiredo, A.C., 2013. Bioactivity against *Bursaphelenchus xylophilus*: Nematotoxics from essential oils, essential oils fractions and decoction waters. Phytochemistry. 94, 220–228. https://doi.org/10.1016/j.phytochem.2013.06.005

Faria, J.M.S., Rodrigues, A.M., Sena, I., Moiteiro, C., Bennett, R.N., Mota, M., Figueiredo, A.C., 2016a. Bioactivity of *Ruta graveolens* and *Satureja montana* essential oils on *Solanum tuberosum* hairy roots and *Solanum tuberosum* hairy roots with *Meloidogyne chitwoodi* co-cultures. J. Agric. Food Chem. 64, 7452–7458. https://doi.org/ 10.1021/acs.jafc.6b03279

Faria, J.M.S., Sena, I., Ribeiro, B., Rodrigues, A.M., Maleita, C.M.N., Abrantes, I., Bennett, R., Mota, M., Figueiredo, A.C. da S., 2016b. First report on *Meloidogyne chitwoodi* hatching inhibition activity of essential oils and essential oils fractions. J. Pest. Sci. 89, 207–217. https://doi.org/10.1007/s10340-015-0664-0

Farzaei, M.H., Shahpiri, Z., Bahramsoltani, R., nia, M.M., Najafi, F., Rahimi, R., 2017. Efficacy and tolerability of phytomedicines in Multiple Sclerosis patients: A review. CNS Drugs. 31, 867–889. https://doi.org/10.1007/s40263-017-0466-4

Fekete, E., Allain, T., Siddiq, A., Sosnowski, O., Buret, A.G., 2021. *Giardia* spp. and the gut microbiota: Dangerous liaisons. Front. Microbiol. 11, 618106. https://doi.org/10.3389/fmicb.2020.618106

Feng, W.Y., Tanaka, R., Inagaki, Y., Saitoh, Y., Chang, M.O., Amet, T., Yamamoto, N., Yamaoka, S., Yoshinaka, Y., 2008. Pycnogenol®, a procyanidin-rich extract from French maritime pine, inhibits intracellular replication of HIV-1 as well as its binding to host cells. Jpn. J. Infect. Dis. 61, 279–285.

Fernando, I.T., Perera, K.I., Athauda, S.B.P., Sivakanesan, R., Kumar, N.S., Jayasinghe, L., 2019. Heat stability of the *in vitro* inhibitory effect of spices on lipase, amylase, and glucosidase enzymes. Food Sci. Nutr. 7, 425–432. https://doi.org/10.1002/fsn3.797

Ferreira, S., Domingues, F., 2016. The antimicrobial action of resveratrol against *Listeria monocytogenes* in food-based models and its antibiofilm properties. J. Sci. Food Agric. 96, 4531–4535. https://doi.org/10.1002/jsfa.7669

Ferreira-Santos, P., Genisheva, Z., Botelho, C., Santos, J., Ramos, C., Teixeira, J.A., Rocha, C.M.R., 2020. Unravelling the biological potential of *Pinus pinaster* bark extracts. Antioxidants. 9, 1–21. https://doi.org/10.3390/antiox9040334

Figueiredo, A., Barroso, J., Pedro, L., Salgueiro, L., Miguel, M., Faleiro, M., 2008. Portuguese *Thymbra* and *Thymus* species volatiles: Chemical composition and biological activities. Curr. Pharm. Des. 14, 3120–3140. https://doi.org/10.2174/138161208786404218

Figueiredo, A.C., Barroso, J.G., Pedro, L.G., 2010. Volatiles from *Thymbra* and *Thymus* species of the Western Mediterranean basin, Portugal and Macaronesia. Nat. Prod. Commun. 5, 1465–1476. https://doi.org/10.1177/1934578x1000500924

Fkiri, S., Ghazghazi, H., Rigane, G., Ridha, B.S., Mezni, F., Khaldi, A., Khouja, M.L., Nasr, Z., 2019. Chemical compositions and biological activities essential oil from the needles of North African *Pinus pinaster* VAR. Rev. Roum. Chim. 64, 511–517. https://doi.org/10.33224/rrch.2019.64.6.07

Fratianni, F., De Martino, L., Melone, A., De Feo, V., Coppola, R., Nazzaro, F., 2010. Preservation of chicken breast meat treated with thyme and balm essential oils. J. Food Sci. 75, M528–M535. https://doi.org/10.1111/j.1750-3841.2010.01791.x

Froiio, F., Mosaddik, A., Morshed, M.T., Paolino, D., Fessi, H., Elaissari, A., 2019. Edible polymers for essential oils encapsulation: Application in food preservation. Ind. Eng. Chem. Res. 58, 20932–20945. https://doi.org/10.1021/acs.iecr.9b02418

Gahukar, R.T., 2018. Management of pests and diseases of important tropical/subtropical medicinal and aromatic plants: A review. J. Appl. Res. Med. Aromat. Plants. 9, 1–18. https://doi.org/10.1016/j.jarmap.2018.03.002

Gao, Z., Zhong, W., Chen, K., Tang, P., Guo, J., 2020. Chemical composition and anti-

biofilm activity of essential oil from *Citrus medica* L. var. *sarcodactylis* Swingle against *Listeria monocytogenes*. Ind. Crops Prod. 144, 112036. https://doi.org/10.1016/j.indcrop.2019.112036

Garcia-Oliveira, P., Barral, M., Carpena, M., Gullón, P., Fraga-Corral, M., Otero, P., Prieto, M.A., Simal-Gandara, J., 2021. Traditional plants from Asteraceae family as potential candidates for functional food industry. Food Funct. 12, 2850–2873. https://doi.org/10.1039/d0fo03433a

Gentile, M.T., Russo, R., Pastorino, O., Cioffi, S., Barbieri, F., Illingworth, E.A., Grieco, M., Chambery, A., Colucci-D'Amato, L., 2018. *Ruta graveolens* water extract inhibits cell-cell network formation in human umbilical endothelial cells via MEK-ERK1/2 pathway. Exp. Cell Res. 364, 50–58. https://doi.org/10.1016/j.yexcr.2018.01.025

Ghabraie, M., Vu, K.D., Tata, L., Salmieri, S., Lacroix, M., 2016. Antimicrobial effect of essential oils in combinations against five bacteria and their effect on sensorial quality of ground meat. Lwt-Food Sci. Technol. 66, 332–339. https://doi.org/10.1016/j.lwt.2015.10.055

Ghosh, S., Bishayee, K., Khuda-Bukhsh, A.R., 2014. Graveoline isolated from ethanolic extract of *Ruta graveolens* triggers apoptosis and autophagy in skin melanoma cells: A novel apoptosis-independent autophagic signaling pathway. Phytother Res. 28, 1153–1162. https://doi.org/10.1002/ptr.5107

Giacometti, F., Shirzad-Aski, H., Ferreira, S., 2021. Antimicrobials and food-related stresses as selective factors for antibiotic resistance along the farm to fork continuum. Antibiotics. 10, 671. https://doi.org/10.3390/antibiotics10060671

Giacometti, J., Kovačević, D.B., Putnik, P., Gabrić, D., Bilušić, T., Krešić, G., Stulić, V., Barba, F.J., Chemat, F., Barbosa-cánovas, G., Jambrak, A.R., 2018. Extraction of bioactive compounds and essential oils from mediterranean herbs by conventional and green innovative techniques : A review. Food Res. Int. 113, 245–262. https://doi.org/10.1016/j.foodres.2018.06.036

Gonçalves, E., Figueiredo, A.C., Barroso, J.G., Henriques, J., Sousa, E., Bonifácio, L., 2020. Effect of *Monochamus galloprovincialis* feeding on *Pinus pinaster* and *Pinus pinea*, oleoresin and insect volatiles. Phytochemistry. 169, 112159. https://doi.org/10.1016/j.phytochem.2019.112159

Gonçalves, M.J., Cruz, M.T., Cavaleiro, C., Lopes, M.C., Salgueiro, L., 2010. Chemical, antifungal and cytotoxic evaluation of the essential oil of *Thymus zygis* subsp. *sylvestris*. Ind. Crops Prod. 32, 70–75. https://doi.org/10.1016/j.indcrop.2010.03.005

Gong, H., He, L., Zhao, Z., Mao, X., Zhang, C., 2021. The specific effect of (R)-(+)-pulegone on growth and biofilm formation in multi-drug resistant *Escherichia coli* and molecular mechanisms underlying the expression of pgaABCD genes. Biomed. Pharmacother. 134, 111149. https://doi.org/10.1016/j.biopha.2020.111149

Gong, H., Li, W., Sun, J., Jia, L., Guan, Q., Guo, Y., Wang, Y., 2022. A review on plant polysaccharide based on drug delivery system for construction and application, with emphasis on traditional Chinese medicine polysaccharide. Int. J. Biol. Macromol. 211, 711–728. https://doi.org/10.1016/j.ijbiomac.2022.05.087

Gordo, J., Máximo, P., Cabrita, E., Lourenço, A., Oliva, A., Almeida, J., Filipe, M., Cruz, P., Barcia, R., Santos, M., Cruz, H., 2012. *Thymus mastichina*: Chemical constituents and their anti-cancer activity. Nat. Prod. Commun. 7, 1491–1494. https://doi.org/10.1177/1934578x1200701120

Gouveia, A.R., Alves, M., Silva, J.A., Saraiva, C., 2016. The antimicrobial effect of rosemary and thyme essential oils against *Listeria monocytogenes* in *sous vide* cook-chill beef during storage. Procedia Food Sci. 7, 173–176. https://doi.org/10.1016/j.profoo.2016.10.001

Grether-Beck, S., Marini, A., Thomas, J., Krutmann, J., 2016. French maritime pine bark extract (Pycnogenol®) effects on human skin: Clinical and molecular evidence. Skin Pharmacol. Physiol. 29, 13–17. https://doi.org/10.1159/000441039

Gülçin, İ., Gören, A.C., Taslimi, P., Alwasel, S.H., Kılıc, O., Bursal, E., 2020. Anticholinergic, antidiabetic and antioxidant activities of Anatolian pennyroyal (*Mentha pulegium*) - Analysis of its polyphenol contents by LC-MS/MS. Biocatal. Agric. Biotechnol. 23. https://doi.org/10.1016/j.bcab.2019.101441

Hachi, M., Ouafae, B., Hachi, T., Mohamed, E.B., Imane, B., Atmane, R., Zidane, L., 2016. Contribution to the ethnobotanical study of antidiabetic medicinal plants of the Central Middle Atlas region (Morocco). Lazaroa. 37, 135–144. https://doi.org/10.5209/LAZAROA.51854

Haddouchi, F., Chaouche, T.M., Ksouri, R., Medini, F., Sekkal, F.Z., Benmansour, A., 2014. Antioxidant activity profiling by spectrophotometric methods of aqueous methanolic extracts of *Helichrysum stoechas* subsp. *rupestre* and *Phagnalon saxatile* subsp. *saxatile*. Chin. J. Nat. Med. 12, 415–422. https://doi.org/10.1016/S1875-5364(14)60065-0

Haddouchi, F., Chaouche, T.M., Zaouali, Y., Ksouri, R., Attou, A., Benmansour, A., 2013. Chemical composition and antimicrobial activity of the essential oils from four *Ruta*

species growing in Algeria. Food Chem. 141, 253–258. https://doi.org/10.1016/j.foodchem.2013.03.007

Hale, A.L., Meepagala, K.M., Oliva, A., Aliotta, G., Duke, S.O., 2004. Phytotoxins from the leaves of *Ruta graveolens*. J. Agric. Food Chem. 52, 3345–3349. https://doi.org/10.1021/jf0497298

Hammami, I., Smaoui, S., Hsouna, A. Ben, Hamdi, N., Triki, M.A., 2015. *Ruta montana* L. leaf essential oil and extracts: Characterization of bioactive compounds and supression of Crown Gall disease. Excli J. 14, 83–94. https://doi.org/ 10.17179/excli2014-655

Hao, Y., Kang, J., Guo, X., Yang, R., Chen, Y., Li, J., Shi, L., 2021. Comparison of nutritional compositions and essential oil profiles of different parts of a dill and two fennel cultivars. Foods. 10, 1784. https://doi.org/10.3390/foods10081784

Harat, Z.N., Sadeghi, M.R., Sadeghipour, H.R., Kamalinejad, M., Eshraghian, M.R., 2008. Immobilization effect of *Ruta graveolens* L. on human sperm: A new hope for male contraception. J. Ethnopharmacol. 115, 36–41. https://doi.org/10.1016/j.jep.2007.09.004

Harati, K., Slodnik, P., Chromik, A.M., Behr, B., Goertz, O., Hirsch, T., Kapalschinski, N., Klein-Hitpass, L., Kolbenschlag, J., Uhl, W., Lehnhardt, M., Daigeler, A., 2015. Pro-apoptotic effects of pycnogenol on HT1080 human fibrosarcoma cells. Int. J. Oncol. 46, 1629–1636. https://doi.org/10.3892/ijo.2015.2854

Hassan, N.A., Alshamari, A.K., Hassan, A.A., Elharrif, M.G., Alhajri, A.M., Sattam, M., Khattab, R.R., 2022. Advances on therapeutic strategies for Alzheimer's disease: From medicinal plant to nanotechnology. Molecules. 27, 4839. https://doi.org/10.3390/molecules27154839

Herman, A., Herman, A.P., 2021. Herbal products and their active constituents used alone and in combination with antifungal drugs against drug-resistant *Candida* sp. Antibiotics. 10. https://doi.org/10.3390/antibiotics10060655

Hernández, A., García García, B., Jordán, M.J., Hernández, M.D., 2015. Study of the dose of thyme essential oil in feed to prolong the shelf life of gilthead seabream (*Sparus aurata*). Aquacult. Nutr. 21, 740–749. https://doi.org/10.1111/anu.12196

Hilfiger, L., Triaux, Z., Marcic, C., Héberlé, E., Emhemmed, F., Darbon, P., Marchioni, E., Petitjean, H., Charlet, A., 2021. Anti-hyperalgesic properties of menthol and pulegone. Front. Pharmacol. 12, 1–13. https://doi.org/10.3389/fphar.2021.753873

Hingston, P., Chen, J., Dhillon, B.K., Laing, C., Bertelli, C., Gannon, V., Tasara, T., Allen, K., Brinkman, F.S.L., Hansen, L.T., Wang, S., 2017. Genotypes associated with *Listeria*

monocytogenes isolates displaying impaired or enhanced tolerances to cold, salt, acid, or desiccation stress. Front. Microbiol. 8, 1–20. https://doi.org/10.3389/fmicb.2017.00369

Hmamouchi, M., Hamamouchi, J., Zouhdi, M., 2001. Chemical and antimicrobial properties of essential oils of five Moroccan Pinaceae. J. Essent. Oil Res. 13, 298–302. https://doi.org/10.1080/10412905.2001.9699699

Hochma, E., Yarmolinsky, L., Khalfin, B., Nisnevitch, M., Ben-Shabat, S., Nakonechny, F., 2021. Antimicrobial effect of phytochemicals from edible plants. Processes. 9, 2089. https://doi.org/10.3390/PR9112089

Huang, W.W., Yang, J.S., Lin, C.F., Ho, W.J., Lee, M.R., 2005. Pycnogenol induces differentiation and apoptosis in human promyeloid leukemia HL-60 cells. Leuk. Res. 29, 685–692. https://doi.org/10.1016/j.leukres.2004.10.006

Hwa-Jung Choi, 2018. Chemical constituents of essential oils possessing anti-Influenza A/WS/33 virus activity. Osong Public Health Res. Perspect. 3, 348–353.

Ibrahim, N., Moussa, A.Y., 2021. A comparative volatilomic characterization of Florence fennel from different locations: antiviral prospects. Food Funct. 12, 1498–1515. https://doi.org/10.1039/d0fo02897e

Idrees, M., Sawant, S., Karodia, N., Rahman, A., 2021. *Staphylococcus aureus* biofilm: Morphology, genetics, pathogenesis and treatment strategies. Int. J. Environ. Res. Public Health. 18, 7602. https://doi.org/10.3390/ijerph18147602

Ishrat, T., Parveen, K., Hoda, M.N., Khan, M.B., Yousuf, S., Ansari, M.A., Saleem, S., Islam, F., 2009. Effects of Pycnogenol and vitamin E on cognitive deficits and oxidative damage induced by intracerebroventricular streptozotocin in rats. Behav. Pharmacol. 20, 567–575. https://doi.org/10.1097/FBP.0b013e32832c7125

Jebali, J., Ghazghazi, H., Aouadhi, C., ELBini-Dhouib, I., Salem, R. Ben, Srairi-Abid, N., Marrakchi, N., Rigane, G., 2022. Tunisian native *Mentha pulegium* L. extracts: Phytochemical composition and biological activities. Molecules. 27, 314. https://doi.org/10.3390/ Molecules.27010314

Jianu, C., Goleţ, I., Stoin, D., Cocan, I., Bujancă, G., Mişcă, C., Mioc, M., Mioc, A., Şoica, C., Lukinich-gruia, A.T., Rusu, L.C., Muntean, D., Horhat, D.I., 2021. Chemical profile of *Ruta graveolens*, evaluation of the antioxidant and antibacterial potential of its essential oil, and molecular docking simulations. Appl. Sci. 11, 11753. https://doi.org/10.3390/app112411753

Jiao, M., Liu, X., Ren, Y., Wang, Y., Cheng, L., Liang, Y., Li, Y., Zhang, T., Wang, W., Mei,

Z., 2022. Comparison of herbal medicines used for women's menstruation diseases in different areas of the world. Front. Pharmacol. 12, 751207. https://doi.org/10.3389/fphar.2021.751207

Jones, I.A., Joshi, L.T., 2021. Biocide use in the antimicrobial era: A review. Molecules. 26, 2276. https://doi.org/10.3390/Molecules.26082276

Jordán, M.J., Martínez, R.M., Martínez, C., Moñino, I., Sotomayor, J.A., 2009. Polyphenolic extract and essential oil quality of *Thymus zygis* ssp. *gracilis* shrubs cultivated under different watering levels. Ind. Crops Prod. 29, 145–153. https://doi.org/10.1016/j.indcrop.2008.04.021

Ju, J., Xie, Y., Yu, H., Guo, Y., Cheng, Y., Qian, H., Yao, W., 2020. Synergistic interactions of plant essential oils with antimicrobial agents: A new antimicrobial therapy. Crit. Rev. Food Sci. Nutr. 62, 1740-1751. https://doi.org/10.1080/10408398.2020.1846494

Kachur, K., Suntres, Z., 2020. The antibacterial properties of phenolic isomers, carvacrol and thymol. Crit. Rev. Food Sci. Nutr. 60, 3042–3053. https://doi.org/10.1080/10408398.2019.1675585

Kakhki, M.T., Sedaghat, N., Mohsenzadeh, M., 2020. Chemical composition, antioxidative, antibacterial, and time-kill activities of some selected plant essential oils against foodborne pathogenic and spoilage organisms. Vet. Res. Forum. 11, 339–346. https://doi.org/10.30466/vrf.2018.91902.2223

Kala, C.P., 2015. Medicinal and aromatic plants: Boon for enterprise development. J. Appl. Res. Med. Aromat. Plants. 2, 134–139. https://doi.org/10.1016/j.jarmap.2015.05.002

Kamkar, A., Javan, A.J., Asadi, F., Kamalinejad, M., 2010. The antioxidative effect of Iranian *Mentha pulegium* extracts and essential oil in sunflower oil. Food Chem. Toxicol. 48, 1796–1800. https://doi.org/10.1016/j.fct.2010.04.003

Kamuren, Z.T., McPeek, C.G., Sanders, R.A., Watkins III, J.B., 2006. Effects of low-carbohydrate diet and Pycnogenol® treatment on retinal antioxidant enzymes in normal and diabetic rats. J. Ocul. Pharmacol. Ther. 22, 10–19.

Kang, J.-H., Song, K. Bin, 2019. Antibacterial activity of the noni fruit extract against *Listeria monocytogenes* and its applicability as a natural sanitizer for the washing of fresh-cut produce. Food Microbiol. 84, 103260. https://doi.org/10.1016/j.fm.2019.103260

Kannan, S., Balakrishnan, J., Govindasamy, A., 2020. *Listeria monocytogens* - Amended understanding of its pathogenesis with a complete picture of its membrane vesicles, quorum sensing, biofilm and invasion. Microb. Pathog. 149, 104575.

https://doi.org/10.1016/j.micpath.2020.104575

Karthika, C., Sureshkumar, R., 2021. Incorporation of natural assumption to deal with cancer. Environ. Sci. Pollut. Res. 28, 4902–4917. https://doi.org/10.1007/s11356-020-11479-4

Kaur, G.J., Arora, D.S., 2009. Antibacterial and phytochemical screening of *Anethum graveolens*, *Foeniculum vulgare* and *Trachyspermum ammi*. BMC Complementary Altern. Med. 9, 30. https://doi.org/10.1186/1472-6882-9-30

Kaye, K.S., Gales, A.C., Dubourg, G., 2017. Old antibiotics for multidrug-resistant pathogens: From *in vitro* activity to clinical outcomes. Int. J. Antimicrob. Agents. 49, 542–548. https://doi.org/10.1016/j.ijantimicag.2016.11.020

Khadivzadeh, T., Najafi, M.N., Kargarfard, L., Ghazanfarpour, M., Dizavandi, F.R., Khorsand, I., 2018. Effect of fennel on the health status of menopausal women: A systematic and meta-analysis. J. Menopausal Med. 24, 67. https://doi.org/10.6118/jmm.2018.24.1.67

Khaleil, M.M., Alnoman, M.M., Elrazik, E.S.A., Zagloul, H., Khalil, A.M.A., 2021. Essential oil of *Foeniculum vulgare* Mill. as a green fungicide and defense-inducing agent against fusarium root rot disease in *Vicia faba* L. Biology. 10, 696. https://doi.org/10.3390/biology10080696

Kherbache, A., Senator, A., Laouicha, S., Al-Zoubi, R.M., Bouriche, H., 2020. Phytochemical analysis, antioxidant and anti-inflammatory activities of *Helichrysum stoechas* (L.) Moench extracts. Biocatal. Agric. Biotechnol. 29, 1–8. https://doi.org/10.1016/j.bcab.2020.101826

Khori, V., Nayebpour, M., Semnani, S., Golalipour, M.J., Marjani, A., 2008. Prolongation of AV nodal refractoriness by *Ruta graveolens* in isolated rat hearts. Potential role as an anti-arrhythmic agent. Saudi Med. J. 29, 357–363.

Khouja, M., Alves, R.C., Melo, D., Costa, A.S.G., Nunes, M.A., Khaldi, A., Oliveira, M.B.P.P., Messaoud, C., 2021. Morphological and chemical differentiation between Tunisian populations of *Pinus halepensis*, *Pinus brutia*, and *Pinus pinaster*. Chem. Biodivers. 18, 14–17. https://doi.org/10.1002/cbdv.202100071

Kim, S.A., Jang, Y.J., Heo, J.E., Li, L., Moon, J.S., Han, N.S., 2018. Complete genome sequence of *Leuconostoc citreum* EFEL2700, a host strain for transformation of pCB vectors. J. Biotechnol. 287, 52–58. https://doi.org/10.1016/j.jbiotec.2018.08.008

Kim, S.-W., Lee, H.-R., Jang, M.-J., Jung, C.-S., Park, I.-K., 2016. Fumigant toxicity of Lamiaceae plant essential oils and blends of their constituents against adult rice weevil

Sitophilus oryzae. Molecules. 21, 1–10. https://doi.org/10.3390/molecules21030361

Koblovská, R., Macková, Z., Vítková, M., Kokoška, L., Klejdus, B., Lapčík, O., 2008. Isoflavones in the Rutaceae family: Twenty selected representatives of the genera *Citrus*, *Fortunella*, *Poncirus*, *Ruta* and *Severinia*. Phytochem. Anal. 19, 64–70. https://doi.org/10.1002/pca.1016

Kooti, W., Moradi, M., Ali-Akbari, S., Sharafi-Ahvazi, N., Asadi-Samani, M., Ashtary-Larky, D., 2015. Therapeutic and pharmacological potential of *Foeniculum vulgare* Mill: A review. J. HerbMed Pharmacol. 4, 1–9.

Korem, M., Gov, Y., Shirron, N., Shuster, A., Rosenberg, M., 2007. Alcohol increases hemolysis by staphylococci. FEMS Microbiol. Lett. 269, 153–159. https://doi.org/10.1111/j.1574-6968.2006.00625.x

Korinek, M., Handoussa, H., Tsai, Y.H., Chen, Y.Y., Chen, M.H., Chiou, Z.W., Fang, Y., Chang, F.R., Yen, C.H., Hsieh, C.F., Chen, B.H., El-Shazly, M., Hwang, T.L., 2021. Anti-inflammatory and antimicrobial volatile oils: Fennel and cumin inhibit neutrophilic inflammation via regulating calcium and MAPKs. Front. Pharmacol. 12, 1–20. https://doi.org/10.3389/fphar.2021.674095

Koss-Mikołajczyk, I., Todorovic, V., Sobajic, S., Mahajna, J., Gerić, M., Tur, J.A., Bartoszek, A., 2021. Natural products counteracting cardiotoxicity during cancer chemotherapy: The special case of doxorubicin, a comprehensive review. Int. J. Mol. Sci. 22, 10037. https://doi.org/10.3390/ijms221810037

Kostova, I., Ivanova, A., Mikhova, B., Klaiber, I., 1999. Alkaloids and coumarins from *Ruta graveolens*. Monatsh. Chem. 130, 703–707. https://doi.org/10.1007/PL00010251

Kutluk, I., Aslan, M., Orhan, I.E., Özçelik, B., 2018. Antibacterial, antifungal and antiviral bioactivities of selected *Helichrysum* species. S. Afr. J. Bot. 119, 252–257. https://doi.org/10.1016/j.sajb.2018.09.009

Lagha, R., Abdallah, F. Ben, AL-Sarhan, B.O., Al-Sodany, Y., 2019. Antibacterial and biofilm inhibitory activity of medicinal plant essential oils against *Escherichia coli* isolated from UTI patients. Molecules. 24, 1–12. https://doi.org/10.3390/molecules24061161

Langeveld, W.T., Veldhuizen, E.J.A., Burt, S.A., 2014. Synergy between essential oil components and antibiotics: A review. Crit. Rev. Microbiol. 40, 76–94. https://doi.org/10.3109/1040841X.2013.763219

Lans, C., Turner, N., Khan, T., Brauer, G., 2007. Ethnoveterinary medicines used to treat endoparasites and stomach problems in pigs and pets in British Columbia, Canada. Vet. Parasitol. 148, 325–340. https://doi.org/ 10.1016/j.vetpar.2007.06.014

Laquale, S., Candido, V., Avato, P., Argentieri, M.P., D'Addabbo, T., 2015. Essential oils as soil biofumigants for the control of the root-knot nematode *Meloidogyne incognita* on tomato. Ann. Appl. Biol. 167, 217–224. https://doi.org/ 10.1111/aab.12221

Leal, F., Coelho, A.C., Soriano, T., Alves, C., Matos, M., 2013. Fungicide activity of *Thymus mastichina* and *Mentha rotundifolia* in plants *in vitro*. J. Med. Food. 16, 273. https://doi.org/10.1089/jmf.2012.0164

Lee, G., Kim, Y., Kim, H., Beuchat, L.R., Ryu, J.H., 2018. Antimicrobial activities of gaseous essential oils against *Listeria monocytogenes* on a laboratory medium and radish sprouts. Int. J. Food Microbiol. 265, 49–54. https://doi.org/10.1016/j.ijfoodmicro.2017.11.001

Lee, H.W., Ang, L., Kim, E., Lee, M.S., 2021. Fennel (*Foeniculum vulgare* Miller) for the management of menopausal women's health: A systematic review and meta-analysis. Complement. Ther. Clin. Pract. 43, 101360. https://doi.org/10.1016/j.ctcp.2021.101360

Lee, J.H., Kim, Y.G., Park, J.G., Lee, J., 2017. Supercritical fluid extracts of *Moringa oleifera* and their unsaturated fatty acid components inhibit biofilm formation by *Staphylococcus aureus*. Food Control. 80, 74–82. https://doi.org/10.1016/j.foodcont.2017.04.035

Lee, K., Lee, J.H., Kim, S. Il, Cho, M.H., Lee, J., 2014. Anti-biofilm, anti-hemolysis, and anti-virulence activities of black pepper, cananga, myrrh oils, and nerolidol against *Staphylococcus aureus*. Appl. Microbiol. Biotechnol. 98, 9447–9457. https://doi.org/10.1007/s00253-014-5903-4

Lee, S., Kim, H., Beuchat, L.R., Kim, Y., Ryu, J.H., 2020. Synergistic antimicrobial activity of oregano and thyme thymol essential oils against *Leuconostoc citreum* in a laboratory medium and tomato juice. Food Microbiol. 90, 103489. https://doi.org/10.1016/j.fm.2020.103489

Lemon, K.P., Freitag, N.E., Kolter, R., 2010. The virulence regulator PrfA promotes biofilm formation by *Listeria monocytogenes*. J. Bacteriol. 192, 3969–3976. https://doi.org/10.1128/JB.00179-10

Les, F., Venditti, A., Cásedas, G., Frezza, C., Guiso, M., Sciubba, F., Serafini, M., Bianco, A., Valero, M.S., López, V., 2017. Everlasting flower (*Helichrysum stoechas* Moench) as a

potential source of bioactive molecules with antiproliferative, antioxidant, antidiabetic and neuroprotective properties. Ind. Crops Prod. 108, 295–302. https://doi.org/10.1016/j.indcrop.2017.06.043

Liao, X., Ma, Y., Daliri, E.B.M., Koseki, S., Wei, S., Liu, D., Ye, X., Chen, S., Ding, T., 2020. Interplay of antibiotic resistance and food-associated stress tolerance in foodborne pathogens. Trends Food Sci. Technol. 95, 97–106. https://doi.org/10.1016/j.tifs.2019.11.006

Lomonaco, S., Nucera, D., Filipello, V., 2015. The evolution and epidemiology of *Listeria monocytogenes* in Europe and the United States. Infect., Genet. Evol. 35, 172–183. https://doi.org/10.1016/j.meegid.2015.08.008

Lourens, A.C.U., Viljoen, A.M., Van Heerden, F.R., 2008. South African *Helichrysum* species: A review of the traditional uses, biological activity and phytochemistry. J. Ethnopharmacol. 119, 630–652. https://doi.org/10.1016/j.jep.2008.06.011

Lucera, A., Costa, C., Conte, A., Del Nobile, M.A., 2012. Food applications of natural antimicrobial compounds. Front. Microbiol. 3, 1–13. https://doi.org/10.3389/fmicb.2012.00287

Luís, Â., Domingues, F., 2021. Screening of the potential bioactivities of pennyroyal (*Mentha pulegium* L.) essential oil. Antibiotics. 10, 1–12. https://doi.org/10.3390/antibiotics10101266

Luís, Â., Duarte, A.P., Pereira, L., Domingues, F., 2017. Chemical profiling and evaluation of antioxidant and anti-microbial properties of selected commercial essential oils: A comparative study. Medicines. 4, 36. https://doi.org/10.3390/medicines4020036

Luís, Â., Silva, F., Sousa, S., Duarte, A.P., Domingues, F., 2014. Antistaphylococcal and biofilm inhibitory activities of gallic, caffeic, and chlorogenic acids. Biofouling. 30, 69–79. https://doi.org/10.1080/08927014.2013.845878

Luís, Â., Sousa, S., Wackerlig, J., Dobusch, D., Duarte, A.P., Pereira, L., Domingues, F., 2019. Star anise (*Illicium verum* Hook. f.) essential oil: Antioxidant properties and antibacterial activity against *Acinetobacter baumannii*. Flavour Fragrance J. 34, 1–12. https://doi.org/10.1002/ffj.3498

Luz, I. da S., Neto, N.J.G., Tavares, A.G., Magnani, M., de Souza, E.L., 2012. Exposure of *Listeria monocytogenes* to sublethal amounts of *Origanum vulgare* L. essential oil or carvacrol in a food-based medium does not induce direct or cross protection. Food Res. Int. 48, 667–672. https://doi.org/10.1016/j.foodres.2012.05.026

Machado, M., Dinis, A.M., Salgueiro, L., Cavaleiro, C., Custódio, J.B.A., Do Céu Sousa, M.,

2010. Anti-*Giardia* activity of phenolic-rich essential oils: Effects of *Thymbra capitata*, *Origanum virens*, *Thymus zygis* subsp. *sylvestris*, and *Lippia graveolens* on trophozoites growth, viability, adherence, and ultrastructure. Parasitol. Res. 106, 1205–1215. https://doi.org/10.1007/s00436-010-1800-7

Machado, T.F., Nogueira, N.A.P., Pereira, R. de C.A., de Sousa, C.T., Batista, V.C.V., 2014. The antimicrobial efficacy of *Lippia alba* essential oil and its interaction with food ingredients. Braz. J. Microbiol. 45, 699–705. https://doi.org/10.1590/S1517-83822014000200045

Machado, T.Q., Felisberto, J.R.S., Guimarães, E.F., De Queiroz, G.A., Da Fonseca, A.C.C. da, Ramos, Y.J., Marques, A.M., Moreira, D.D.L., Robbs, B.K., 2021. Apoptotic effect of β-pinene on oral squamous cell carcinoma as one of the major compounds from essential oil of medicinal plant *Piper rivinoides* Kunth. Nat. Prod. Res, 1–5. https://doi.org/10.1080/14786419.2021.1895148

Mahady, G.B., Pendland, S.L., Stoia, A., Hamill, F.A., Fabricant, D., Dietz, B.M., Chadwick, L.R., 2005. *In vitro* susceptibility of *Helicobacter pylori* to botanical extracts used traditionally for the treatment of gastrointestinal disorders. Phytother. Res. 19, 988–991. https://doi.org/10.1002/ptr.1776

Mahmoud, E.A., Elansary, H.O., El-Ansary, D.O.D., Al-Mana, F.A., 2020. Elevated bioactivity of *Ruta graveolens* against cancer cells and microbes using seaweeds. Processes. 8, 1–15. https://doi.org/10.3390/pr8010075

Maimoona, A., Naeem, I., Saddiqe, Z., Jameel, K., 2011. A review on biological, nutraceutical and clinical aspects of French maritime pine bark extract. J. Ethnopharmacol. 133, 261–277. https://doi.org/10.1016/j.jep.2010.10.041

Mancuso, G., Borgonovo, G., Scaglioni, L., Bassoli, A., 2015. Phytochemicals from *Ruta graveolens* activate TAS2R bitter taste receptors and TRP channels involved in gustation and nociception. Molecules. 20, 18907–18922. https://doi.org/10.3390/molecules201018907

Mandras, N., Roana, J., Scalas, D., Del Re, S., Cavallo, L., Ghisetti, V., Tullio, V., 2021. The inhibition of non-albicans candida species and uncommon yeast pathogens by selected essential oils and their major compounds. Molecules. 26, 4937. https://doi.org/10.3390/Molecules.26164937

Marchese, A., Orhan, I.E., Daglia, M., Barbieri, R., Di Lorenzo, A., Nabavi, S.F., Gortzi, O., Izadi, M., Nabavi, S.M., 2016. Antibacterial and antifungal activities of thymol: A brief review of the literature. Food Chem. 210, 402–414. https://doi.org/10.1016/j.foodchem.2016.04.111

Marini, E., Magi, G., Ferretti, G., Bacchetti, T., Giuliani, A., Pugnaloni, A., Rippo, M.R., Facinelli, B., 2018. Attenuation of *Listeria monocytogenes* virulence by *Cannabis sativa* L. essential oil. Front. Cell. Infect. Microbiol. 8, 1–11. https://doi.org/10.3389/fcimb.2018.00293

Marinković, J., Ćulafić, D.M., Nikolić, B., Đukanović, S., Marković, T., Tasić, G., Ćirić, A., Marković, D., 2020. Antimicrobial potential of irrigants based on essential oils of *Cymbopogon martinii* and *Thymus zygis* towards *in vitro* multispecies biofilm cultured in *ex vivo* root canals. Arch. Oral Biol. 117, 104842. https://doi.org/10.1016/j.archoralbio.2020.104842

Martínez-Graciá, C., González-Bermúdez, C.A., Cabellero-Valcárcel, A.M., Santaella-Pascual, M., Frontela-Saseta, C., 2015. Use of herbs and spices for food preservation: Advantages and limitations. Curr. Opin. Food Sci. 6, 38–43. https://doi.org/10.1016/j.cofs.2015.11.011

Martins, M.R., Tinoco, M.T., Almeida, A.S., 2012. Chemical composition, antioxidant and antimicrobial properties of three essential oils from portuguese flora. J. Pharmacogn. 3, 39–44.

Matereke, L.T., Okoh, A.I., 2020. *Listeria monocytogenes* virulence, antimicrobial resistance and environmental persistence: A review. Pathogens. 9, 1–12. https://doi.org/10.3390/pathogens9070528

Matle, I., Mbatha, K.R., Lentsoane, O., Magwedere, K., Morey, L., Madoroba, E., 2019. Occurrence, serotypes, and characteristics of *Listeria monocytogenes* in meat and meat products in South Africa between 2014 and 2016. J. Food Saf. 39, 1–14. https://doi.org/10.1111/jfs.12629

Meade, E., Slattery, M.A., Garvey, M., 2021. Biocidal resistance in clinically relevant microbial species: A major public health risk. Pathogens. 10, 598. https://doi.org/10.3390/pathogens10050598

Melo, G.F. do A., da Costa, A.C.V., Garino Junior, F., Medeiros, R.S., Madruga, M.S., Neto, V.Q., 2013. The sensitivity of bacterial foodborne pathogens to *Croton blanchetianus* Baill essential oil. Braz. J. Microbiol. 44, 1189–1194. https://doi.org/10.1590/S1517-83822014005000009

Meloni, Marilena, Reid, A., Conti, E., 2013. Characterization of microsatellites for the endangered *Ruta oreojasme* (Rutaceae) and cross-amplification in related species. Appl. Plant Sci. 1, 1200347. https://doi.org/10.3732/apps.1200347

Mesfin, M., Asres, K., Shibeshi, W., 2014. Evaluation of anxiolytic activity of the essential

oil of the aerial part of *Foeniculum vulgare* Miller in mice. BMC Complementary Altern. Med. 14, 1–7. https://doi.org/10.1186/1472-6882-14-310

Miao, X., Liu, H., Zheng, Y., Guo, D., Shi, C., Xu, Y., Xia, X., 2019. Inhibitory effect of thymoquinone on *Listeria monocytogenes* ATCC 19115 biofilm formation and virulence attributes critical for human infection. Front. Cell. Infect. Microbiol. 9, 304. https://doi.org/10.3389/fcimb.2019.00304

Miara, M.D., Bendif, H., Rebbas, K., Rabah, B., Hammou, M.A., Maggi, F., 2019. Medicinal plants and their traditional uses in the highland region of Bordj Bou Arreridj (Northeast Algeria). J. Herb. Med. 16, 100262. https://doi.org/10.1016/j.hermed.2019.100262

Miguel, G., Simões, M., Figueiredo, A.C., Barroso, J.G., Pedro, L.G., Carvalho, L., 2004. Composition and antioxidant activities of the essential oils of *Thymus caespititius*, *Thymus camphoratus* and *Thymus mastichina*. Food Chem. 86, 183–188. https://doi.org/10.1016/j.foodchem.2003.08.031

Mitra, S., Prova, S.R., Sultana, S.A., Das, R., Nainu, F., Emran, T. Bin, Tareq, A.M., Uddin, M.S., Alqahtani, A.M., Dhama, K., Simal-Gandara, J., 2021. Therapeutic potential of indole alkaloids in respiratory diseases: A comprehensive review. Phytomedicine. 90, 153649. https://doi.org/10.1016/j.phymed.2021.153649

Mittal, R.P., Rana, A., Jaitak, V., 2018. Essential oils: An impending substitute of synthetic antimicrobial agents to overcome antimicrobial resistance. Curr. Drug Targets. 20, 605–624. https://doi.org/10.2174/1389450119666181031122917

Moghaddam, M., Taheri, P., Pirbalouti, A.G., Mehdizadeh, L., 2015. Chemical composition and antifungal activity of essential oil from the seed of *Echinophora platyloba* DC. against phytopathogens fungi by two different screening methods. LWT-Food Sci. Technol. 61, 536–542. https://doi.org/10.1016/j.lwt.2014.12.008

Molinos, A.C., Abriouel, H., López, R.L., Omar, N. Ben, Valdivia, E., Gálvez, A., 2009. Enhanced bactericidal activity of enterocin AS-48 in combination with essential oils, natural bioactive compounds and chemical preservatives against *Listeria monocytogenes* in ready-to-eat salad. Food Chem. Toxicol. 47, 2216–2223. https://doi.org/10.1016/j.fct.2009.06.012

Moore-Neibel, K., Gerber, C., Patel, J., Friedman, M., Jaroni, D., Ravishankar, S., 2013. Antimicrobial activity of oregano oil against antibiotic-resistant *Salmonella enterica* on organic leafy greens at varying exposure times and storage temperatures. Food Microbiol. 34, 123–129. https://doi.org/10.1016/j.fm.2012.12.001

Morton, C., Telmer, C., 2014. New subfamily classification for the Rutaceae. Ann. Mo. Bot. Gard. 99, 620–641. https://doi.org/ 10.3417/2010034

Munekata, P.E.S., Pateiro, M., Rodríguez-Lázaro, D., Domínguez, R., Zhong, J., Lorenzo, J.M., 2020. The role of essential oils against pathogenic *Escherichia coli* in food products. Microorganisms. 8, 1–16. https://doi.org/10.3390/microorganisms8060924

Murray, C.J., Ikuta, K.S., Sharara, F., Swetschinski, L., Robles Aguilar, G., Gray, A., Han, C., Bisignano, C., Rao, P., Wool, E., Johnson, S.C., Browne, A.J., Chipeta, M.G., Fell, F., Hackett, S., Haines-Woodhouse, G., Kashef Hamadani, B.H., Kumaran, E.A.P., McManigal, B., Agarwal, R., Akech, S., Albertson, S., Amuasi, J., Andrews, J., Aravkin, A., Ashley, E., Bailey, F., Baker, S., Basnyat, B., Bekker, A., Bender, R., Bethou, A., Bielicki, J., Boonkasidecha, S., Bukosia, J., Carvalheiro, C., Castañeda-Orjuela, C., Chansamouth, V., Chaurasia, S., Chiurchiù, S., Chowdhury, F., Cook, A.J., Cooper, B., Cressey, T.R., Criollo-Mora, E., Cunningham, M., Darboe, S., Day, N.P.J., De Luca, M., Dokova, K., Dramowski, A., Dunachie, S.J., Eckmanns, T., Eibach, D., Emami, A., Feasey, N., Fisher-Pearson, N., Forrest, K., Garrett, D., Gastmeier, P., Giref, A.Z., Greer, R.C., Gupta, V., Haller, S., Haselbeck, A., Hay, S.I., Holm, M., Hopkins, S., Iregbu, K.C., Jacobs, J., Jarovsky, D., Javanmardi, F., Khorana, M., Kissoon, N., Kobeissi, E., Kostyanev, T., Krapp, F., Krumkamp, R., Kumar, A., Kyu, H.H., Lim, C., Limmathurotsakul, D., Loftus, M.J., Lunn, M., Ma, J., Mturi, N., Munera-Huertas, T., Musicha, P., Mussi-Pinhata, M.M., Nakamura, T., Nanavati, R., Nangia, S., Newton, P., Ngoun, C., Novotney, A., Nwakanma, D., Obiero, C.W., Olivas-Martinez, A., Olliaro, P., Ooko, E., Ortiz-Brizuela, E., Peleg, A.Y., Perrone, C., Plakkal, N., Ponce-de-Leon, A., Raad, M., Ramdin, T., Riddell, A., Roberts, T., Robotham, J.V., Roca, A., Rudd, K.E., Russell, N., Schnall, J., Scott, J.A.G., Shivamallappa, M., Sifuentes-Osornio, J., Steenkeste, N., Stewardson, A.J., Stoeva, T., Tasak, N., Thaiprakong, A., Thwaites, G., Turner, C., Turner, P., van Doorn, H.R., Velaphi, S., Vongpradith, A., Vu, H., Walsh, T., Waner, S., Wangrangsimakul, T., Wozniak, T., Zheng, P., Sartorius, B., Lopez, A.D., Stergachis, A., Moore, C., Dolecek, C., Naghavi, M., 2022. Global burden of bacterial antimicrobial resistance in 2019: A systematic analysis. Lancet. 399, 629–655. https://doi.org/10.1016/S0140-6736(21)02724-0

Nahar, L., El-Seedi, H.R., Khalifa, S.A.M., Mohammadhosseini, M., Sarker, S.D., 2021. *Ruta* essential oils: Composition and bioactivities, Molecules. 26, 4766. https://doi.org/10.3390/molecules26164766

Naoi, M., Maruyama, W., Shamoto-Nagai, M., 2022. Disease-modifying treatment of Parkinson's disease by phytochemicals: Targeting multiple pathogenic factors. J.

Neural Transm. 129, 737–753. https://doi.org/10.1007/s00702-021-02427-8

Nazzaro, F., Fratianni, F., Coppola, R., De Feo, V., 2017. Essential oils and antifungal activity. Pharmaceuticals. 10, 1–20. https://doi.org/10.3390/ph10040086

Neto, N.J.G., Luz, I.S., Honório, W.G., Tavares, A.G., de Souza, E.L., 2012. *Rosmarinus officinalis* L. essential oil and the related compound 1,8-cineole do not induce direct or cross-protection in *Listeria monocytogenes* ATCC 7644 cultivated in meat broth. Can. J. Microbiol. 58, 973–981. https://doi.org/10.1139/W2012-070

NicAogáin, K., O'Byrne, C.P., 2016. The role of stress and stress adaptations in determining the fate of the bacterial pathogen *Listeria monocytogenes* in the food chain. Front. Microbiol. 7, 1–16. https://doi.org/10.3389/fmicb.2016.01865

Nieto, G., Díaz, P., Bañón, S., Garrido, M.D., 2010. Effect on lamb meat quality of including thyme (*Thymus zygis* ssp. *gracilis*) leaves in ewes' diet. Meat Sci. 85, 82–88. https://doi.org/10.1016/j.meatsci.2009.12.009

Nogueira, J.C.R., Diniz, M. de F.M., Lima, E.O., 2008. *In vitro* antimicrobial activity of plants in Acute Otitis Externa. Braz. J. Otorhinolar. 74, 118–124. https://doi.org/10.1016/S1808-8694(15)30761-8

Ogawara, H., 2019. Comparison of antibiotic resistance mechanisms in antibiotic-producing and pathogenic bacteria. Molecules. 24, 1–55. https://doi.org/10.3390/Molecules.24193430

Olaimat, A.N., Al-Holy, M.A., Shahbaz, H.M., Al-Nabulsi, A.A., Abu Ghoush, M.H., Osaili, T.M., Ayyash, M.M., Holley, R.A., 2018. Emergence of antibiotic resistance in *Listeria monocytogenes* isolated from food products: A comprehensive review. Compr. Rev. Food Sci. Food Saf. 17, 1277–1292. https://doi.org/10.1111/1541-4337.12387

Oliva, A., Garzoli, S., Sabatino, M., Tadić, V., Costantini, S., Ragno, R., Božović, M., 2020. Chemical composition and antimicrobial activity of essential oil of *Helichrysum italicum* (Roth) G. Don fil. (Asteraceae) from Montenegro. Nat. Prod. Res. 34, 445–448. https://doi.org/10.1080/14786419.2018.1538218

Oliveira, A.R., Domingues, F.C., Ferreira, S., 2017. The influence of resveratrol adaptation on resistance to antibiotics, benzalkonium chloride, heat and acid stresses of *Staphylococcus aureus* and *Listeria monocytogenes*. Food Control. 73, 1420–1425. https://doi.org/10.1016/j.foodcont.2016.11.011

Orhan, N., Onaran, M., Şen, I., Işik Gönül, I., Aslan, M., 2015. Preventive treatment of calcium oxalate crystal deposition with immortal flowers. J. Ethnopharmacol. 163, 60–67. https://doi.org/10.1016/j.jep.2015.01.009

Orlanda, J.F.F., Nascimento, A.R., 2015. Chemical composition and antibacterial activity of *Ruta graveolens* L. (Rutaceae) volatile oils, from São Luís, Maranhão, Brazil. South Afr. J. Bot. 99, 103–106. https://doi.org/ 10.1016/j.sajb.2015.03.198

Ornano, L., Venditti, A., Sanna, C., Ballero, M., Maggi, F., Lupidi, G., Bramucci, M., Quassinti, L., Bianco, A., 2015. Chemical composition and biological activity of the essential oil from *Helichrysum microphyllum* Cambess. ssp. *tyrrhenicum* Bacch., Brullo e Giusso growing in La Maddalena Archipelago, Sardinia. J. Oleo Sci. 64, 19–26. https://doi.org/10.5650/jos.ess14171

Ortega-Ramirez, L.A., Silva-Espinoza, B.A., Vargas-Arispuro, I., Gonzalez-Aguilar, G.A., Cruz-Valenzuela, M.R., Nazzaro, F., Ayala-Zavala, J.F., 2016. Combination of *Cymbopogon citrates* and *Allium cepa* essential oils increased antibacterial activity in leafy vegetables. J. Sci. Food Agric. 97, 2166–2173. https://doi.org/10.1002/jsfa.8025

Ostad, S.N., Soodi, M., Shariffzadeh, M., Khorshidi, N., Marzban, H., 2001. The effect of fennel essential oil on uterine contraction as a model for dysmenorrhea, pharmacology and toxicology study. J. Ethnopharmacol. 76, 299–304. https://doi.org/10.1016/S0378-8741(01)00249-5

Otto, M., 2014. *Staphylococcus aureus* toxins. Curr. Opin. Microbiol. 17, 32–37. https://doi.org/10.1016/j.mib.2013.11.004

Oviedo-Sarmiento, J.S., Bustos Cortes, J.J., Delgado Ávila, W.A., Cuca Suárez, L.E., Herrera Daza, E., Patiño-Ladino, O.J., Prieto-Rodríguez, J.A., 2021. Fumigant toxicity and biochemical effects of selected essential oils toward the red flour beetle, *Tribolium castaneum* (Coleoptera: Tenebrionidae). Pestic. Biochem. Physiol. 179, 104941. https://doi.org/10.1016/j.pestbp.2021.104941

Owen, L., Laird, K., 2018. Synchronous application of antibiotics and essential oils: Dual mechanisms of action as a potential solution to antibiotic resistance. Crit. Rev. Microbiol. 44, 414–435. https://doi.org/10.1080/1040841X.2018.1423616

Owlia, P., Saderi, H., Rasooli, I., Sefidkon, F., 2009. Antimicrobial characteristics of some herbal oils on *Pseudomonas aeruginosa* with special reference to their chemical compositions. Iran. J. Pharm. Res. 8, 107–114.

Özbek, H., Uğraş, S., Dülger, H., Bayram, I., Tuncer, I., Öztürk, G., Öztürk, A., 2003. Hepatoprotective effect of *Foeniculum vulgare* essential oil. Fitoterapia. 74, 317–319. https://doi.org/10.1016/S0367-326X(03)00028-5

Palaniappan, K., Holley, R.A., 2010. Use of natural antimicrobials to increase antibiotic susceptibility of drug resistant bacteria. Int. J. Food Microbiol. 140, 164–168.

https://doi.org/10.1016/j.ijfoodmicro.2010.04.001

Park, C.G., Jang, M., Yoon, K.A., Kim, J., 2016. Insecticidal and acetylcholinesterase inhibitory activities of Lamiaceae plant essential oils and their major components against *Drosophila suzukii* (Diptera: Drosophilidae). Ind. Crops Prod. 89, 507–513. https://doi.org/10.1016/j.indcrop.2016.06.008

Pateiro, M., Munekata, P.E.S., Sant'Ana, A.S., Domínguez, R., Rodríguez-Lázaro, D., Lorenzo, J.M., 2021. Application of essential oils as antimicrobial agents against spoilage and pathogenic microorganisms in meat products. Int. J. Food Microbiol. 337, 108966. https://doi.org/10.1016/j.ijfoodmicro.2020.108966

Pavela, R., 2005. Insecticidal activity of some essential oils against larvae of *Spodoptera littoralis*. Fitoterapia. 76, 691–696. https://doi.org/10.1016/j.fitote.2005.06.001

Pavela, R., 2008. Insecticidal properties of several essential oils on the house fly (*Musca domestica* L.). Phytother. Res. 22, 274–278. https://doi.org/10.1002/ptr.2300

Peñalver, P., Huerta, B., Borge, C., Astorga, R., Romero, R., Perea, A., 2005. Antimicrobial activity of five essential oils against origin strains of the *Enterobacteriaceae* family. Apmis. 113, 1–6. https://doi.org/10.1111/j.1600-0463.2005.apm1130101.x

Perera, A.G.W.U., Karunaratne, M.M.S.C., Chinthaka, S.D.M., 2022. Prolonged repellent activity of *Ruta graveolens* essential oil adsorbed on different mineral matrices against *Sitophilus zeamais* (L.) (Coleoptera: Curculionidae). J. Stored Prod. Res. 97, 101976. https://doi.org/10.1016/j.jspr.2022.101976

Pérez-Sánchez, R., Infante, F., Gálvez, C., Ubera, J.L., 2007. Fungitoxic activity against phytopathogenic fungi and the chemical composition of *Thymus zygis* essential oils. Food Sci. Technol. Int. 13, 341–347. https://doi.org/10.1177/1082013207085687

Perricone, M., Arace, E., Corbo, M.R., Sinigaglia, M., Bevilacqua, A., 2015. Bioactivity of essential oils: A review on their interaction with food components. Front. Microbiol. 6, 1–7. https://doi.org/10.3389/fmicb.2015.00076

Perumal, A.B., Huang, L., Nambiar, R.B., He, Y., Li, X., Sellamuthu, P.S., 2022. Application of essential oils in packaging films for the preservation of fruits and vegetables: A review. Food Chem. 375, 131810. https://doi.org/10.1016/j.foodchem.2021.131810

Pina-Vaz, C., Rodrigues, A.G., Pinto, E., Costa-de-Oliveira, S., Tavares, C., Salgueiro, L., Cavaleiro, C., Gonçalves, M., Martinez-de-Oliveira, J., 2004. Antifungal activity of *Thymus* oils and their major compounds. J. Eur. Acad. Dermatol. Venereol. 18, 73–8. https://doi.org/10.1111/j.1468-3083.2004.00886.x

Piras, A., Porcedda, S., Falconieri, D., Maxia, A., Gonçalves, M.J., Cavaleiro, C., Salgueiro,

L., 2019. Antifungal activity of essential oil from *Mentha spicata* L. and *Mentha pulegium* L. growing wild in Sardinia island (Italy). Nat. Prod. Res. 35, 993-999. https://doi.org/10.1080/14786419.2019.1610755

Poimenidou, S. V., Bikouli, V.C., Gardeli, C., Mitsi, C., Tarantilis, P.A., Nychas, G.J., Skandamis, P.N., 2016. Effect of single or combined chemical and natural antimicrobial interventions on *Escherichia coli* O157:H7, total microbiota and color of packaged spinach and lettuce. Int. J. Food Microbiol. 220, 6–18. https://doi.org/10.1016/j.ijfoodmicro.2015.12.013

Pollio, A., De Natale, A., Appetiti, E., Aliotta, G., Touwaide, A., 2008. Continuity and change in the Mediterranean medical tradition: *Ruta* spp. (Rutaceae) in Hippocratic medicine and present practices. J. Ethnopharmacol. 116, 469–482. https://doi.org/10.1016/j.jep.2007.12.013

Qiu, J., Li, H., Su, H., Dong, J., Luo, M., Wang, J., Leng, B., Deng, Y., Liu, J., Deng, X., 2012. Chemical composition of fennel essential oil and its impact on *Staphylococcus aureus* exotoxin production. World J. Microbiol. Biotechnol. 28, 1399–1405. https://doi.org/10.1007/s11274-011-0939-4

Quave, C.L., Horswill, A.R., 2014. Flipping the switch: Tools for detecting small molecule inhibitors of staphylococcal virulence. Front. Microbiol. 5, 1–10. https://doi.org/10.3389/fmicb.2014.00706

Rahimi, R., Ardekani, M.R.S., 2013. Medicinal properties of *Foeniculum vulgare* Mill. in traditional Iranian medicine and modern phytotherapy. Chin. J. Integr. Med. 19, 73–79. https://doi.org/10.1007/s11655-013-1327-0

Rahman, M., Alam, M.U., Luies, S.K., Kamal, A., Ferdous, S., Lin, A., Sharior, F., Khan, R., Rahman, Z., Parvez, S.M., Amin, N., Hasan, R., Tadesse, B.T., Taneja, N., Islam, M.A., Ercumen, A., 2022. Contamination of fresh produce with antibiotic-resistant bacteria and associated risks to human health: A scoping review. Int. J. Environ. Res. Public Health. 19, 1–15. https://doi.org/10.3390/ijerph19010360

Ranjbar, R., Halaji, M., 2018. Epidemiology of *Listeria monocytogenes* prevalence in foods, animals and human origin from Iran: A systematic review and meta-analysis. BMC Public Health. 18, 1057. https://doi.org/10.1186/s12889-018-5966-8

Rao, J., Chen, B., Mcclements, D.J., 2019. Improving the efficacy of essential oils as antimicrobials in foods: Mechanisms of action. Annu. Rev. Food Sci. Technol. 10, 365–387. https://doi.org/10.1146/annurev-food-032818-121727

Ratheesh, M., Shyni, G.L., Helen, A., 2009. Methanolic extract of *Ruta graveolens* L. inhibits inflammation and oxidative stress in adjuvant induced model of arthritis in rats. Inflammopharmacology. 17, 100–105. https://doi.org/10.1007/s10787-009-8044-0

Rather, M.A., Dar, B.A., Sofi, S.N., Bhat, B.A., Qurishi, M.A., 2016. *Foeniculum vulgare*: A comprehensive review of its traditional use, phytochemistry, pharmacology, and safety. Arabian J. Chem. 9, S1574–S1583. https://doi.org/10.1016/j.arabjc.2012.04.011

Raut, J.S., Karuppayil, S.M., 2014. A status review on the medicinal properties of essential oils. Ind. Crops Prod. 62, 250–264. https://doi.org/10.1016/j.indcrop.2014.05.055

Ren, M.-X., Tang, J.-Y., 2012. Up and down: Stamen movements in *Ruta graveolens* (Rutaceae) enhance both outcrossing and delayed selfing. Ann. Bot. 110, 1017–1025. https://doi.org/ 10.1093/aob/mcs181

Ribeiro, A.M., Estevinho, B.N., Rocha, F., 2021. Preparation and incorporation of functional ingredients in edible films and coatings. Food Bioprocess Technol. 14, 209–231. https://doi.org/10.1007/s11947-020-02528-4

Ribeiro-Santos, R., Andrade, M., Melo, N.R. de, Sanches-Silva, A., 2017. Use of essential oils in active food packaging: Recent advances and future trends. Trends Food Sci. Technol. 61, 132–140. https://doi.org/10.1016/j.tifs.2016.11.021

Ribeiro-Santos, R., Andrade, M., Sanches-Silva, A., de Melo, N.R., 2018. Essential oils for food application: Natural substances with established biological activities. Food Bioprocess Technol. 11, 43–71. https://doi.org/10.1007/s11947-017-1948-6

Ribes, S., Fuentes, A., Talens, P., Barat, J.M., 2018. Prevention of fungal spoilage in food products using natural compounds: A review. Critical Reviews in Food Sci. Nutr. 58, 2002–2016. https://doi.org/10.1080/10408398.2017.1295017

Rivera, D., Verde, A., Fajardo, J., Obón, C., Consuegra, V., García-Botía, J., Ríos, S., Alcaraz, F., Valdés, A., del Moral, A., Laguna, E., 2019. Ethnopharmacology in the Upper Guadiana River area (Castile-La Mancha, Spain). J. Ethnopharmacol. 241, 111968. https://doi.org/10.1016/j.jep.2019.111968

Rodrigues, A.M., Mendes, M.D., Lima, A.S., Barbosa, P.M., Ascensão, L., Barroso, J.G., Pedro, L.G., Mota, M.M., Figueiredo, A.C., 2017. *Pinus halepensis*, *Pinus pinaster*, *Pinus pinea* and *Pinus sylvestris* essential oils chemotypes and monoterpene hydrocarbon enantiomers, before and after inoculation with the pinewood nematode *Bursaphelenchus xylophilus*. Chem. Biodivers. 14, e1600153. https://doi.org/10.1002/cbdv.201600153

Rodrigues, M., Lopes, A.C., Vaz, F., Filipe, M., Alves, G., Ribeiro, M.P., Coutinho, P., Araujo, A.R.T.S., 2020. *Thymus mastichina*: Composition and biological properties with a focus on antimicrobial activity. Pharmaceuticals. 13, 1–26. https://doi.org/10.3390/ph13120479

Roller, S., Seedhar, P., 2002. Carvacrol and cinnamic acid inhibit microbial growth in fresh-cut melon and kiwifruit at 4° and 8 °C. Lett. Appl. Microbiol. 35, 390–394. https://doi.org/10.1046/j.1472-765X.2002.01209.x

Roosta, R.A., Moghaddasi, R., Hosseini, S.S., 2017. Export target markets of medicinal and aromatic plants. J. Appl. Res. Med. Aromat. Plants. 7, 84–88. https://doi.org/10.1016/j.jarmap.2017.06.003

Rota, M.C., Herrera, A., Martínez, R.M., Sotomayor, J.A., Jordán, M.J., 2008. Antimicrobial activity and chemical composition of *Thymus vulgaris*, *Thymus zygis* and *Thymus hyemalis* essential oils. Food Control. 19, 681–687. https://doi.org/10.1016/j.foodcont.2007.07.007

Roudashti, S., Zeighami, H., Mirshahabi, H., Bahari, S., Soltani, A., Haghi, F., 2017. Synergistic activity of sub-inhibitory concentrations of curcumin with ceftazidime and ciprofloxacin against *Pseudomonas aeruginosa* quorum sensing related genes and virulence traits. World J. Microbiol. Biotechnol. 33, 1–8. https://doi.org/10.1007/s11274-016-2195-0

Roussis, V., Tsoukatou, M., Chinou, I.B., Harvala, C., 2002. Composition and antibacterial activity of the essential oils of two *Helichrysum stoechas* varieties growing in the island of Crete. J. Essent. Oil Res. 14, 459–461. https://doi.org/10.1080/10412905.2002.9699920

Roy, A., Park, H.J., Abdul, Q.A., Jung, H.A., Choi, J.S., 2018. Pulegone exhibits anti-inflammatory activities through the regulation of NF-κb and Nrf-2 signaling pathways in LPS-stimulated RAW 264.7 cells. Nat. Prod. Sci. 24, 28–35. https://doi.org/10.20307/nps.2018.24.1.28

Ruas, A., Graça, A., Marto, J., Gonçalves, L., Oliveira, A., da Silva, A.N., Pimentel, M., Moura, A.M., Serra, A.T., Figueiredo, A.C., Ribeiro, H.M., 2022. Chemical characterization and bioactivity of commercial essential oils and hydrolates obtained from Portuguese forest logging and thinning. Molecules. 27, 3572. https://doi.org/10.3390/molecules27113572

Saeed, K., Pasha, I., Jahangir Chughtai, M.F., Ali, Z., Bukhari, H., Zuhair, M., 2022. Application of essential oils in food industry: Challenges and innovation. J. Essent. Oil Res. 34, 97–110. https://doi.org/10.1080/10412905.2022.2029776

Sahal, G., Nasseri, B., Ebrahimi, A., Bilkay, I.S., 2019. Electrospun essential oil-polycaprolactone nanofibers as antibiofilm surfaces against clinical *Candida tropicalis* isolates. Biotechnol. Lett. 41, 511–522. https://doi.org/10.1007/s10529-019-02660-y

Salehi, B., Mishra, A.P., Shukla, I., Sharifi-Rad, M., Contreras, M. del M., Segura-Carretero, A., Fathi, H., Nasrabadi, N.N., Kobarfard, F., Sharifi-Rad, J., 2018. Thymol, thyme, and other plant sources: Health and potential uses. Phytother. Res. 32, 1688–1706. https://doi.org/10.1002/ptr.6109

Salvo, G., Bacchetta, G., Ghahremaninejad, F., Conti, E., 2008. Phylogenetic relationships of Ruteae (Rutaceae): New evidence from the chloroplast genome and comparisons with non-molecular data. Mol. Phylogenet. Evol. 49, 736–748. https://doi.org/10.1016/j.ympev.2008.09.004

Sánchez-Hidalgo, M., Montalbán-López, M., Cebrián, R., Valdivia, E., Martínez-Bueno, M., Maqueda, M., 2011. AS-48 bacteriocin: Close to perfection. Cell. Mol. Life Sci. 68, 2845–2857. https://doi.org/10.1007/s00018-011-0724-4

Sangha, J.S., Astatkie, T., Cutler, G.C., 2017. Ovicidal, larvicidal, and behavioural effects of some plant essential oils on diamondback moth (Lepidoptera: Plutellidae). Can. Entomol. 149, 639–648. https://doi.org/10.4039/tce.2017.13

Santoyo, S., Jaime, L., García-Risco, M.R., Lopez-Hazas, M., Reglero, G., 2014. Supercritical fluid extraction as an alternative process to obtain antiviral agents from thyme species. Ind. Crops Prod. 52, 475–480. https://doi.org/10.1016/j.indcrop.2013.10.028

Sapper, M., Bonet, M., Chiralt, A., 2019. Wettability of starch-gellan coatings on fruits, as affected by the incorporation of essential oil and/or surfactants. LWT-Food Sci. Technol. 116, 108574. https://doi.org/10.1016/j.lwt.2019.108574

Sapper, M., Wilcaso, P., Santamarina, M.P., Roselló, J., Chiralt, A., 2018. Antifungal and functional properties of starch-gellan films containing thyme (*Thymus zygis*) essential oil. Food Control. 92, 505–515. https://doi.org/10.1016/j.foodcont.2018.05.004

Saraiva, M. de M.S., Lim, K., Do Monte, D.F.M., Givisiez, P.E.N., Alves, L.B.R., Neto, O.C. de F., Kariuki, S., Júnior, A.B., De Oliveira, C.J.B., Gebreyes, W.A., 2022. Antimicrobial resistance in the globalized food chain: A One Health perspective applied to the poultry industry. Braz. J. Microbiol. 53, 465–486. https://doi.org/10.1007/s42770-021-00635-8

Sarengaowa, Hu, W., Feng, K., Xiu, Z., Jiang, A., Lao, Y., 2019. Tandem mass tag-based quantitative proteomic analysis reveal the inhibition mechanism of thyme essential oil against flagellum of *Listeria monocytogenes*. Food Res. Int. 125, 108508.

https://doi.org/10.1016/j.foodres.2019.108508

Scherer, R., Godoy, H.T., 2009. Antioxidant activity index (AAI) by the 2,2-diphenyl-1-picrylhydrazyl method. Food Chem. 112, 654–658. https://doi.org/10.1016/j.foodchem.2008.06.026

Schött, G., Liesegang, S., Gaunitz, F., Gleß, A., Basche, S., Hannig, C., Speer, K., 2017. The chemical composition of the pharmacologically active *Thymus* species, its antibacterial activity against *Streptococcus mutans* and the antiadherent effects of *T. vulgaris* on the bacterial colonization of the *in situ* pellicle. Fitoterapia. 121, 118–128. https://doi.org/10.1016/j.fitote.2017.07.005

Seak, C., Lin, C., 2007. *Ruta graveolens* intoxication. Clin. Toxicol. 45, 173–175. https://doi.org/ 10.1080/15563650600956667

Servi, H., Şen, A., Yildirim Servi, E., Doğan, A., 2021. Chemical composition and biological activities of essential oils of *Foeniculum vulgare* Mill. and *Daucus carota* L. growing wild in Turkey. J. Res. Pharm. 25, 142–152. https://doi.org/10.29228/jrp.5

Shah, B., Davidson, P.M., Zhong, Q., 2012. Nanocapsular dispersion of thymol for enhanced dispersibility and increased antimicrobial effectiveness against *Escherichia coli* O157:H7 and *Listeria monocytogenes* in model food systems. Appl. Environ. Microbiol. 78, 8448–8453. https://doi.org/10.1128/AEM.02225-12

Sharma, K., Babaei, A., Oberoi, K., Aayush, K., Sharma, R., Sharma, S., 2022. Essential oil nanoemulsion edible coating in food industry: A review. Food Bioprocess Tech. 15, 2375-2395. https://doi.org/10.1007/s11947-022-02811-6

Sharma, N., Tan, M.A., An, S.S.A., 2021. Mechanistic aspects of apiaceae family spices in ameliorating Alzheimer's disease. Antioxidants. 10, 1571. https://doi.org/10.3390/antiox10101571

Shi, C., Zhao, X., Yan, H., Meng, R., Zhang, Y., Li, W., Liu, Z., Guo, N., 2016. Effect of tea tree oil on *Staphylococcus aureus* growth and enterotoxin production. Food Control. 62, 257–263. https://doi.org/10.1016/j.foodcont.2015.10.049

Silva, C. de S., Figueiredo, H.M. de, Stamford, T.L.M., Silva, L.H.M. da, 2019. Inhibition of *Listeria monocytogenes* by *Melaleuca alternifolia* (tea tree) essential oil in ground beef. Int. J. Food Microbiol. 293, 79–86. https://doi.org/10.1016/j.ijfoodmicro.2019.01.004

Silva, F., Ferreira, S., Duarte, A., Mendona, D.I., Domingues, F.C., 2011. Antifungal activity of *Coriandrum sativum* essential oil, its mode of action against *Candida* species and potential synergism with amphotericin B. Phytomedicine. 19, 42–47.

https://doi.org/10.1016/j.phymed.2011.06.033

Silva, L., Rodrigues, A.M., Ciriani, M., Falé, P.L.V., Teixeira, V., Madeira, P., Machuqueiro, M., Pacheco, R., Florêncio, M.H., Ascensão, L., Serralheiro, M.L.M., 2017. Antiacetylcholinesterase activity and docking studies with chlorogenic acid, cynarin and arzanol from *Helichrysum stoechas* (Lamiaceae). Med. Chem. Res. 26, 2942–2950. https://doi.org/10.1007/s00044-017-1994-7

Silvestre, W.P., Livinalli, N.F., Baldasso, C., Tessaro, I.C., 2019. Pervaporation in the separation of essential oil components: A review. Trends Food Sci. Technol. 93, 42–52. https://doi.org/10.1016/j.tifs.2019.09.003

Singh, J., Rasane, P., Nanda, V., Kaur, S., 2022. Bioactive compounds of corn silk and their role in management of glycaemic response. J. Food Sci. Technol. https://doi.org/10.1007/s13197-022-05442-z

Singh, P., Pandey, A.K., 2018. Prospective of essential oils of the genus *Mentha* as biopesticides: A Review. Front. Plant Sci. 9, 1295. https://doi.org/10.3389/fpls.2018.01295

Singh, S.P., 2019. A comprehensive review on pharmacological activity of *Foeniculum vulgare*. Global J. Pharm. Pharm. Sci. 7, 1–5. https://doi.org/10.19080/GJPPS.2019.07.555703

Singh, V., Phukan, U.J., 2019. Interaction of host and *Staphylococcus aureus* protease-system regulates virulence and pathogenicity. Med. Microbiol. Immunol. 208, 585–607. https://doi.org/10.1007/s00430-018-0573-y

Soares, C., Morales, H., Faria, J., Figueiredo, A.C., Pedro, L.G., Venâncio, A., 2016. Inhibitory effect of essential oils on growth and on aflatoxins production by *Aspergillus parasiticus*. World Mycotoxin J. 9, 525–534. https://doi.org/10.3920/WMJ2015.1987

Solarte, A.L., Astorga, R.J., Aguiar, F., Galán-Relaño, Á., Maldonado, A., Huerta, B., 2017. Combination of antimicrobials and essential oils as an alternative for the control of *Salmonella enterica* multiresistant strains related to foodborne disease. Foodborne Pathog. Dis. 14, 558–563. https://doi.org/10.1089/fpd.2017.2295

Solarte, A.L., Astorga, R.J., De Aguiar, F.C., De Frutos, C., Barrero-Domínguez, B., Huerta, B., 2018. Susceptibility distribution to essential oils of *Salmonella enterica* strains involved in animal and public health and comparison of the Typhimurium and Enteritidis serotypes. J. Med. Food. 21, 946–950. https://doi.org/10.1089/jmf.2017.0103

Soncu, E.D., Özdemir, N., Arslan, B., Küçükkaya, S., Soyer, A., 2020. Contribution of surface application of chitosan–thyme and chitosan–rosemary essential oils to the volatile composition, microbial profile, and physicochemical and sensory quality of dry-fermented sausages during storage. Meat Sci. 166, 108127. https://doi.org/10.1016/j.meatsci.2020.108127

Song, C., Zhao, J., Zheng, R., Hao, C., Yan, X., 2022. Chemical composition and bioactivities of thirteen non-host plant essential oils against *Plutella xylostella* L. (Lepidoptera: Plutellidae). J. Asia-Pac. Entomol. 25, 101881. https://doi.org/10.1016/j.aspen.2022.101881

Sousa, R.M.O.F., Cunha, A.C., Fernandes-Ferreira, M., 2021. The potential of Apiaceae species as sources of singular phytochemicals and plant-based pesticides. Phytochemistry. 187, 112714. https://doi.org/10.1016/j.phytochem.2021.112714

Stanković, M.S., Radić, Z.S., Blanco-Salas, J., Vázquez-Pardo, F.M., Ruiz-Téllez, T., 2017. Screening of selected species from Spanish flora as a source of bioactive substances. Ind. Crops Prod. 95, 493–501. https://doi.org/10.1016/j.indcrop.2016.09.070

Stepanović, S., Ćirković, I., Ranin, L., Švabić-Vlahović, M., 2004. Biofilm formation by *Salmonella* spp. and *Listeria monocytogenes* on plastic surface. Lett. Appl. Microbiol. 38, 428–432. https://doi.org/10.1111/j.1472-765X.2004.01513.x

Stepanycheva, E., Petrova, M., Chermenskaya, T., Pavela, R., 2019. Fumigant effect of essential oils on mortality and fertility of thrips *Frankliniella occidentalis* Perg. Environ. Sci. Pollut. Res. 26, 30885–30892. https://doi.org/10.1007/s11356-019-06239-y

Strambu-Dima, A., 2022. Food-related consumer behavior endorsing European food chain sustainability—A marketing study on the romanian consumer. Sustainability. 14, 9045. https://doi.org/10.3390/su14159045

Štrbac, F., Bosco, A., Maurelli, M.P., Ratajac, R., Stojanović, D., Simin, N., Orčić, D., Pušić, I., Krnjajić, S., Sotiraki, S., Saralli, G., Cringoli, G., Rinaldi, L., 2022. Anthelmintic properties of essential oils to control gastrointestinal nematodes in sheep — *In vitro* and *in vivo* studies. Vet. Sci. 9, 93. https://doi.org/10.3390/vetsci9020093

Su, X., Cao, G., Zhang, J., Pan, H., Zhang, D., Kuang, D., Yang, X., Xu, X., Shi, X., Meng, J., 2019. Characterization of internalin genes in *Listeria monocytogenes* from food and humans, and their association with the invasion of Caco-2 cells. Gut Pathog. 11, 1–11. https://doi.org/10.1186/s13099-019-0307-8

Suresh, M.K., Biswas, R., Biswas, L., 2019. An update on recent developments in the

prevention and treatment of *Staphylococcus aureus* biofilms. Int. J. Med. Microbiol. 309, 1–12. https://doi.org/10.1016/j.ijmm.2018.11.002

Taghouti, M., Martins-Gomes, C., Schäfer, J., Santos, J.A., Bunzel, M., Nunes, F.M., Silva, A.M., 2020. Chemical characterization and bioactivity of extracts from *Thymus mastichina*: A *Thymus* with a distinct salvianolic acid composition. Antioxidants. 9, 34. https://doi.org/10.3390/antiox9010034

Takó, M., Beáta Kerekes, E., Zambrano, C., Kotogán, A., Papp, T., Krisch, J., Vágvölgyi, C., 2020. Plant phenolics and phenolic-enriched extracts as antimicrobial agents against food-contaminating microorganisms. Antioxidants. 9, 1–21. https://doi.org/10.3390/antiox9020165

Talib, W.H., Awajan, D., Hamed, R.A., Azzam, A.O., Mahmod, A.I., AL-Yasari, I.H., 2022. Combination anticancer therapies using selected phytochemicals. Molecules. 27, 5452. https://doi.org/10.3390/molecules27175452

Talić, S., Odak, I., Bevanda, A.M., Crnjac, N., Paštar, M., 2019. *Helichrysum italicum* (Roth) G. Don subsp. *italicum* from Herzegovina: Volatile composition, variations during seasons, total polyphenols, acetylcholinesterase inhibition and antioxidant activity. Croat. Chem. Acta. 92, 69–77. https://doi.org/10.5562/cca3475

Tanveer, M., Shehzad, A., Butt, M.S., Shahid, M., 2021. Pharmacodynamics of *Foeniculum vulgare* against ulcer, inflammation, hepatotoxicity and nephrotoxicity. J. Anim. Plant Sci. 31, 841–853. https://doi.org/10.36899/JAPS.2021.3.0274

Teixeira, B., Marques, A., Ramos, C., Batista, I., Serrano, C., Matos, O., Neng, N.R., Nogueira, J.M.F., Saraiva, J.A., Nunes, M.L., 2012. European pennyroyal (*Mentha pulegium*) from Portugal: Chemical composition of essential oil and antioxidant and antimicrobial properties of extracts and essential oil. Ind. Crops Prod. 36, 81–87. https://doi.org/10.1016/j.indcrop.2011.08.011

Teixeira, B., Marques, A., Ramos, C., Neng, N.R., Nogueira, J.M.F., Saraiva, J.A., Nunes, M.L., 2013. Chemical composition and antibacterial and antioxidant properties of commercial essential oils. Ind. Crops Prod. 43, 587–595. https://doi.org/10.1016/j.indcrop.2012.07.069

Thielmann, J., Muranyi, P., Kazman, P., 2019. Screening essential oils for their antimicrobial activities against the foodborne pathogenic bacteria *Escherichia coli* and *Staphylococcus aureus*. Heliyon. 5, e01860. https://doi.org/10.1016/j.heliyon.2019.e01860

Tognolini, M., Ballabeni, V., Bertoni, S., Bruni, R., Impicciatore, M., Barocelli, E., 2007.

Protective effect of *Foeniculum vulgare* essential oil and anethole in an experimental model of thrombosis. Pharmacol. Res. 56, 254–260. https://doi.org/10.1016/j.phrs.2007.07.002

Tomazelli Júnior, O., Kuhn, F., Padilha, P.J.M., Vicente, L.R.M., Costa, S.W., Boligon, A.A., Scapinello, J., Nesi, C.N., Dal Magro, J., Lamo Castellví, S., 2018. Microencapsulation of essential thyme oil by spray drying and its antimicrobial evaluation against *Vibrio alginolyticus* and *Vibrio parahaemolyticus*. Braz. J. Biol. 78, 311–317. https://doi.org/10.1590/1519-6984.08716

Tong, C., Hu, H., Chen, G., Li, Z., Li, A., Zhang, J., 2021. Disinfectant resistance in bacteria: Mechanisms, spread, and resolution strategies. Environ. Res. 195, 110897. https://doi.org/10.1016/j.envres.2021.110897

Trifan, A., Luca, S.V., Greige-Gerges, H., Miron, A., Gille, E., Aprotosoaie, A.C., 2020. Recent advances in tackling microbial multidrug resistance with essential oils: Combinatorial and nano-based strategies. Crit. Rev. Microbiol. 46, 338–357. https://doi.org/10.1080/1040841X.2020.1782339

Trombetta, D., Castelli, F., Sarpietro, M.G., Venuti, V., Cristani, M., Daniele, C., Saija, A., Mazzanti, G., Bisignano, G., 2005. Mechanisms of antibacterial action of three monoterpenes. Antimicrob. Agents Chemother. 49, 2474–2478. https://doi.org/10.1128/AAC.49.6.2474-2478.2005

Tümen, İ., Akkol, E.K., Süntar, I., Kurtca, M., 2018. Research on the antioxidant, wound healing, and anti-inflammatory activities and the phytochemical composition of maritime pine (*Pinus pinaster* Ait). J. Ethnopharmacol. 211, 235–246. https://doi.org/10.1016/j.jep.2017.09.009

Uluseker, C., Kaster, K.M., Thorsen, K., Basiry, D., Shobana, S., Jain, M., Kumar, G., Kommedal, R., Pala-Ozkok, I., 2021. A review on occurrence and spread of antibiotic resistance in wastewaters and in wastewater treatment plants: Mechanisms and perspectives. Front. Microbiol. 12, 717809. https://doi.org/10.3389/fmicb.2021.717809

U.S. Food & Drug Administration (FDA), 9 June 2019. https://www.fda.gov/food/food-ingredients-packaging/generally-recognized-safe-gras. (accessed 25 November 21).

U.S. Food & Drug Administration (FDA), 7 May 2021. https://www.fda.gov/food/food-ingredients-packaging/generally-recognized-safe-gras (accessed 6 June 22).

Van Haute, S., Raes, K., Van der Meeren, P., Sampers, I., 2016. The effect of cinnamon, oregano and thyme essential oils in marinade on the microbial shelf life of fish and meat

products. Food Control. 68, 30–39. https://doi.org/10.1016/j.foodcont.2016.03.025

Vandenesch, F., Lina, G., Henry, T., 2012. *Staphylococcus aureus* hemolysins, bi-component leukocidins, and cytolytic peptides: A redundant arsenal of membrane-damaging virulence factors? Front. Cell. Infect. Microbiol. 2, 12. https://doi.org/10.3389/fcimb.2012.00012

Varela, M.F., Stephen, J., Lekshmi, M., Ojha, M., Wenzel, N., Sanford, L.M., Hernandez, A.J., Parvathi, A., Kumar, S.H., 2021. Bacterial resistance to antimicrobial agents. Antibiotics. 10, 593. https://doi.org/10.3390/antibiotics10050593

Vázquez-Armenta, F.J., Silva-Espinoza, B.A., Cruz-Valenzuela, M.R., González-Aguilar, G.A., Nazzaro, F., Fratianni, F., Ayala-Zavala, J.F., 2017. Antibacterial and antioxidant properties of grape stem extract applied as disinfectant in fresh leafy vegetables. J. Food Sci. Technol. 54, 3192–3200. https://doi.org/10.1007/s13197-017-2759-5

Vázquez-Ucha, J.C., Martínez-Guitián, M., Lasarte-Monterrubio, C., Conde-Pérez, K., Arca-Suárez, J., Álvarez-Fraga, L., Pérez, A., Crecente-Campo, J., Alonso, M.J., Bou, G., Poza, M., Beceiro, A., 2020. *Syzygium aromaticum* (clove) and *Thymus zygis* (thyme) essential oils increase susceptibility to colistin in the nosocomial pathogens *Acinetobacter baumannii* and *Klebsiella pneumoniae*. Biomed. Pharmacother. 130, 110606. https://doi.org/10.1016/j.biopha.2020.110606

Verraes, C., Van Boxstael, S., Van Meervenne, E., Van Coillie, E., Butaye, P., Catry, B., de Schaetzen, M.A., Van Huffel, X., Imberechts, H., Dierick, K., Daube, G., Saegerman, C., De Block, J., Dewulf, J., Herman, L., 2013. Antimicrobial resistance in the food chain: A review. Int. J. Environ. Res. Public Health. 10, 2643–2669. https://doi.org/10.3390/ijerph10072643

Viegas, D.A., Palmeira-De-Oliveira, A., Salgueiro, L., Martinez-De-Oliveira, J., Palmeira-De-Oliveira, R., 2014. *Helichrysum italicum*: From traditional use to scientific data. J. Ethnopharmacol. 151, 54–65. https://doi.org/10.1016/j.jep.2013.11.005

Vieira, M., Bessa, L.J., Martins, M.R., Arantes, S., Teixeira, A.P.S., Mendes, Â., Da Costa, P.M., Belo, A.D.F., 2017. Chemical composition, antibacterial, antibiofilm and synergistic properties of essential oils from *Eucalyptus globulus* LABILL. and seven Mediterranean aromatic plants. Chem. Biodivers. 14, e1700006. https://doi.org/10.1002/cbdv.201700006

Walczak, M., Michalska-Sionkowska, M., Olkiewicz, D., Tarnawska, P., Warżyńska, O., 2021. Potential of carvacrol and thymol in reducing biofilm formation on technical surfaces. Molecules. 26, 2723. https://doi.org/10.3390/molecules26092723

Wałecka, E., Molenda, J., Karpíšková, R., Bania, J., 2011. Effect of osmotic stress and culture density on invasiveness of *Listeria monocytogenes* strains. Int. J. Food Microbiol. 144, 440–445. https://doi.org/10.1016/j.ijfoodmicro.2010.10.032

Wang, L.H., Zhang, Z.H., Zeng, X.A., Gong, D.M., Wang, M.S., 2017. Combination of microbiological, spectroscopic and molecular docking techniques to study the antibacterial mechanism of thymol against *Staphylococcus aureus*: Membrane damage and genomic DNA binding. Anal. Bioanal. Chem. 409, 1615–1625. https://doi.org/10.1007/s00216-016-0102-z

Wang, X., Shen, Y., Thakur, K., Han, J., Zhang, J.G., Hu, F., Wei, Z.J., 2020. Antibacterial activity and mechanism of ginger essential oil against *Escherichia coli* and *Staphylococcus aureus*. Molecules. 25, 3955. https://doi.org/10.3390/molecules25173955

Wei, L., Wang, Y.Z., Li, Z.Y., 2012. Floral ontogeny of Ruteae (Rutaceae) and its systematic implications. Plant Biol. 14, 190–197. https://doi.org/10.1111/j.1438-8677.2011.00475.x

Wei, L., Xiang, X.G., Wang, Y.Z., Li, Z.Y., 2015. Phylogenetic relationships and evolution of the androecia in Ruteae (Rutaceae). PloS One. 10, 10–14. https://doi.org/ 10.1371/journal.pone.0137190

World Health Organization (WHO), 31 July 2020. Antibiotic resistance. https://www.who.int/news-room/fact-sheets/detail/antibiotic-resistance (accessed 12 September 2022).

World Health Organization (WHO), 17 November 2021. WHO's first global report on antibiotic resistance reveals serious, worldwide threat to public health. https://www.who.int/news/item/30-04-2014-who-s-first-global-report-on-ntibiotic-resistancereveals-serious-worldwide-threat-to-public-health (accessed 9 December 2021).

World Health Organization (WHO), 19 May 2022. Food safety. https://www.who.int/news-room/fact-sheets/detail/food-safety (accessed 10 September 2022).

Wiktorczyk-Kapischke, N., Skowron, K., Grudlewska-Buda, K., Wałecka-Zacharska, E., Korkus, J., Gospodarek-Komkowska, E., 2021. Adaptive response of *Listeria monocytogenes* to the stress factors in the food processing environment. Front. Microbiol. 12, 710085. https://doi.org/10.3389/fmicb.2021.710085

Wu, S.C., Liu, F., Zhu, K., Shen, J.Z., 2019. Natural products that target virulence factors in antibiotic-resistant *Staphylococcus aureus*. J. Agric. Food Chem. 67, 13195–13211.

https://doi.org/10.1021/acs.jafc.9b05595

Yakoubi, R., Megateli, S., Hadj Sadok, T., Bensouici, C., Bağci, E., 2021a. A synergistic interactions of Algerian essential oils of *Laurus nobilis* L., *Lavandula stoechas* L. and *Mentha pulegium* L. on anticholinesterase and antioxidant activities. Biocatal. Agric. Biotechnol. 31, 101891. https://doi.org/10.1016/j.bcab.2020.101891

Yakoubi, R., Megateli, S., Hadj Sadok, T., Gali, L., 2021b. Photoprotective, antioxidant, anticholinesterase activities and phenolic contents of different Algerian *Mentha pulegium* extracts. Biocatal. Agric. Biotechnol. 34, 102038. https://doi.org/10.1016/j.bcab.2021.102038

Yaldiz, G., Camlica, M., 2019. Variation in the fruit phytochemical and mineral composition, and phenolic content and antioxidant activity of the fruit extracts of different fennel (*Foeniculum vulgare* L.) genotypes. Ind. Crops Prod. 142, 111852. https://doi.org/10.1016/j.indcrop.2019.111852

Yang, I.-H., Shin, J.-A., Cho, S.-D., 2014. Pycnogenol induces nuclear translocation of apoptosis-inducing factor and caspase-independent apoptosis in MC3 human mucoepidermoid carcinoma cell line. J. Cancer Prev. 19, 265–272. https://doi.org/10.15430/jcp.2014.19.4.265

Yang, I.H., Shin, J.A., Kim, L.H., Kwon, K.H., Cho, S.D., 2016. The caspase 3-dependent apoptotic effect of Pycnogenol in human oral squamous cell carcinoma HSC-3 cells. J. Clin. Biochem. Nutr. 58, 40–47. https://doi.org/10.3164/jcbn.15-7

Yang, Q., Liu, Q., Lv, H., Wang, F., Liu, R., Zeng, N., 2019. Effect of pulegone on the NLPR3 inflammasome during inflammatory activation of THP-1 cells. Exp. Ther. Med. 19, 1304–1312. https://doi.org/10.3892/etm.2019.8327

Yang, V.W., Clausen, C.A., 2007. Antifungal effect of essential oils on southern yellow pine. Int. Biodeterior. Biodegrad. 59, 302–306. https://doi.org/10.1016/j.ibiod.2006.09.004

Youdim, K.A., Deans, S.G., Finlayson, H.J., 2002. The antioxidant properties of thyme (*Thymus zygis* L.) essential oil: An inhibitor of lipid peroxidation and a free radical scavenger. J. Essent. Oil Res. 14, 210–215. https://doi.org/10.1080/10412905.2002.9699825

Yousefi, M., Khorshidian, N., Hosseini, H., 2020. Potential application of essential oils for mitigation of *Listeria monocytogenes* in meat and poultry products. Front. Nutr. 7, 577287. https://doi.org/10.3389/fnut.2020.577287

Yuan, Z., Dai, Y., Ouyang, P., Rehman, T., Hussain, S., Zhang, T., Yin, Z., Fu, H., Lin, J., He,

C., Lv, C., Liang, X., Shu, G., Song, X., Li, L., Zou, Y., Yin, L., 2020. Thymol inhibits biofilm formation, eliminates pre-existing biofilms, and enhances clearance of methicillin-resistant *Staphylococcus aureus* (MRSA) in a mouse peritoneal implant infection model. Microorganisms. 8, 1–17. https://doi.org/10.3390/microorganisms8010099

Zang, E., Jiang, L., Cui, H., Li, X., Yan, Y., Liu, Q., Chen, Z., Li, M., 2022. Only plant-based food additives: An overview on application, safety, and key challenges in the food industry. Food Rev. Int. 1–32. https://doi.org/10.1080/87559129.2022.2062764

Zengin, G., Cvetanović, A., Gašić, U., Tešić, Ž., Stupar, A., Bulut, G., Sinan, K.I., Uysal, S., Picot-Allain, M.C.N., Mahomoodally, M.F., 2020. A comparative exploration of the phytochemical profiles and biopharmaceutical potential of *Helichrysum stoechas* subsp. *barrelieri* extracts obtained via five extraction techniques. Process Biochem. 91, 113–125. https://doi.org/10.1016/j.procbio.2019.12.002

Zhang, C., Li, C., Abdel-Samie, M.A., Cui, H., Lin, L., 2020. Unraveling the inhibitory mechanism of clove essential oil against *Listeria monocytogenes* biofilm and applying it to vegetable surfaces LWT-Food Sci. Technol 134, 110210. https://doi.org/10.1016/j.lwt.2020.110210

Zhang, Y., Wang, J., Dong, J., Wei, J., Wang, Y., Dai, X., Wang, X., Luo, M., Tan, W., Deng, X., Niu, X., 2013. Inhibition of α-toxin production by subinhibitory concentrations of naringenin controls *Staphylococcus aureus* pneumonia. Fitoterapia. 86, 92–99. https://doi.org/10.1016/j.fitote.2013.02.001

Zheng, X., Feyaerts, A.F., Dijck, P. Van, Bossier, P., 2020. Inhibitory activity of essential oils against *Vibrio campbellii* and *Vibrio parahaemolyticus*. Microorganisms. 8, 1–19. https://doi.org/10.3390/microorganisms8121946

Zibadi, S., Yu, Q., Rohdewald, P.J., Larson, D.F., Watson, R.R., 2007. Impact of Pycnogenol® on cardiac extracellular matrix remodeling induced by L-NAME administration to old mice. Cardiovasc. Toxicol. 7, 10–18. https://doi.org/10.1007/s12012-007-0001-9

Annex I

Genus *Ruta*: A natural source of high value products with biological and pharmacological properties

This annex corresponds to a published manuscript with the following reference:

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Abstract

Ethnopharmacological relevance: *Ruta* genus is constituted by ten species, of which the most commonly described are *R. chalepensis* and *R. graveolens*. *Ruta* plants are perennial shrubs belonging to the family Rutaceae, which are traditionally used in folk medicine, since ancient times mostly for the treatment of various ailments of the womb.

Aim of the study: To provide a review of the different uses of *Ruta* species in traditional medicine, as well as, on their multifactorial biological and pharmacological properties.

Material and methods: A search of the literature on genus *Ruta* and *Ruta* species was performed using various scientific databases and search engines and the information of articles were reviewed and compiled.

Results: Different parts of the plants belonging to *Ruta* genus are used in folk medicine to treat a wide range of different diseases. The principal use of these is in gynaecological field, but the treatment of pain, fever, nausea, inflammation, infections, nervous disorders, among others, are also described. These plants have been used to fertility regulation, as anti-fertility agent, to control menstrual flux and bleedings, as abortifacient and as contraceptive. The phytochemical composition of these plants consists mainly in essential oil (EO), but phenolic compounds were also reported, like coumarins and flavonoids, as well as alkaloids. *Ruta* species products like extracts and EOs have shown broad pharmacological activities, such as antimicrobial and antifungal activities, as well as, antiviral and antiparasitic. Moreover, *Ruta* plants products present antioxidant, neuroprotective, anti-inflammatory, anti-cancer and anti-diabetic activities and demonstrated contraceptive and abortifacient effects. These plants were also tested to be used for non-therapeutic approaches, as bio-insecticides in the control of different insect pests showing to be able to reduce infestation.

Conclusions: *Ruta* species could be a potential source of natural products with biological activities. *Ruta* extracts, essential oils and isolated compounds have shown a diverse potential for use in the treatment of different diseases, as well as, for pests control, contributing to the valorisation of these plants. Nonetheless, this review indicates that more studies are needed to demonstrate the full potential of *Ruta* species, and to further explore the toxicology and safety of these plants.

Keywords: *Ruta* species; phytochemistry; folk medicine; biological activities

1. Introduction

Medicinal plants are widely used in the folk medicine and have an interesting chemical composition and the therapeutic interest was increased. Plant-based natural product is one of the potential sources in discovering new agents and/or drugs.

Rutaceae family plants (citrus family) are well-known for their economic importance and also for the cultivated citrus fruits, timber and aromatic oils, being a potential source of many medicinal substances (Wei et al., 2012; Wei et al., 2015; Samuel et al., 2001; Koblovská et al., 2008). Extensive research on this family has been done and shows the potential application of these natural products in the treatment of such diverse conditions as Alzheimer's disease, depression and cancer, as well as in treating infections, due to their anti-bacterial, anti-fungal, anti-leishmanial and anti-plasmodial properties (Adamska-Szewczyk et al., 2016). One of the genus of Rutaceae family plants studied is the genus *Ruta*. *Ruta* (common name rue), belongs to tribe Ruteae and is the type genus of the subfamily Rutoideae (Morton and Telmer, 2014; Wei et al., 2012, 2015). *Ruta* is a strongly scented subshrubs native to the Mediterranean region (Hammami et al., 2015). The genus *Ruta* includes ten species of perennial shrubs, of which *R. chalepensis* and *R. graveolens* are recognized as the most common (Meloni et al., 2013b).

Despite, plants of the Rutaceae family have been reviewed by other authors; the genus *Ruta* have not been comprehensively analysed. This review aims to provide an overview of the botanical characteristics, uses in traditional medicine, chemical composition, bioactive activity, therapeutic applications and some aspects of the toxicity of plants of the genus *Ruta*.

2. Material and methods

An extensive search for literature was made for all the time periods using the genus name "*Ruta*" and the species names "*Ruta angustifolia*", "*Ruta chalepensis*", "*Ruta corsica*", "*Ruta graveolens*", "*Ruta lamarmorae*", "*Ruta microcarpa*", "*Ruta montana*", "*Ruta oreojasme*", "*Ruta pinnata*" and "*Ruta tuberculata*". The search was conducted in available online scientific databases such PubMed, ScienceDirect, Web of Science and Scopus. Studies on using homeopathic medicines from plant material were not included in this review.

3. Botany

3.1 Geographical distribution

Ruta is a genus of strongly fragrant evergreen subshrubs mainly found in temperate and tropical regions, native to the Mediterranean region (Appelhans et al., 2016; Hammami et al., 2015; Wei et al., 2012). The complex, but well-known, history of microplate movements and climatic oscillations, provides to the Mediterranean region the geographic backdrop for the diversification of *Ruta* (Salvo et al., 2010). The limits of the geographic distribution of *Ruta* broadly correspond to this region, however, the genus often occurs in other places where elements characteristic of the Mediterranean vegetation were present (Salvo et al., 2010). Despite its Mediterranean origin, *Ruta* species are vastly distributed around de globe (Table 1).

Table 1. Origin and geographical distribution of *Ruta* species.

***Ruta* species**	**Origin**	**Distribution**	**Place characteristics**	**Reference**
R. angustifolia	Mediterranean (Southern Europe and North Africa)	Europe and Southeast Asia	Mountains to an elevation of 1000 m above sea level	(Richardson et al., 2016; Salvo et al., 2008)
R. chalepensis	Mediterranean	Eurasia, North Africa, America	Calcareous rocky slopes and in temperate and tropical countries	(Acquaviva et al., 2011; Akkari et al., 2015; Alotaibi et al., 2018; Bouabidi et al., 2015; Iauk et al., 2004; Mejri et al., 2010; Salvo et al., 2008; Soudani et al., 2018)
R. corsica	Island of Corsica	ND	ND	(Salvo et al., 2008)
R. graveolens	Mediterranean (Southern Europe)	Europe and many Asian countries, including China, India, and Japan	ND	(Gentile et al., 2018; Hale et al., 2004; Kostova et al., 1999; Ratheesh et al., 2009; Salvo et al., 2008)
R. lamarmorae	Island of Sardinia	ND	ND	(Salvo et al., 2008)
R. microcarpa	Canary Islands	North of the island of La Gomera	Hilly, open areas or steep rocky slopes, including screes, although some populations have colonized abandoned cultivation areas along with other xeric species	(Meloni et al., 2013a; Salvo et al., 2008)
R. montana	Mediterranean (North Africa)	ND	ND	(Farid et al., 2017; Kambouche et al., 2008; Salvo et al., 2008)

Table 1. Origin and geographical distribution of *Ruta* species (continuation).

***Ruta* species**	**Origin**	**Distribution**	**Place characteristics**	**Reference**
R. oreojasme	Canary Islands	South of the island of Gran Canaria	rocky cliffs and escarpments at an altitude of 300–800 m	(Meloni et al., 2013b; Meloni et al., 2015; Salvo et al., 2008)
R. pinnata	Canary Islands	ND	ND	(Salvo et al., 2008)
R. tuberculata	ND	Northern Sahara	ND	(Haddouchi et al., 2013)

ND – not described

3.2 Botanical characteristics

Ruta plants are subshrubs with 20–60 cm tall whose flowers have two whorls of stamens and are bisexual (Hammami et al., 2015; Wei et al., 2012). The botanical traits of *Ruta* most common species, are presented in Table 2, and include as main features, leaves with the presence of secretory cavities containing aromatic essential oils with deterrent odour and bitter taste (Bennaoum et al., 2017; Khadhri et al., 2014).

R. corsica is a shrub, with a powerful odour emitted by bluish-green leaves and have a bitter taste (Pollio et al., 2008) and *R. tuberculata* is characterised by its leaves lanceolate or often elongated and small flowers with four yellow petals and in Algeria, the flowering period is from June (Haddouchi et al., 2013). *R. montana* is an evergreen shrub that is 20–60 cm tall and it is tetraploid as is their mainland sister species *R. pinnata* (Farid et al., 2017; Meloni et al., 2013a).

The relevance of *Ruta* genus due to its biological and pharmacological properties and as a source of high value products, is focused in the phytochemistry and pharmacological potential of its species (Haddouchi et al., 2013; Pollio et al., 2008).

Table 2. Botanical characteristics of *Ruta* common species.

	Type of plant	Height	General characteristics of the plant	Flowering period	Reference
R. angustifolia	Shrub	Grows up to 1.5 m	Slender stem. Leaves are light green oval; 2–3 times divided into segments oblong; powerful odour and have a bitter taste. Flowers are yellow with a very strong foetid odour. Sepals with ciliate fringed.	In Algeria is at June	(Faria et al., 2016a,b; Haddouchi et al., 2013; Pollio et al., 2008)
R. chalepensis	Very strong smelling perennial herbaceous shrub	Grows up to 80 cm	Leaves are glabrous, alternate bi-pinnatisect; narrow-oblong, lanceolate, or obovate segments; with oil glands with strong deterrent odour and bitter taste. Flowers have cymes with four to five free or connate, below oleate or obovate sepals, four to five bright yellow petals, eight to ten stamens, and a superior ovary. Petals are fringed, matching only half the width of the petals and bracts much larger than the stem to which they are attached.	In Lebanon is from March to May; in Algeria is at June; in Tunisia is at April	(Acquaviva et al., 2011; Bouabidi et al., 2015; Günaydin and Savci, 2005; Haddouchi et al., 2013; Iauk et al., 2004; Khoury et al., 2014; Pollio et al., 2008; De Sa et al., 2000; Soudani et al., 2018)
R. graveolens	Perennial shrub	30-80 cm	Stems are much ramified. Leaves have 4-11 cm long and 3-7 cm wide; small, oblong, deeply divided, pinnate, glandular dotted, bluish-green and emit a powerful odour and have a bitter taste. Flowers are small, yellow, borne in dichasial cymes. It is branded by its petals not fringed; have 4 petals, except for the central flower, which has 5 petals. Possesses ten stamens. Fruits are rounded, brown, small and lobulated. It presents woody root and a crooked, branched rhizom.	In Algeria is June; in Chine is from March to June	(Farzaei et al., 2017; Gentile et al., 2018; Haddouchi et al., 2013; Hale et al., 2004; Harat et al., 2008; Khori et al., 2008; Pollio et al., 2008; Ren and Tang, 2012)

Table 2. Botanica characteristics of *Ruta* common species (continuation).

	Type of plant	Height	General characteristics of the plant	Flowering period	Reference
R. microcarpa	Shrub	0.80–1.5 m	Dense branches stems. Leaves are remotely toothed. Small, yellowish, tetramerous and hermaphroditic flowers. Small fruits and fruting in May-June.	In La Gomera (Canary Island) is from March to May	(Meloni et al., 2013a)
R. oreojasme	Small shrub	40 cm	Branched stems Pinnate leaves Yellow, tetramerous, hermaphroditic and protandrous flowers Lights brown fruits and fruting in June-August. Small and black seeds	In Canary Island is from April to June	(Meloni et al., 2015)

4. Folk medicine

Ruta species have been used in traditional medicine in a wide range of different problems/diseases (Table 3); however, the major therapeutic uses of *Ruta* spp. is in the field of gynaecology. *Ruta* spp. were mainly used for the treatment of various ailments of the womb. These species were also prescribed for menses disturbance, as their regulation, amenorrhea or excess. It was administered both as an abortive and to help conception. These plants were recommended to treat various disturbances that may occur during the pregnancy or the delivery, to expel the placenta, and against puerperal fever (Pollio et al., 2008). There have been previous reports of the abortive action of *R. chalepensis* and *R. graveolens*. A study presents several cases of herbal-induced multiple organ system failure which that were misdiagnosed as post abortion sepsis. These results strongly suggest that most of the abortions happen in the context of herbal intoxication, most frequently following the ingestion of *Ruta* plants. The ingestion of plants to induce abortion involves the risk of severe intoxication that could result in death or future reproductive complications (Ciganda and Laborde, 2003).

Among the treatments of the different diseases, the most used species are *R. chalepensis* and *R. graveolens*, which are used throughout the world. The preparations of these plants are mainly produced in the form of decoctions and infusions of aerial parts.

Table 3. Uses of *Ruta* species in folk medicine.

Species	Uses	Part used	Mode of preparation	Country	Reference
R. angustifolia	Liver disease and jaundice	ND	ND	Indonesia	(Richardson et al., 2016; Wahyuni et al., 2014)
	Cure cramps, flatulence and fever	Whole plant	Decoction	Malaysia, Vietnam and Java	(Richardson et al., 2016)
R. chalepensis	Fever	Leaves	Aqueous decoction	Africa	(Acquaviva et al., 2011; Iauk et al., 2004; Khadhri et al., 2014)
	Veterinarian uses (carminative, ruminant antistatic, postpartum coadjuvant (antiseptic), tranquilizer, flea repellent, to heal wounds, anti-inflammatory and anthelminthic)	Aerial parts	Direct ingestion, direct application or embrocation	Catalan Pyrenean regions and Balearic Islands areas	(Carrio et al., 2012)
	Gastric disorders, headache and rheumatism, as well as for their diuretic, anti-inflammatory and anti-spasmodic properties	Fresh leaves	Infusions	Chile	(Tampe et al., 2016)
	Anti-venom	Roots	Decoction	China	(Acquaviva et al., 2011; Iauk et al., 2004; Khadhri et al., 2014)
	Fertility regulation, anti-fertility agent		Hot water crude extracts	China and Turkey	(Khadhri et al., 2017)
	Stomach ache and common cold	Leaves with succulent stem	ND	Ethiopia	(Berhane et al., 2014)
R. chalepensis	Dropsy, neuralgia, rheumatism and menstrual and other bleeding disorders			India	(Acquaviva et al., 2011; Iauk et al., 2004; Khadhri et al., 2014)
	Laxative, analgesic, antispasmodic, abortifacient, antiepileptic, emmenagogue, for dermatopathy treatment, fever, anti-inflammatory, to treatment of gastric, diuretic, headache, rheumatism disorders, snake bites, and wounds	Whole plant	ND	Many countries	(Bouajaj et al., 2014; Soudani et al., 2018; Alotaibi et al., 2018; Emam et al., 2009)

Table 3. Uses of *Ruta* species in folk medicine (continuation).

Species	Uses	Part used	Mode of preparation	Country	Reference
	Asthma, renal colic, arthritis, rheumatism, backache, skin bacterial and fungal diseases, eye inflammation and ear infection, as antitussive, antispasmodic, anti-inflamatory, anti-lice, sedative, and bronchodilator and also for treatment of snake bites	Whole plant	ND	Palestine	(Jaradat et al., 2017)
	Analgesic and antipyretic and for the treatment of rheumatism and mental disorders	Aerial parts	Decoction	Saudi Arabia	(Acquaviva et al., 2011; Iauk et al., 2004; Khoury et al., 2014)
	Hysteria, epilepsy, vertigo, colic, intestinal worms, poisonings, anxiety and eye problems, antispasmodic, analgesic, antipyretic, anti-inflammatory, sedative, menstrual problems, rheumatism, mental disorders	Whole plant/leaves	Decoction	ND	(Acquaviva et al., 2011; Iauk et al., 2004; Khadhri et al., 2017; Khoury et al., 2014)
	Treatment of convulsion and other nervous disorders in children	Leaves	Infused with vinegar	ND	(Iauk et al., 2004)
	Epilepsy, as an insect repellent	Aerial parts	ND	ND	(Acquaviva et al., 2011; Iauk et al., 2004; Khoury et al., 2014)
R. chalepensis	Psoriasis, otitis	Aerial parts	Macerated in olive oil	ND	(Acquaviva et al., 2011; Iauk et al., 2004; Khoury et al., 2014)
	Earaches and headaches and as antiseptic drops for ear and eye infections	Heated leaves	Juice	ND	(Acquaviva et al., 2011; Iauk et al., 2004; Khoury et al., 2014)
	Treat pains of kidney, bladder, stomach and spine	Leaves	Infusion	Sete Cidades village, São Miguel Island, Azores, Portugal	(Silva et al., 2019)
	To treat stomach pain, common cold, nausea and headache during pregnancy	Leaves	Put the leaves in infusions or coffee or the leaves can be sniffed	Addis Ababa and Bati, Ethiopia	(Berhane et al., 2014; Nega et al., 2019)

Table 3. Uses of *Ruta* species in folk medicine (continuation).

Species	Uses	Part used	Mode of preparation	Country	Reference
R. graveolens	Aching pain, eye problems, rheumatism and dermatitis	ND	ND	Asian countries; Italy and Croatia	(Gentile et al., 2018; Mancuso et al., 2015; Ratheesh et al., 2009)
	Heart disorders	Aerial parts	Fresh juice, infusion	Bosnia and Herzegovina	(Redžić, 2007)
	Menstrual cramps, colic, intestinal infection, stomach problems, renal problems, headache, otalgia, pains in general (including diverted pain), hematoma, fever, indigestion, thrombosis, healing, anti-tetanus (infections), antiseptic, amenorrhea, to restore menstrual flux to normal levels, abortive, antiemetic, arthrosis, increase organism defenses, calming, conjunctivitis, postpartum depression, depurative, toothache, earache, diarrhea, emollient, wound, furuncle, influenza, eye infection, inflammation, ear inflammation, ovary inflammation, eye inflammation, uterine inflammation, eye cleaning, uterine cleaning, indigestion, discomfort, nervousness, lice, intestinal problems, renal problems, uterine problems, rheumatism, cough, thrombosis, helminthiasis	Leaves	Decoction, infusion, poultice, juice or juice with milk	Brazil	(Cartaxo et al., 2010)
		Whole plant	ND		(De Medeiros et al., 2013)
		Leaves	Infusion		(De Freitas et al., 2005; Yazbek et al., 2016)
R. graveolens	Veterinarian uses (Roundworms, tapeworms, treatment for endoparasites)	Aerial parts	ND	Canada	(Lans et al., 2007)
	Contraception, anti-inflammatory, sedative, antifungal, analgesic, antispasmodic, antihelmintic and abortive agent	Leaves	Fresh extract (solvent ND) or infusion	Iran	(Harat et al., 2008)
	Used as antidotes for some snake and scorpion venoms and to treat many infections and inflammation	Whole plant	Extracts (solvent ND)	Italy and Croatia	(Mancuso et al., 2015)
	Stomachache, vomit, diarrhea, snake bite, wounds, cough, fever, headache, inflammation, nervousness, flu, toothache, body pain, ear pain	Whole plant	Infusion	México	(Alonso-Castro et al., 2012)

Table 3. Uses of *Ruta* species in folk medicine (continuation).

Species	Uses	Part used	Mode of preparation	Country	Reference
	Emmenagogues, antifertility and abortifacient	ND	ND	Persia	(Madari and Jacobs, 2004)
	Bladder and kidneys, convulsions, diabetes, fever, headache, stomach complaints, worms, sinus and to treat hysteria	Whole plant	Leaf juice or the oil	South Africa	(Stafford et al., 2008)
		Leaves	Infusion		(Thring and Weitz, 2006)
	Palpitations and heart protection	Aerial parts	Decoction	Taiwan	(Seak and Lin, 2007)
	Inflammatory conditions, eczema, ulcers, arthritis, fibromyalgia, antidote for venoms, insect repellent, abortifacient drug, faintness, cramp, hysteria, diseases of the womb	Whole plant	Infusion	Montevideo, Uruguay	(Ghosh et al., 2014; Koblovská et al., 2008; Ciganda and Laborde, 2003)
R. montana	Hysteria, worms, or colic, as an antiseptic, stimulant, emmenagogue, and abortifacient and for its antifertility activity	ND	ND	Algeria	(Kabouche et al., 2003; Kambouche et al., 2008)
	Tonic, febrifuge, treatment of malaria, inflammatory, antioxidant, microbial processes, digestive, gastrointestinal motility, child fevers and as an abortive drug—but with great care because of the toxic effect due to the presence of xanthotoxin	ND	Infusion/decoction	Many countries	(Kabouche et al., 2003; Khadhri et al., 2017)
R. tuberculata	Aching bones and joint pain, dysmenorrhea, infertility in women, difficult delivery, post-partum care, liver and bowels complaints, child convulsions, fever, anaemia and headache	Aerial parts	Decotion and infusion	Saharo-arabic region	(Hammiche and Maiza, 2006)

ND – not described

5. Phytochemistry

The efficient use of plants by agricultural, food and pharmaceutical industries is due to detailed knowledge of their secondary metabolites (Koblovská et al., 2008). Several members from the Rutaceae family have been studied, in the last years, for their phytochemical and pharmacological properties (Adamska-Szewczyk et al., 2016). The biochemical networks and pathways of the Rutaceae produce a tremendous spectrum of secondary metabolites. Some of these chemical compounds, including limonoids, flavonoids, coumarins and alkaloids have been described as possessing herbicidal, antimicrobial, insecticidal, trypanocidal and antimalarial activities (Morton and Telmer, 2014). The phytochemical screening of *Ruta* species showed a large number of chemical constituents that have been isolated, such as alkaloids, flavonoids, coumarins, such bergapten, tannins, volatile oil, glycosides, sterols and triterpenes (Khadhri et al., 2017; Wahyuni et al., 2014). In fact, glycosides, flavonoids, and tannins are considered potent inhibitors of pro-inflammatory signaling molecules, which may explain in part the bioactivities observed (Khadhri et al., 2017). *Ruta* species present many compounds, like benzoquinones, flavone glycosides, triterpenoids, sterols, such as stigmasterol, lupeol, 5-methoxyarborinine, 5-hydroxyarborinine, kokusaginine, psoralen, xanthotoxin, ostruthin, isopimpinellin, integriquinolone, dictamnine, limonoid obacunone, furoquinolin alkaloid, xanthyletin and xanthoxyletin (Wahyuni et al., 2014). Many of these metabolites have attracted biological and pharmacological interest, demonstrating antifungal, phytotoxic, and antidotal activities (Sampaio et al., 2018). Table 4 shows the wide variety of compounds that was described for each species. These compounds have been found both in extracts of plants and in their essential oils.

Table 4. Phytochemical composition of *Ruta* species.

Compounds/group of compounds	***Ruta* species**	**Reference**
11-Dodecen-2-one	*R. chalepensis* (EO)	(Haddouchi et al., 2013)
1,8-Cineole	*R. graveolens* (EO)	(Chaftar et al., 2015)
1,8-Nonadiene	*R. montana* (EO)	(Hammami et al., 2015)
12-Methoxy-19-norpodocarpa-3,5,8,11,13-pentaen-7-one	*R. chalepensis* (EO); *R. graveolens* (EO)	(Haddouchi et al., 2013)
1-Dodecanol	*R. graveolens* (EO)	(Chaftar et al., 2015)
1-Dodecene	*R. chalepensis* (EO)	(Khadhri et al., 2014)
1-Methyl-4-methoxy-2-quinolone	*R. montana* (chloroform extract)	(Touati et al., 2000)
1-Nonene	*R. chalepensis* (EO); *R. graveolens* (EO); *R. montana* (EO)	(Haddouchi et al., 2013; Khadhri et al., 2014)

Table 4. Phytochemical composition of *Ruta* species (continuation).

Compounds/group of compounds	*Ruta* species	Reference
1-Tetradecanol methyacrylate	*R. chalepensis* (EO); *R. graveolens* (EO)	(Haddouchi et al., 2013; Yosra et al., 2019)
1-Butene	*R. montana* (EO)	(Hammami et al., 2015)
2-Butanone	*R. montana* (EO)	(Hammami et al., 2015)
2-Butene	*R. montana* (EO)	(Hammami et al., 2015)
2-Decanone	*R. angustifolia* (EO); *R. chalepensis* (EO); *R. graveolens* (EO, ethanolic extract); *R. montana* (EO)	(Akkari et al., 2015; Amdouni et al., 2016; Boutoumi et al., 2009; Chaftar et al., 2015; Fekhar et al., 2017; Ghabbari et al., 2018; Haddouchi et al., 2013; Kambouche et al., 2008; Khadhri et al., 2014; Laquale et al., 2015; Mejri et al., 2010; Orlanda and Nascimento, 2015; Da Silva et al., 2014)
2-(Decan-9-one)-N-methyl-4-quinolone	*R. montana* (chloroform extract)	(Touati et al., 2000)
2-Decyl acetate		
2-Dodecanone	*R. angustifolia* (EO); *R. chalepensis* (EO); *R. graveolens* (EO, ethanolic extract); *R. montana* (EO)	(Bagchi et al., 2004; Boutoumi et al., 2009; Chaftar et al., 2015; Fekhar et al., 2017; Ghabbari et al., 2018; Haddouchi et al., 2013; Kambouche et al., 2008; Khadhri et al., 2014; Khadhri et al., 2014; Mejri et al., 2010; Orlanda and Nascimento, 2015; Da Silva et al., 2014)
2-Methyloctyl acetate	*R. chalepensis* (EO)	(Conti et al., 2013; Tzakou et al., 2001)
2-Nonanol	*R. chalepensis* (EO); *R. graveolens* (ethanolic extract); *R. montana* (EO)	(Ghabbari et al., 2018; Khadhri et al., 2014; Tzakou et al., 2001)
2-Nonanol acetate	*R. montana* (EO)	(Kambouche et al., 2008)
2-Nonanone	*R. angustifolia* (EO); *R. chalepensis* (EO, hydrosol); *R. graveolens* (EO, ethanolic extract); *R. montana* (EO)	(Amdouni et al., 2016; Bagchi et al., 2004; Boutoumi et al., 2009; Chaftar et al., 2015; Conti et al., 2013; Fakhfakh et al., 2012; Fekhar et al., 2017; Ghabbari et al., 2018; Haddouchi et al., 2013; Kambouche et al., 2008; Khadhri et al., 2014; Khoury et al., 2014; Knaak et al., 2013; Krayni et al., 2015; Laquale et al., 2015; Mancuso et al., 2015; Orlanda and Nascimento, 2015; Rustaiyan et al., 2002; Da Silva et al., 2014; Tampe et al., 2016; Traka et al., 2018)
2-{6′-(2H-benzo[d]1′′,3′′-dioxolen-5′′-yl)hexyl}-hydroquinolin-4-one	*R. chalepensis* (ethanolic extract)	(Sayed et al., 2000)
2-{6′-(2H-benzo[*d*]1′′,3′′-dioxolen-5′′-yl)hexyl}-4-methoxy-quinoline	*R. chalepensis* (ethanolic extract)	(Sayed et al., 2000)

Table 4. Phytochemical composition of *Ruta* species (continuation).

Compounds/group of compounds	*Ruta* species	Reference
2-(Nonan-8-one)-N-methyl-4-quinolone	*R. montana* (chloroform extract)	(Touati et al., 2000)
2-(Nonan-8-one)-4-methoxy-quinoline	*R. montana* (chloroform extract)	(Touati et al., 2000)
2-(Nonan-8-one)-(1H)-4-quinolone	*R. montana* (chloroform extract)	(Touati et al., 2000)
2-Nonyl acetate	*R. chalepensis* (EO)	(Bagchi et al., 2004)
2-Octanol acetate	*R. chalepensis* (EO); *R. montana* (EO)	(Haddouchi et al., 2013; Kambouche et al., 2008)
2-Octanone	*R. chalepensis* (EO); *R. graveolens* (EO)	(Haddouchi et al., 2013; Da Silva et al., 2014)
2-Tridecanone	*R. angustifolia* (EO); *R. chalepensis* (EO); *R. graveolens* (EO, ethanolic extract); *R. montana* (EO)	(Boutoumi et al., 2009; Chaftar et al., 2015; Fekhar et al., 2017; Ghabbari et al., 2018; Haddouchi et al., 2013; Kambouche et al., 2008; Mejri et al., 2012; Da Silva et al., 2014)
2-Undecanol	*R. graveolens* (ethanolic extract); *R. montana* (EO)	(Ghabbari et al., 2018; Khadhri et al., 2014)
2-Undecanol acetate	*R. montana* (EO)	(Kambouche et al., 2008)
2-Undecanone	*R. angustifolia* (EO); *R. chalepensis* (EO, hydrosol); *R. graveolens* (EO, ethanolic extract); *R. montana* (EO); *R. tuberculata* (EO)	(Akkari et al., 2015; Bagchi et al., 2004; Boutoumi et al., 2009; Chaftar et al., 2015; Conti et al., 2013; Fakhfakh et al., 2012; Faria et al., 2016; Fekhar et al., 2017; Ghabbari et al., 2018; Haddouchi et al., 2013; Kambouche et al., 2008; Khadhri et al., 2014; Khoury et al., 2014; Knaak et al., 2013; Krayni et al., 2015; Laquale et al., 2015; Mancuso et al., 2015; Mejri et al., 2010; Orlanda and Nascimento, 2015; Rustaiyan et al., 2002; Da Silva et al., 2014; Soares et al., 2016; Tampe et al., 2016; Traka et al., 2018)
3-Carene	*R. tuberculata* (EO)	(Haddouchi et al., 2013)
3-Phenylpropanal	*R. montana* (EO)	(Hammami et al., 2015)
3-Octanone	*R. montana* (EO)	(Kambouche et al., 2008)
4-Methoxy-1-methyl-2(1H)-quinolinone	*R. chalepensis* (ethanolic extract)	(Sayed et al., 2000)
4-*O*-Feruloylquinic acid	*R. graveolens* (hydroalcoholic extract)	(Pacifico et al., 2016)
4-*O*-*p*-cumaroylquinic acid	*R. graveolens* (hydroalcoholic extract)	(Pacifico et al., 2016)
4-Terpineol	*R. montana* (EO)	(Kambouche et al., 2008; Khadhri et al., 2014)
5-(1′,1′-Dimethylallyl)-8-hydroxyfuro[2-3-b] quinoline	*R. chalepensis* (ethanolic extract)	(Emam et al., 2010)
5,6-Diethenyl-1-methyl-cyclohexene	*R. graveolens* (EO)	(Yosra et al., 2019)

Table 4. Phytochemical composition of *Ruta* species (continuation).

Compounds/group of compounds	***Ruta* species**	**Reference**
6,8-*C*-dihexosyl-apigenin	*R. graveolens* (hydroalcoholic extract)	(Pacifico et al., 2016)
7,30-*O*-Dimethyl-gossypetin-3-*O*-rutinoside	*R. graveolens* (hydroalcoholic extract	(Pacifico et al., 2016)
8-(3,5-Dimethil-4-Hydroxyphenyl)-2-octene	*R. chalepensis* (EO); *R. montana* (EO)	(Khadhri et al., 2014)
8-Phenyl-2-octanone	*R. graveolens* (EO)	(Soares et al., 2016)
acacetin	*R. graveolens* (hydroalcoholic extract)	(Pacifico et al., 2016)
Acetophenone	*R. montana* (EO)	(Hammami et al., 2015)
Alkaloids	*R. chalepensis* (EO, ethanolic and methanolic extracts); *R. graveolens* (EO)	(Haddouchi et al., 2013; (Hamdiken et al., 2018; Iauk et al., 2004; Sayed et al., 2000)
Amino acids	*R. chalepensis* (EO; ethanolic extract);	(Alotaibi et al., 2018; Haddouchi et al., 2013)
	R. graveolens (EO)	
Angustifolin	*R. angustifolia* (chloroform extract)	(Wahyuni et al., 2014)
Arborinine	*R. angustifolia* (dichloromethane extract)	(Wahyuni et al., 2014)
Bergapten	*R. graveolens* (EO, aqueous extract)	(Mancuso et al., 2015)
Benzene acetic acid	*R. montana* (EO)	(Hammami et al., 2015)
Benzoic acid	*R. montana* (EO)	(Hammami et al., 2015)
Biscoumarin daphnoretin	*R. chalepensis* (ethanolic extract)	(Emam et al., 2010)
Borneol	*R. graveolens* (EO)	(Chaftar et al., 2015)
Bornyl acetate	*R. tuberculata* (EO)	(Haddouchi et al., 2013)
Butanoic acid	*R. montana* (EO)	(Hammami et al., 2015)
Cadinene	*R. montana* (EO)	(Khadhri et al., 2014)
Camphor	*R. chalepensis* (EO); *R. graveolens* (EO)	(Chaftar et al., 2015; Mejri et al., 2010)
Carvacrol	*R. graveolens* (EO); *R. montana* (EO)	(Khadhri et al., 2014; Laquale et al., 2015)
Caryophyllene oxide	*R. montana* (EO)	(Hammami et al., 2015)
Cedrol	*R. graveolens* (EO)	(Chaftar et al., 2015)
Chalepin	*R. angustifolia* (dichloromethane extract)	(Wahyuni et al., 2014)
Chrysanthenone	*R. graveolens* (EO)	(Chaftar et al., 2015)
Cinnamic acid	*R. graveolens* (methanolic extract)	(Szopa et al., 2012)
Cis-p-menth-1-en-3-ol (*cis*-piperitol)	*R. tuberculata* (EO)	(Haddouchi et al., 2013)
Cis-p-menth-2-en-1-ol	*R. tuberculata* (EO)	(Haddouchi et al., 2013)
Cnidioside A	*R. graveolens* (hydroalcoholic extract)	(Pacifico et al., 2016)

Table 4. Phytochemical composition of *Ruta* species (continuation).

Compounds/group of compounds	*Ruta* species	Reference
Coumarins	*R. chalepensis* (EO); *R. graveolens* (EO)	(Haddouchi et al., 2013)
Cyclopentane oxide	*R. montana* (EO)	(Hammami et al., 2015)
Dicoumarinyl ether	*R. montana* (ND)	(Kabouche et al., 2003)
Dictamnine	*R. chalepensis* (ethanolic extract)	(Sayed et al., 2000)
Diethyl phthalate	*R. graveolens* (EO)	(Orlanda and Nascimento, 2015)
Dihydroxymaltol	*R. graveolens* (ethanolic extract)	(Ghabbari et al., 2018)
Dodecanoic acid	*R. graveolens* (ethanolic extract)	(Ghabbari et al., 2018)
Elemolo	*R. graveolens* (ethanolic extract)	(Ghabbari et al., 2018)
Epigallocatechin-3-*O*-gallate	*R. graveolens* (aqueous extract)	(Gentile et al., 2018)
Esters	*R. chalepensis* (ethanolic extract)	(Alotaibi et al., 2018)
Eugenol	*R. montana* (EO)	(Hammami et al., 2015)
Evolitrine	*R. montana* (chloroform extract)	(Touati et al., 2000)
Farnesene	*R. graveolens* (EO)	(Da Silva et al., 2014)
Farnesol	*R. montana* (EO)	(Hammami et al., 2015)
Ferulic acid	*R. graveolens* (methanolic extract)	(Szopa et al., 2012)
Flavonoids	*R. chalepensis* (EO; ethanolic and methanolic extracts); *R. graveolens* (EO)	(Haddouchi et al., 2013; Hamdiken et al., 2018; Iauk et al., 2004)
Furanocoumarin (e.g. isorutarin)	*R. graveolens* (hydroalcoholic extract)	(Pacifico et al., 2016)
Furocoumarins	*R. chalepensis* (ethanolic extract)	(Iauk et al., 2004)
Gentiobiose	*R. graveolens* (hydroalcoholic extract)	(Pacifico et al., 2016)
Germacrene-B	*R. tuberculata* (EO)	(Haddouchi et al., 2013)
Geyrene	*R. chalepensis* (EO); *R. graveolens* (EO, ethanolic extract); *R. montana* (EO)	(Amdouni et al., 2016; Ghabbari et al., 2018; Haddouchi et al., 2013; Khadhri et al., 2014; Da Silva et al., 2014)
Glycosides	*R. chalepensis* (EO; ethanolic extract); *R. graveolens* (EO)	(Alotaibi et al., 2018; Haddouchi et al., 2013)
Graveoline	*R. chalepensis* (ethanolic extract); *R. graveolens* (ethanolic extract; ethyl acetate extract; hexane extract)	(Ghosh et al., 2014; Hale et al., 2004; Oliva et al., 2003; Sampaio et al., 2018; Sayed et al., 2000)
Graveolinine	*R. chalepensis* (ethanolic extract)	(Sayed et al., 2000)
Heraclenol	*R. montana* (ND)	(Kabouche et al., 2003)
Hexadecanoic acid	*R. montana* (EO)	(Khadhri et al., 2014)
Isobornyl acetate	*R. graveolens* (EO)	(Chaftar et al., 2015)

Table 4. Phytochemical composition of *Ruta* species (continuation).

Compounds/group of compounds	*Ruta* species	Reference
Isomaturnin	*R. chalepensis* (EO); *R. montana* (EO)	(Khadhri et al., 2014)
Isopimpinellin	*R. graveolens* (aqueous extract); *R. montana* (ND)	(Mancuso et al., 2015; Kabouche et al., 2003)
Isorhamnetin	*R. graveolens* (aqueous extract)	(Gentile et al., 2018)
Isorhamnetin-3-*O*-rutinoside	*R. graveolens* (aqueous extract; hydroalcoholic extract; methanolic extract)	(Gentile et al., 2018; Ivanova et al., 2005; Pacifico et al., 2016)
Isorutarin	*R. graveolens* (methanolic extract)	(Ivanova et al., 2005)
Isovaleric acid	*R. montana* (EO)	(Hammami et al., 2015)
Kokusaginine	*R. angustifolia* (dichloromethane extract); *R. graveolens* (aqueous extract)	(Mancuso et al., 2015; Wahyuni et al., 2014)
Lactones	*R. chalepensis* (ethanolic extract)	(Alotaibi et al., 2018)
Limonene	*R. graveolens* (EO); *R. montana* (EO)	(Kambouche et al., 2008; Khadhri et al., 2014; Da Silva et al., 2014)
Linalool	*R. chalepensis* (EO); *R. montana* (EO)	(Ghazghazi et al., 2015; Hammami et al., 2015)
Maculosidine	*R. chalepensis* (ethanolic extract)	(Sayed et al., 2000)
Menthol	*R. chalepensis* (EO)	(Ghazghazi et al., 2015)
Methylcyclopropane	*R. montana* (EO)	(Hammami et al., 2015)
Methyl decanoate	*R. chalepensis* (EO)	(Amdouni et al., 2016)
Methyl ester	*R. graveolens* (methanolic extract)	(Ivanova et al., 2005)
Monoterpene alcohols	*R. tuberculata* (EO)	(Haddouchi et al., 2013)
Monoterpene hydrocarbons	*R. tuberculata* (EO)	(Haddouchi et al., 2013)
Mycrene	*R. chalepensis* (EO)	(Tzakou et al., 2001)
Naphthalene	*R. montana* (EO)	(Hammami et al., 2015)
Nonanal	*R. angustifolia* (EO); *R. chalepensis* (EO); *R. montana* (EO)	(Haddouchi et al., 2013; Kambouche et al., 2008)
Octyl acetate	*R. chalepensis* (EO); *R. graveolens* (EO)	(Amdouni et al., 2016; Fakhfakh et al., 2012; Krayni et al., 2015; Orlanda and Nascimento, 2015)
O-Methyl-daphnoretin	*R. graveolens* (aqueous extract)	(Mancuso et al., 2015)
p-Coumaric acid	*R. graveolens* (methanolic extract)	(Szopa et al., 2012)
p-Cymene	*R. graveolens* (EO); *R. montana* (EO); *R. tuberculata* (EO)	(Chaftar et al., 2015; Haddouchi et al., 2013; Kambouche et al., 2008)
Pentadecanolide acetate	*R. graveolens* (EO)	(Orlanda and Nascimento, 2015)
Pentylfuran	*R. montana* (EO)	(Hammami et al., 2015)

Table 4. Phytochemical composition of *Ruta* species (continuation).

Compounds/group of compounds	*Ruta* species	Reference
Phenols	*R. chalepensis* (ethanolic extract)	(Iauk et al., 2004)
Phytyl acetate	*R. chalepensis* (EO)	(Amdouni et al., 2016)
Piperazine	*R. chalepensis* (EO)	(Akkari et al., 2015)
Piperitone	*R. tuberculata* (EO)	(Haddouchi et al., 2013)
Piperonyl acetone	*R. graveolens* (ethanolic extract)	(Ghabbari et al., 2018)
Pregeijerene	*R. chalepensis* (EO); *R. montana* (EO)	(Khadhri et al., 2014)
Propanoic acid	*R. montana* (EO)	(Hammami et al., 2015)
Protocatechuic acid	*R. graveolens* (methanolic extract)	(Szopa et al., 2012)
Pseudane IX	*R. angustifolia* (dichloromethane extract)	(Wahyuni et al., 2014)
Psoralen	*R. montana* (EO)	(Kambouche et al., 2008)
Pteleine	*R. chalepensis* (ethanolic extract)	(Sayed et al., 2000)
Pulegone	*R. chalepensis* (EO)	(Mejri et al., 2010)
Pyrrolidine	*R. montana* (EO)	(Hammami et al., 2015)
Quercetin-3-*O*-glucosyl-rhamnosyl-galactoside	*R. graveolens* (aqueous extract)	(Gentile et al., 2018)
Quercetin-3-*O*-rutinoside (rutin)	*R. graveolens* (aqueous extract; hydroalcoholic extract; methanolic extract)	(Gentile et al., 2018; Ivanova et al., 2005; Mancuso et al., 2015; Pacifico et al., 2016; Raghav et al., 2006)
Ranupenin-3-*O*-rutinoside	*R. graveolens* (hydroalcoholic extract)	(Pacifico et al., 2016)
Rutacridone	*R. chalepensis* (ethanolic extract)	(Sayed et al., 2000)
Rutamarin	*R. graveolens* (aqueous extract)	(Mancuso et al., 2015)
Rutamontine	*R. montana* (ND)	(Kabouche et al., 2003)
Rutarin	*R. graveolens* (methanolic extract)	(Ivanova et al., 2005)
Sabinene	*R. chalepensis* (EO); *R. tuberculata* (EO)	(Haddouchi et al., 2013)
Saponins	*R. chalepensis* (ethanolic and methanolic extracts)	(Hamdiken et al., 2018; Iauk et al., 2004)
Scopoletin	*R. angustifolia* (dichloromethane extract)	(Wahyuni et al., 2014)
Sec-undecyl acetate	*R. graveolens* (ethanolic extract)	(Ghabbari et al., 2018)
Sinapoylferuloyl dihexoside (e.g. 1-sinapoyl-2-feruloyl gentiobioside)	*R. graveolens* (hydroalcoholic extract)	(Pacifico et al., 2016)
Skimmianine	*R. chalepensis* (ethanolic extract); *R. graveolens* (aqueous extract)	(Mancuso et al., 2015; Sayed et al., 2000)

Table 4. Phytochemical composition of *Ruta* species (continuation).

Compounds/group of compounds	*Ruta* species	Reference
Sogravacridonechlorine	*R. chalepensis* (ethanolic extract)	(Sayed et al., 2000)
Sterols	*R. chalepensis* (EO; methanolic extract); *R. graveolens* (EO)	(Haddouchi et al., 2013; Hamdiken et al., 2018)
Syringic acid	*R. graveolens* (methanolic extract)	(Szopa et al., 2012)
Tannins	*R. chalepensis* (EO; ethanolic and methanolic extracts); *R. graveolens* (EO)	(Alotaibi et al., 2018; Haddouchi et al., 2013; Hamdiken et al., 2018)
Tetrazole	*R. montana* (EO)	(Hammami et al., 2015)
Trans-p-menth-1-en-3-ol (*trans*-piperitol)	*R. tuberculata* (EO)	(Haddouchi et al., 2013)
Trans-p-menth-2-en-1-ol	*R. tuberculata* (EO)	(Haddouchi et al., 2013)
Triterpenes	*R. chalepensis* (EO; ethanolic extract); *R. graveolens* (EO)	(Alotaibi et al., 2018; Haddouchi et al., 2013)
Tridecane	*R. graveolens* (EO)	(Chaftar et al., 2015)
Tridecanol	*R. graveolens* (EO)	(Chaftar et al., 2015)
Valencene	*R. montana* (EO)	(Khadhri et al., 2014)
Vanillic acid	*R. graveolens* (methanolic extract)	(Szopa et al., 2012)
Varamol-106	*R. graveolens* (ethanolic extract)	(Ghabbari et al., 2018)
Xanthotoxin	*R. graveolens* (aqueous extract)	(Mancuso et al., 2015)
α-Decanone	*R. graveolens* (EO)	(Knaak et al., 2013)
α-Humulene	*R. montana* (EO)	(Hammami et al., 2015)
α-Limonene	*R. chalepensis* (EO); *R. graveolens* (EO)	(Haddouchi et al., 2013)
α-Phellandrene	*R. tuberculata* (EO)	(Haddouchi et al., 2013)
α-Pinene	*R. chalepensis* (EO); *R. montana* (EO); *R. tuberculata* (EO)	(Haddouchi et al., 2013; Tzakou et al., 2001; Kambouche et al., 2008)
α-Terpinene	*R. tuberculata* (EO)	(Haddouchi et al., 2013)
α-Terpineol	*R. montana* (EO)	(Khadhri et al., 2014)
α-Thujene	*R. tuberculata* (EO)	(Haddouchi et al., 2013)
α-Thujone	*R. graveolens* (EO)	(Chaftar et al., 2015)
β-Caryophyllene	*R. tuberculata* (EO)	(Haddouchi et al., 2013)
β-Caryophyllene epoxide	*R. tuberculata* (EO)	(Haddouchi et al., 2013)
β-Eudesmol	*R. montana* (EO)	(Khadhri et al., 2014)
β-Myrcecne	*R. montana* (EO); *R. tuberculata* (EO)	(Haddouchi et al., 2013; Kambouche et al., 2008)
β-Pinene	*R. chalepensis* (EO)	(Tzakou et al., 2001)
β-Phellandrene	*R. chalepensis* (EO); *R. tuberculata* (EO)	(Haddouchi et al., 2013; Tzakou et al., 2001)

Table 4. Phytochemical composition of *Ruta* species (continuation).

Compounds/group of compounds	*Ruta* species	Reference
β-Thujone	*R. graveolens* (EO); *R. montana* (EO)	(Chaftar et al., 2015; Kambouche et al., 2008)
γ-Elemene	*R. tuberculata* (EO)	(Haddouchi et al., 2013)
γ-Fagarine	*R. angustifolia* (dichloromethane extract)	(Wahyuni et al., 2014)
y-Terpinene	*R. chalepensis* (EO)	(Tzakou et al., 2001)
δ-Carene	*R. montana* (EO)	(Kambouche et al., 2008)

ND – not described

Essential oils are complex combinations containing many single compounds. Each of these constituents contributes to the beneficial or adverse effects of these oils, thus making the study of the essential oil composition very important to direct its application (Haddouchi et al., 2013). Essential oils of the *Ruta* genus are generally characterized by a mixture of ketones, which can reach 84% of the oil (Bertrand et al., 2003). Nevertheless, there are some significant species-specific disparities in the concentration of these compounds and/or the presence of others in high concentrations. It is associated with the fact that the composition of essential oil of *Ruta* species is vastly influenced by intrinsic and extrinsic factors, amongst which genetic and environmental factors can be pointed (Jaradat et al., 2017; Khadhri et al., 2014). These chemical differences are probably explained by the variability of the plant species and the existence of different chemotypes, which change from each region, depending on environmental factors, harvest time, as well as, geological and climatic conditions through the season (Bennaoum et al., 2017; Da Silva et al., 2014; Jaradat et al., 2017). In fact, different substances can be produced in different proportions depending on factors, such as temperature, humidity, duration and intensity of solar radiation, rainy season, as well as interaction with pollinators and predators (Da Silva et al., 2014). The light has also a decisive effect on the composition of the oil since it affects the metabolism of the plants. Thus, plants growing in shade will have different properties from another exposed to sunlight (Haddouchi et al., 2013). It is noteworthy that these factors can greatly affect the essential oil activity (Da Silva et al., 2014).

The analysis of the essential oil composition of several *Ruta* species indicates that 2-undecanone, 2-nonanone and 2-dodecanone are its main constituents; nonetheless variations may occur among species but also within the same species (Khadhri et al., 2014). For example, the essential oil of the aerial parts of R. *chalepensis* in Greece had forty-four identified components, with the main components being β-phellandrene (10.7%) and 2-methyloctyl acetate (44.0%) (Tzakou and Couladis, 2001). In South-East of Tunisia, the essential oil contained octyl acetate, 2-undecanone and 2-nonanone as the major

components (Fakhfakh et al., 2012). In another study of *R. chalepensis* essential oil from Tunisia, the major components were menthol (43.92%) and linalool (42.10%) (Ghazghazi et al., 2015). Regarding *R. corsica*, the main essential oil components were represented by alkyl acetates and the 2-nonyl acetate was the most characteristic one (42.9%). Bertrand et al. (2003) showed that the mixture of 2-ketones represents only 6% of *R. corsica* oil and the high concentration of 2-alkyl acetates seems to be the originality of this species (Bertrand et al., 2003). The major components in the essential oil of leaves of *R. montana* collected in Tunisia were 1-butene (38.33%), methylcyclopropane (15.47%), 2-butene (22.56%) and caryophyllene oxide (8.18%). Terpenoid hydrocarbons were the characteristic constituents of the oil of *R. montana* (Hammami et al., 2015). When considering *R. tuberculata* harvested in Algeria, it was demonstrated the presence of monoterpene alcohols that represent 40.79% of the total oil, followed by the monoterpene hydrocarbons (29.81%) and piperitone (Haddouchi et al., 2013).

As mentioned, the constitution and quantity of each component can vary considering different parts of the plant and also according to its stage (Akkari et al., 2015; Krayni et al., 2015; Mejri et al., 2010, 2012). For instance, the analysis of the essential oils from *R. chalepensis* from North Indian showed a maximum concentration of 2-undecanone in the flower essential oil followed by fruit and leaf essential oils. The quantity of 2-undecanone was highest in the leaves when the plants were young and in the vegetative stage, and it gradually decreased when the plants started flowering and the fruiting. On the other hand, the percentage of 2-nonanone was at its maximum in the leaf essential oil followed by flower and fruit oils, which increased from the vegetative to the flowering stage and was highest during fruiting stage. The concentration of 2-nonyl acetate was observed to be highest in the leaves during the vegetative stage, while 2-dodecanone was at its maximum in the fruits. Linalool, an important aromatic compound, is highest in flowers (Bagchi et al., 2003).

The effect of the harvest periods of aerial parts of *R. chalepensis* oils in Tunisa, also showed significant variations in the yield of essential oils. The oil collected during April (flowering stage which is characterized by a high metabolic activity) showed the best yields whereas oil collected during December showed the lower values (Bouabidi et al., 2015). The month of the collection also changed the oil composition of the different parts of the plant (Bejaoui and Karmous, 2012).

Regarding to extracts, it must be aware that the factors that affect the composition of essential oils also influence the extracts composition. The hydroalcoholic extracts from *R. graveolens* leaves collected in all seasons in Durazzano (Italy) show some differences in phenolic content. The extracts obtained in summer, autumn, winter and spring have gentiobiose, rutin, isorhamnetin-3-*O*-rutinoside and disinapoyldihexoside. However, the

quantity of these compounds was different in the extracts. The summer extract presents higher quantity of gentiobiose, but the spring extract has more of the other three compounds. 6,8-C-dihexosyl-apigenin, cnidioside A and acacetin are present only in the spring season and 4-*O*-*p*-cumaroylquinic acid, 4-*O*-feruloylquinic acid, isorutarin and 7,30-*O*-dimethyl-gossypetin-3-*O*-rutinoside only found in the winter extract. These data show alterations of the plant composition having regard to time of year at which samples are taken (Pacifico et al., 2016). The methanol extracts of leaves, fruits, stems and seeds of *R. graveolens* from Valcamonica Valley, Lombardy, Italy were analyzed separately. In this work, the results showed differences in the composition according to the part of the plant used (Mancuso et al., 2015). Szopa et al. (2012), studied the influence of the light conditions in the composition of *R. graveolens* methanolic extract of *in vitro* cultures. They concluded that white light and blue light have a beneficial effect on the accumulation of phenolic acids and furanocoumarins (Szopa et al., 2012).

So, it can be concluded that R*uta* essential oils and extracts have slight differences in composition. These differences can be associated to several factors and consequently it is so important to analyze the composition in order to relate the compounds present to their biological activities.

6. Biological activities and therapeutic applications of *Ruta* extracts

Plants have been the basis of medical treatments over the years. The research on herbal infusions, ointment and balms used in traditional medicine, allows to realize the scientific evidence of active plant compounds (Forsatkar et al., 2016). In fact, the screening of plant extracts allows the detection of potential sources of new bioactive molecules (Emam et al*.,* 2010; Orlanda and Nascimento, 2015). The evaluation of the effectiveness and safety of plant extracts and/or their metabolites may point to its applications in medicine or the control of agricultural pests (Emam et al., 2010).

Ruta species have been described as possessing several pharmaceutical properties, associated to their bioactive compounds, in this topic the biological activities associated with the extracts and essential oils of the different *Ruta* species were reviewed.

6.1 Antibacterial and antifungal activities

The research to find efficient antimicrobial agents is a constant ongoing work, where plants are a potential focus to discover new chemical structures that may help to overcome drug resistance, as well as, side effects of certain antibiotics (Chaftar et al., 2016; Haddouchi et

al., 2013). Furthermore, despite the effectiveness of antimicrobial drugs, their persistence in the environment, harmful effects on non-target organisms, toxicity to humans and other mammals and development of resistant strains of pathogens, trigger the public concern (Emam et al., 2010). To combat these effects, the use of new, safer, and ecologically compatible natural active compounds may be an alternative (Emam et al., 2010; Ghazghazi et al., 2015). Antimicrobials from plant sources can significantly help in the treatment of resistant microbial strains because they may have activity through different mechanisms of those of the currently used synthetic drugs (Orlanda and Nascimento, 2015).

There are several studies describing the antibacterial and antifungal potential of *Ruta* species, with *R. chalepensis* and *R. graveolens* being the most frequently studied.

6.1.1 Crude extracts

There are various reports on the antimicrobial activity of some *Ruta* species, where the effect of the solvent used for extraction, the part of the plant used, the growing conditions or the harvesting point were evaluated. The *R. angustifolia* methanolic extract has been evaluated regarding its antibacterial activity, however, this species showed no inhibition against *Staphylococcus aureus* ATCC 25923, *Bacillus cereus* ATCC 11778, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 8739 (1.0 mg/disc) (Shuib et al., 2015).

The crude methanolic extracts of leaves, roots and stems of *R. chalepensis* exhibited antibacterial activity against *E. coli* and *Pseudomonas aeruginosa* with inhibition zones and minimal inhibition concentration values ranging from 18 to 35 mm and 0.78-1.56 mg/mL, respectively, while against other tested bacteria the antimicrobial activity was moderate to null. Differences between the antimicrobial activities of leaves, stems and roots methanolic extracts were found, with the leaf extract being most active than stems and roots extracts. The authors related these differences with the different phenols and flavonoid content of each part of the plant. The methanolic extract of leaves presented more phenolic and flavonoid content (12.82 mg GAE/g DW and 0.757 mg RE/g, respectively), followed by stems (10.6 mg GAE/g DW and 0.539 mg RE/g) and finally the roots (10.3 mg GAE/g DW and 0.423 mg RE/g) (Ghazghazi et al., 2015). *R. chalepensis* buffered methanol (BM) and acetone extract of leaves from Yemen showed inhibition zones against *Bacillus cereus, Listeria monocytogenes* and *S. aureus* (12-17 mm using 800 µg per disc and MIC of 1.32-2.64 mg/mL) (Alzoreky and Nakahara, 2003). In another study, the antibacterial activity of different extracts of *R. chalepensis* was also evaluated against Gram-positive (*Actinomyces sp., Bacillus thuringiensis, Enterococcus faecalis* and *Micrococcus luteus*) and Gram-negative (*E. coli, Klebsiella pneumoniae* and *P. aeruginosa*) bacteria. Globally, the most effective antibacterial activity was found with the ethanolic extract against majority of the

tested bacteria. The most vulnerable bacteria for the ethanolic extract were *B. thuringiensis* and *Actinomyces sp.* with MIC value of 195 μg/mL and the most resistant species with the highest MIC value were found to be *M. luteus*, *E. coli* and *P. aeruginosa* (1562 μg/mL). The methanolic extract was active against all tested bacteria, presenting a lower MIC (390 μg/mL) than the ethanolic extract for *P. aeruginosa* (1562 μg/mL). Methanol/water and the ethyl acetate extracts showed a more or less significant inhibition and *P. aeruginosa* was the most sensitive to methanol/water extract with (780 μg/mL), whereas, *B. thuringiensis* was the most sensitive to the ethyl acetate extract (390 μg/mL). However, the hexane and water extracts did not show any antibacterial effect to on most of the tested bacteria (Kacem et al., 2015). Through different works, it can be observed the differences in biological activity depending on the solvent used for the extraction (Alotaibi et al., 2018).

The influence of solvent used in extraction was also demonstrated for *R. graveolens*, with methanol, petroleum ether, ethyl acetate and water–methanole extracts. The water-methanol extract was less active showing lower MIC values in some species tested and no activity in others. The petroleum ether and the ethyl acetate extracts presented higher MIC values. Only the ethyl acetate extract inhibited the growth of *Staphylococcus epidermidis* 1093 (13.3 mm) (Ivanova et al., 2005). In another study, the buffered methanolic and acetone extracts of *R. graveolens* were active against *B. cereus*, *L. monocytogenes* and *S. aureus* with 12-17 mm of inhibition zone (800 μg per disc) and MIC of 0.66-2.64 mg/mL (Alzoreky and Nakahara, 2003). Despite the differences observed due to the extraction process, in general it can be summed that *R. graveolens* extracts have higher antibacterial activity against Gram-positive bacteria.

Also, the growing conditions and harvesting site may influence the antimicrobial activity, so the methanolic and ethanolic extracts of wild growing and cultivated *R. graveolens* collected at the beginning and the ending of the flowering season have been evaluated. For the antimicrobial assays, three Gram-positive (*B. cereus* ATCC 10876, *L. monocytogenes* ATCC 15313 and *S. aureus* ATCC 6538), three Gram-negative (*E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and *Salmonella enteritidis* ATCC 13076) and two fungal strains (*Candida albicans* ATCC 10231 and *Aspergillus niger* ATCC 16404) were used. The samples exhibited moderate antimicrobial activity against most of the bacterial and fungal strains tested, particularly against Gram-positive bacteria, being the methanolic extract of wild growing plants collected at the beginning of the flowering season and the ethanolic extract of wild-growing plants collected at the beginning of the flowering season the most active (MIC 6.25-25 mg/mL). With this study the authors showed the existing differences between cultivated and wild rue, solvents used for extraction and harvest period, with the antimicrobial activity being higher for ethanolic extracts of wild rue harvested at the beginning of blossoming stage (Pavlović et al., 2014). A similar behaviour was found for *R.*

chalepensis, with Ouerghemmi et al. (2017) concluding that spontaneous *Ruta* stems methanolic extract was particularly effective against *S. aureus* and *P. aeruginosa* with diameters of inhibition of 16.3 mm and 17.7 mm (50 µL of 5 mg of extract per disc) when compared with cultivated *R. chalepensis* extracts. Nonetheless, cultivated *Ruta* stem extracts also exhibited antibacterial activity with the highest being against *E. coli* (17.3 mm) (Ouerghemmi et al., 2017).

Further studies were taken considering that nanoparticles could be an alternative to the antibiotics drug resistance, due to its unique mode of action (Sivakamavalli et al., 2014). *R. graveolens* was used for the synthesis of green chemistry-based silver nanoparticles (AgNPs) and its efficiency as antibacterial, antifungal and antibiofilm agents was evaluated against multiple antibiotic resistant bacteria and yeast (*S. aureus* MTCC 9542, *P. aeruginosa* MTCC: 4673 and *C. albicans* MTCC 7315). The *R. graveolens* AgNPs showed good antimicrobial activity against the pathogens *S. aureus* and *P. aeruginosa* (5 and 4 µg/mL, respectively) and effective antifungal and antibiofilm activity against the fungal pathogen *C. albicans* (5 µg/mL and 40–60 µg/mL, respectively) (Sivakamavalli et al., 2014).

Concerning crude extracts of *R. montana* leaves, they showed an inhibitory effect between 5.0–58.0 %, being the most active the methanolic extract (40-58 %), followed by chloroform and ethyl acetate extracts (20–42.1 % and 18–53 %) and at last the hexane extract (12.4–18.3 %). The MIC values of the extracts ranged from 250 to 3000 µg/mL for methanol, ethyl acetate and chloroform extracts, and hexane extract have not demonstrated any potential antifungal effect (Hammami et al., 2015).

6.1.2 Essential oils

Also, essential oils have been studied regarding its antimicrobial activity taking into account the factors that may affect its activity. Regarding to harvest places, the antibacterial activity of *R. chalepensis* EO from plants obtained in three different regions against *E. coli, P. aeruginosa, S. aureus* and Methicillin-resistant *S. aureus* demonstrated that the best activity was from Jerusalem region (0.75-7 mg/mL) followed by the EO obtained from Hebron region (1.5-9 mg/mL). Also, the weakest antibacterial activity was for the EO obtained from Jenin region (10-22 mg/mL). *P. aeruginosa* was the specie more resistant in all regions. Regarding the antifungal activity against *C. albicans*, the lowest MIC found for rue's EO was for the plants collected from Jenin region, followed by Jerusalem and Hebron regions (2.75-4 mg/mL) (Jaradat et al., 2017). Thus, the different places of harvest can influence the antimicrobial activity, with EO presenting different profiles of antibacterial and antifungal activities (Jaradat et al., 2017). Also, the antimicrobial activity of the EO of

R. chalepensis collected in North Lebanon revealed moderate *in vitro* antifungal activity against *Trichophyton rubrum* and *C. albicans* (MIC of 512 µg/mL), while the bacterial strains of *S. aureus* and *E. coli* were not susceptible to this EO (MIC > 512 µg/mL) (Khoury et al., 2014).

The different parts of plants also play a role in the influence of their antimicrobial activity. A study about *R. chalepensis* essential oil, conclude that leaves EO to have a significant higher antibacterial activity against all test microorganisms (50-400 µg/mL), followed by stems' EO (100-783.33 µg/mL) and at last the roots' EO (350-800 µg/mL) (Amdouni et al., 2016).

As for crude extracts, also the composition of the essential oils is influenced by several factors. Bouajaj et al. (2014) proposed that the antifungal activity of *R. chalepensis* EO against phytopathogenic strains is related to the proportions of both monoterpenes and sesquiterpenes. However, other major or trace components in the oil could also give rise to part of the antifungal activity, due to a possible synergistic or antagonistic interaction between the EO components (Bouajaj et al., 2014).

Such as extracts, *R. graveolens* EO had a more relevant antibacterial activity. When evaluating several Gram-positive and Gram-negative bacteria (*B. cereus* ATCC 11778, *S. aureus* ATCC 25923, *M. flavus* ATCC 25923, *Micrococcus luteus* ATCC 9341, *E.* coli ATCC 25922, *Enterobacter aerogenes* ATCC 13048, *P. aeruginosa* ATCC 10145 and *Salmonella* Typhi ATCC 19430), the MIC values ranged from 0.75 to 75 µg/mL and the most susceptible bacterium was *M. flavus* and the less susceptible was *P. aeruginosa* (Orlanda and Nascimento, 2015). Chaftar et al. (2016) showed that EO from *R. graveolens* were active against all the tested species amongst the 11 bacterial and seven fungal species, except for *Legionella pneumophila* and *Bacillus megaterium* (MIC < 0.02 and 0.50 mg/mL, respectively) (Chaftar et al., 2016). The results of Owlia and co-authors (2009) presented no antimicrobial activity of *R. graveolens* EO against *P. aeruginosa* (ATCC 27853) (Owlia et al., 2009). However, the *R. graveolens* EO from Tunisian plants demonstrated a strong anti-*Legionella* activity (MICs ≤ 0.06 mg/mL) against six of seven isolates from Tunisian spas and two reference strains. Authors suggested that the anti-*Legionella* activity may be due to the high content of 2-undecanone, being however necessary more studies to understand if or even other major or less abundant components may have a role in this activity (Chaftar et al., 2015). The EO from *R. graveolens* also inhibited all *Staphylococcus* strains and *Candida* species isolated from patients with acute otitis externa, with inhibition halos between 10 and 13 mm in diameter (Nogueira et al., 2008). Mycotoxins are natural contaminants produced by diverse fungal species that can contaminate plant materials used as food and even after processing. So, the effect of *R. graveolens* EO was tested on the

growth and aflatoxin production of *Aspergillus parasiticus*. The use of *R. graveolens* essential oil showed to inhibit the aflatoxin production, even if the fungal growth inhibition was marginal (Soares et al., 2016), thus indicating that despite not inhibiting cellular growth the EO may influence the virulence factors production of microorganisms.

Yosra et al. (2019) compared the EO of *R. graveolens* and *R. montana* from Tunisian plants regarding its antibacterial activity. Using the disc diffusion method, the authors concluded that the antibacterial activity was higher with *R. montana* EO than *R. graveolens* EO. The inhibition zones (10 µL/disc, 6 mm disc) were 21 mm, 17 mm and 21 mm for *P. aeruginosa* ATCC 7624, *S. aureus* and *S. aureus* ATCC 76110, respectively, with *R. montana*, whereas the *R. graveolens* essential oil showed lower values (Yosra et al., 2019). *R. montana* also exhibited a moderate to high antifungal activity against some plant pathogenic fungi (*Fusarium oxysporum, Botrytis cinerea, Aspergillus oryzea, Verticillium dahliae, Rhizoctonia solani* and *Fusarium solani*). The EO showed an inhibitory effect against the growth of these fungi between 40 and 80% and with the MIC values ranging from 160 to 1100 µg/mL, among which *B. cinerea* was found to be the most susceptible fungal pathogen (Mohammedi et al., 2019).

On the other hand, when EO of *R. montana* from Melouane (Northern Algeria) was tested, it did not inhibit the bacterial growth of *E. coli*, however after EO thionation, the activity towards this bacterium was remarkably enhanced. At the same concentration, the EO had no inhibition zone and the thionated EO had 25 mm. Relative to the antifungal activity, the inhibition zone went from 11 mm to 32 mm at the lowest concentration (0.1%) for *C. albicans* ATCC 10231. The antimicrobial activity of *R. montana* EO was improved by thionation of the EO, which alteration of the composition may be the cause for the increase in the biological activity (Fekhar et al., 2017).

In a study evaluating the antimicrobial activity of the essential oils of the antimicrobial different *Ruta* species, inhibition zones (10 µL/6 mm disc) from disc diffusion assays ranged from 6 to 17 mm for bacterial strains, where the *R. chalepensis* essential oil had the highest activity observed against *S. aureus* and *S.* Typhi with the inhibition zones of 15 and 17 mm, respectively. *R. graveolens* and *R. tuberculata* oil exhibited weak to modest antimicrobial activity. The weakest antibacterial activity was found for *R. angustifolia* essential oil, which presented no activity against the tested strains. For fungal strains, the inhibition zones ranged from 8 to 35 mm with the highest antifungal activity found with *R. angustifolia* and *R. graveolens* EO against *C. albicans* (35 and 33 mm) and in *R. chalepensis* and *R. tuberculata* EO against *Cladosporium herbarum* (35 and 34 mm). When deepening the study, the lowest MIC value was found in *R. graveolens* EO against *C. albicans* (18 µg/mL) and for filamentous fungi (*Aspergillus fumigatus* (MNHN 566), *Aspergillus flavus* (MNHN

994294), *C. herbarum* (MNHN 3369), *Fusarium oxysporum* (MNHN 963917), *Alternaria alternaria* (MNHN 843390)) with MIC values between 3.5 and 8.7 µg/mL. The authors analysed the chemical composition of the different EOs and concluded that these differences of antimicrobial activity between the *Ruta* species is probably due to the different chemical composition of the EOs. The presence of 95.63% of ketones in the total *R. angustifolia* oil was probably the cause of no antibacterial activity. The monoterpene hydrocarbons present in *R. graveolens* and *R. chalepensis* can be associated with the modest antibacterial nature and the presence of monoterpene alcohols and monoterpene hydrocarbons can be responsible for the activity of *R. tuberculata* against *E. faecalis* and *B. cereus* (Haddouchi et al., 2013).

Globally, *Ruta* species may be presented as an approach for developing natural fungicide and bactericide compounds.

6.1.3 Isolated compounds

Isolated compounds obtained from extracts of plants of this genus have also been tested. Taking into account the diversity of results presented for the various *Ruta* species and that the composition influences the bioactive properties of plants, it is important to study the activity of the isolated compounds because the interactions between compounds lead to synergistic, additive or antagonistic effects. These effects could contribute to design more potent antimicrobial blends because some antimicrobials when applied as single compounds have a different activity than some mixtures (Hyldgaard et al., 2012). The antifungal activity of new alkaloid 5-(1,1-dimethylallyl)-8-hydroxyfuro[2-3-b] quinoline and the known biscoumarin daphnoretin obtained from the chloroform fraction of the *R. chalepensis* ethanolic extract were evaluated against the phytopathogenic fungi *R. solani, Sclerotium rolfsii* and *F. solani*. The percent germination of *R. solani* and *S. rolfsii* sclerotia decreased with an increasing concentration of the two compounds, where the biscoumarin daphnoretin was responsible for strongest inhibition. The data suggest that alkaloids and coumarins may be some of the rue' compounds having an important role in the chemical defense against plant pathogens (Emam et al., 2010).

The effect of graveoline, isolated from *R. angustifolia,* combined with alkaloid-antibiotics, erythromycin or vancomycin, was studied. The results showed that the combination between graveoline, a natural quinolone alkaloid, and the antibiotics tested demonstrate a synergistic effect against *S. aureus* ATCC 25923 and partial synergy effect against *E. coli* ATCC 25922. This study revealed a potential alternative antimicrobial combination agent to the delay of the emergence of resistance in bacteria (Kamal et al., 2018).

The antimicrobial potential of rutin-loaded chitosan nanoparticles (CRNPs) was evaluated against *B. thuringiensis, Bacillus pumilus, P. aeruginosa, Acinetobacter junii, E. faecalis* in order to explore an antimicrobial potential of rutin. Rutin is a polyphenol compound that possesses strong antioxidant and antimicrobial properties and was isolated from the ethyl acetate fraction from stems of *R. graveolens* ethanolic extract. The load of rutin into the nanoparticles enhanced its potential, with *B. pumilis* and *E. faecalis* being the most sensitive pathogens. The MIC results showed a dominance of CRNPs (7.81–15.62 μg/mL) over bare rutin (31.25–62.50 μg/mL) and chitosan (62.5–250 μg/mL). The possible potential of CRNPs may be associated with synergistic antimicrobial properties between chitosan and rutin enhancing the antimicrobial potential of the nanoparticles (Patil and Jobanputra, 2015). Thus, the use of plant extracts to develop nanomedicine against various human and clinical pathogens could be an alternative approach to combat many infections and drug resistance.

6.2 Antiviral activity

Regarding antiviral potential activities, Wahyuni et al. (2014) determined the possible anti-HCV (Hepatitis C virus) activity of *R. angustifolia* extracts, their subfractions and isolated compounds. The results revealed that a dichloromethane extract of *R. angustifolia* leaves possessed the most potent activity, suggesting that a semi-polar compound(s) extracted by dichloromethane was involved in the anti-HCV activity. In the continuous of this work, they identified chalepin and pseudane IX as anti-HCV compounds and their anti-HCV activities were stronger than that of ribavirin (2.8 ± 0.4 μg/mL), which has been widely used for the treatment of HCV infection. The analysis of the mode of action of chalepin and pseudane IX showed that HCV inhibition occurred at the post-entry step and that these compounds diminished the levels of HCV RNA replication and viral protein synthesis. So, these extracts are possible candidates as anti-hepatitis C virus (Wahyuni et al., 2014). In another study, Wahyuni et al. (2019) combined the ethanolic extract of *R. angustifolia* leaves and with current antiviral drugs, simeprevir and telaprevir. The combination of *R. angustifolia* extract and simeprevir or telaprevir had a synergistic effect on the inhibition of hepatitis C virus. This synergism revealed higher inhibition of virus compared to treatment with either of the drugs alone (Wahyuni et al., 2019).

6.3 Antiparasitic activity

Parasitic diseases remain a major public health problem and the increasing drug resistance has limited the usefulness of some of the existing compounds. Parasitic diseases occur

mostly in lower-income countries, so traditional remedies are essential for the treatment of several diseases and abound in most endemic regions (Tagboto and Townson, 2001).

Among the various biological activities described for *Ruta* species, antiparasitic activity has been reported for crude extracts and EO (Table 5). When considering the tested individual compounds, 2-undecanone show to be the most active compound found in *R. chalepensis* extract presenting nematocidal and anthelmintic activities (Akkari et al., 2015; Ntalli et al., 2011). On the other side, rutin and 8-methoxypsoralen showed no nematocidal activity (Ntalli et al., 2011). The chalepensin from *R. chalepensis* methanolic extracts was found as the bioactive compound responsible for the antiprotozoal activity against *Entamoeba histolytica* (Quintanilla-Licea et al., 2014). For plant pathogens like *Bursaphelenchus xylophilus* and others, the EO fractions with oxygen-containing molecules and components like 2-undecanone, thymol, carvacrol, *p*-cymene and/or *c*-terpinene were the ones with higher nematotoxic activity (Faria et al., 2013, 2016a,b). Also, the different parts of the plant have distinctive activities, with the essential oil of *R. chalepensis* leaves showing higher inhibitory effects than flowers essential oil on egg hatching rate and flowers essential oil inhibited more worms of *Haemonchus contortus* than the leaves essential oil (Akkari et al., 2015).

Moreover, *Ruta* extracts and essential oils showed good anthelmintic activity against gastrointestinal nematodes demonstrating reductions of viability, of potential for invasion and multiplication rate of the parasites (Ortu et al., 2017; Teixeira et al., 2014). Further, the *Ruta* extracts showed low cytotoxicity to host cells in the treatment against *Leishmania* demonstrating the selectivity for these parasites (Ahmed et al., 2011; Faria et al., 2013). Based on these data, the *Ruta* plants exhibited considerable activity for antiparasitic control and EOs are potential environmentally friendly alternatives (De Queiroz et al., 2014; Faria et al., 2013).

Table 5. Insecticidal and larvicidal activities of *Ruta* species.

Biological activity	*Ruta* species	Part used	Mode of preparation	Activity against	Reference
Insecticidal and/or larvicidal activity	*R. chalepensis*	leaves and stems	aqueous extract	Sweet potato whitefly *Bemisia tabaci* immature stages	(Al-mazra'awi and Ateyyat, 2009)
		leaves	aqueous ethanolic extract (80%)	Larvae *Spodoptera littoralis*	(Emam et al., 2009)
		leaves	choroform fraction of the methanolic extract	Rice weevil, *Sitophilus oryaze* L. adults	(Jeon et al., 2013)
		aerial parts	diethyl ether and methanolic extracts	Dengue mosquito *Aedes aegypti*	(De La Torre Rodriguez et al., 2013)
		leaves and stems	methanolic extracts	Adulticidal activities against *Aedes aegypti*	(Al-Massarani et al., 2019)
		aerial parts	hydrosols	Crop pests *Aphis gossypii* and *Tetranychus urticae*	(Traka et al., 2018)
		aerial parts	methanolic extracts	*Culex pipiens* larvae	(Abdel-Sattar et al., 2014)
		whole plant	methanolic extracts	Early larvae of *Culex pipiens pallen*s and against *Aedes aegypti*	(Kim et al., 2002)
		fresh leaves and flower	essential oil	Third and fourth instars larvae of *Orgyia trigotephras*	(Akkari et al., 2015)
		fresh leaves	essential oil	Asian tiger mosquito, *Aedes albopictus* Skuse	(Bedini et al., 2018; Conti et al., 2013)
		aerial parts	essential oil	Flour beetles, *Tribolium castaneum* and *Tribolium confusum*	(Abbad et al., 2014)
	R. montana	aerial parts	essential oil	Adult male, *Blatella germanica* cockroach and adult and larvae *Culex pipiens* mosquitoes	(Boutoumi et al., 2009)
	R. graveolens	leaves	ethanolic extract	Fly *Ceratitis capitata*	(Ghabbari et al., 2018)
		leaves	essential oil	Third-instar larvae of *Aedes aegypti* and juvenile nematodes of *Meloidogyne incognita*	(Da Silva et al., 2014)

Table 5. Insecticidal and larvicidal activities of *Ruta* species (continuation).

Biological activity	*Ruta* species	Part used	Mode of preparation	Activity against	Reference
Repellent effect	*R. chalepensis*	leaves and stems	aqueous extract	Sweet potato whitefly *Bemisia tabaci* adults	(Al-mazra'awi and Ateyyat, 2009)
		fresh leaves	essential oil	Asian tiger mosquito, *Aedes albopictus* Skuse	(Conti et al., 2013)
		aerial parts	essential oil	Weevil *Aegorhinus superciliosus*	(Tampe et al., 2016)
		leaves	essential oil using coconut oil as a solvent	Mosquitoes (mainly *Mansonia*)	(Hadis et al., 2003)
	R. montana	aerial parts	essential oil	Insect pest of stored grains *Ephestia kuehniella*	(Bouzeraa and Labed, 2019)
Biting deterrents	*R. chalepensis*	aerial parts	essential oil	*Aedes aegypti* and *Anopheles quadrimaculatus*	(Ali et al., 2013)
Molluscicide activity	*R. chalepensis*	aerial parts	hexane and dichloromethane extract	*Bulinus truncatus*	(Hmamouchi et al., 2000)
Antiparasitic activity	*R. chalepensis*	aerial parts	methanolic extract	Root knot nematodes *Meloidogyne incognita* and *Meloidogyne javanica*	(Calzada et al., 2006; Jasso Díaz et al., 2017; Molina-Garza et al., 2014; Ntalli et al., 2011; Ortu et al., 2017; Quintanilla-Licea et al., 2014)
				Gastrointestinal nematodes *Teladorsagia* spp., *Haemonchus contortus* and *Trichostrongylus* spp. *Entamoeba histolytica* and *Giardia lamblia* trophozoites	
				Trypanosoma cruzi epimastigotes	
		fresh leaves and flower	essential oil	*Haemonchus contortus* from sheep	(Akkari et al., 2015)
		whole plant	essential oil	*Leishmania major* and *Leishmania infantum*	(Ahmed et al., 2011)
		aerial parts	essential oil	Root knot nematodes *Meloidogyne incognita* and *Meloidogyne javanica*	(Ntalli et al., 2011)
	R. graveolens	aerial parts	aqueous Extracts	*Leishmania amazonensis*	(De Queiroz et al., 2014)
		aerial parts	hydroalcoholic extract	*Leishmania amazonensis* and *Trypanosoma cruzi* Adult worms of *Schistosoma mansoni*	(De Carvalho et al., 2019; Teixeira et al., 2014)

Table 5. Insecticidal and larvicidal activities of *Ruta* species (continuation).

Biological activity	*Ruta* species	Part used	Mode of preparation	Activity against	Reference
		aerial parts	essential oil	Pinewood nematode, *Bursaphelenchus xylophilus* L. Columbia root-knot nematode, *Meloidogyne chitwoodi*	(Faria et al., 2016, 2013)
	R. pinnata	mature fruits	methanolic extract	Caprine apicomplexan *Eimeria ninakohlyakimovae*	(López et al., 2018)
Fumigant activity	*R. graveolens*	leaves	essential oil	Adults stored date pests *Ectomyelois kuehniella* and *E. Ceratonia*	(Chaaban et al., 2019)
	R. montana	aerial parts	essential oil	Insect pest of stored grains *Ephestia kuehniella*	(Bouzeraa and Labed, 2019)

6.4 Insecticidal and larvicidal activities

Considering the vast potential of plants in the combat of insects and as a repellent and its traditional use, *Ruta* species have been studied in regard to its insecticidal and larvicidal activities. Many insect pests that are responsible for plant and fruit damage, problems in stored food products, postharvest grains, and processed goods (Jeon et al., 2013), but also serves as vectors of dangerous diseases, such as dengue, malaria, yellow fever, filariasis, and other viral infections (Al-mazra'awi and Ateyyat, 2009; Ali et al., 2013; Conti et al., 2013; Jeon et al., 2013; Tampe et al., 2016). Synthetic insecticides are effective, although associated with disadvantages, as lack of effectiveness, resistance against pesticides, environmental pollution, and toxicity on non-target organisms (Akkari et al., 2015; Jeon et al., 2013). For these reasons, the investigation for alternative approaches has grown and natural pest control methods have been increasingly explored, looking for bioinsecticides that demonstrate a high degree of insect elimination and low toxicity to humans (Akkari et al., 2015; De La Torre Rodriguez et al., 2013).

Table 5 presents the insecticidal and larvicidal activity of *Ruta* species, where the activity of the crude extract is highlighted; however also isolated compounds showed potential activities. For example, quinoline and its derivatives isolated from *R. chalepensis* leaves proved to be the most toxic compound against *Sitophilus oryzae* and *Spodoptera littoralis* comparing to the results obtained for crude extracts and respective fractions (Emam et al., 2009; Jeon et al., 2013). In another study, the authors concluded that the insecticidal properties of leaves and flower essential oils from *R. chalepensis* can be attributed to the abundance of ketones (Abbad et al., 2014; Akkari et al., 2015; Ali et al., 2013; Tampe et al., 2016). It can be said that the relationship between the chemical structures of the compounds in the tested oils and their proportions influence the pharmacological activities (Akkari et al., 2015).

As previously seen, the composition variation depends on several environmental factors and can influence the biological activity of the plant. For example, EO from *R. graveolens* was investigated with basis on seasonal variation (October 2009 to September 2010) and the results showed that during dry periods the major compounds (2-undecanone and 2-nonanone) were present in higher levels enhancing the nematicidal and larvicidal activities of the essential oil (Da Silva et al., 2014). Also, *R chalepensis* EOs demonstrated similar activity to the commercial Deet (diethyl toluamide) (Ali et al., 2013; Hadis et al., 2003) showing a dose-dependent larvicidal activity which was improved with time exposure (Da Silva et al., 2014; De La Torre Rodriguez et al., 2013). The *Ruta* species can be considered as a potential bioinsecticides that could reduce infestation (Da Silva et al., 2014; De La Torre Rodriguez et al., 2013; Tampe et al., 2016).

6.5 Antioxidant activity

Antioxidants compounds neutralize free radicals and their negative effects, while acting on prevention, interception, and repair, using mechanisms as reducing agents by trapping free radicals, donating hydrogen, acting as chelators and quenching singlet oxygen (Kacem et al., 2014).

Commonly, synthetic chemical antioxidants are used to extend the shelf life of food products, however the increased intake of these chemicals may cause toxic or carcinogenic effects to consumers (Jaradat et al., 2017; Ouerghemmi et al., 2017). The antioxidants are also important in human health, possibly improving the quality of life by preventing or delaying the onset of diseases (Loizzo et al., 2018). In human physiological and pathophysiological processes, the reactive oxygen species (ROS) have a crucial role and can disturb the normal cells function due to an imbalance between the radical formation and protection (Kacem et al., 2014; Loizzo et al., 2018). ROS formed in normal metabolic reactions leads to oxidative damage in biological molecules, such as nucleic acids, proteins, lipid and can be responsible for the initiation or aggravation of diverse pathological states (Kacem et al., 2014). Due to all the side effects of the synthetic antioxidants, the research for natural agents is growing, with plants being presented as a valuable source of natural antioxidants such as phenolic compounds, flavonoids and vitamins (Jaradat et al., 2017; Ouerghemmi et al., 2017).

Ruta species have been a target for these studies presenting good antioxidant potential. The free radical scavenging activity of decoction and ethanol extracts obtained from *R. chalepensis* and *R. monatana* stems and leaves have been studied with the 2,2'-diphenylpicrylhydrazyl (DPPH) test. The extracts from both plants have shown high antioxidant activity, with EC_{50} from 1.47 ± 0.1 μg/mL to 2.26 ± 0 μg/mL, presenting the most promising results. The results were near to the antioxidant activity of the standard gallic acid (EC_{50} = 0.95 ± 0.04 μg/mL). Even when subjected to the action of synthetic gastric and pancreatic juices the antioxidant activity was maintained, indicating a good potential for its use in phytotherapy (Khadhri et al., 2017). As it happened with other biological activities, the antioxidant activity is influenced by diverse factors such as the species, part of the plant and solvent used in the extraction. Kacem et al. (2015) evaluated the effect of the solvent used in the extraction and they demonstrated that the ethanolic extract of *R. chalepensis* aerial parts exhibited the most potent antioxidant activity followed by methanol and methanol/water extracts (Kacem et al., 2015). In another study, the aerial parts of *R. chalepensis* were extracted with ethanol and successively extracted using other solvents. The antioxidant activity of *R. chalepensis* showed DPPH radical scavenging activity in a concentration–dependent manner and the best activity was obtained by ethyl

acetate/*n*-butanol extract (% DPPH scavenging = 94.28%, IC_{50} = 56.6 µg/mL), being followed by ethanol (% DPPH scavenging = 87.51%, IC_{50} = 320.7 µg/mL) and ether/chloroform extracts (% DPPH scavenging = 80.37%, IC_{50} = 414.9 µg/mL). All extracts possessed very promising antioxidant activities compared with the standard ascorbic acid (% DPPH scavenging = 86.36%, IC_{50} = 11.20 µg/mL) (Alotaibi et al., 2018). This activity may be depending on phenolic content, with a direct correlation between phenolic content and antioxidant activity (Gali and Bedjou, 2019).

Comparing the different parts of the plant, it was suggested that the biological activities of the methanolic extracts of leaves, roots and stems of *R. chalepensis* have significant differences in their antioxidant activities. The highest antioxidant activity was recorded in leaves methanolic extract (Ghazghazi et al., 2015). The total antioxidant capacity of spontaneous and cultivated *R. chalepensis* was higher in the leaves and flowers and significantly lower in stems, with a scavenging ability of the wild flower extract (IC_{50} = 23.73 µg/mL) higher than the one found for the standard Butylated hydroxytoluene (BHT, IC_{50} = 25 µg/mL). Regarding their reducing power, the wild and cultivated leaf extracts and the cultivated flower extract demonstrated the highest reducing power (IC_{50} = 0.90 mg/mL, 0.92 mg/mL, and 1.10 mg/mL, respectively), followed by the wild and cultivated stem extracts ($IC_{5\,0}$ = 1.96 mg/mL and 2.05 mg/mL, respectively). It can be concluded, that in general the leaves and flowers of wild and cultivated *R. chalepensis* have a better antioxidant activity (Ouerghemmi et al., 2017).

In another study, the antioxidant activities of *R. chalepensis* methanolic leaf extract were performed using different tests: DPPH, β-carotene bleaching and metal chelating activity assays. The results suggest that the extract was able to reduce free radical DPPH (IC_{50} = 60.2 µg/mL; BHT = 12 µg/mL), showing a promising inhibition of lipid peroxidation (IC_{50} = 16.9 µg/mL; BHT = 8.5 µg/mL), but presenting a low metal chelating activity (14.6%) compared to the control ethylenediamine tetraacetic acid (EDTA) (97.8%) (Loizzo et al., 2018). Another study also investigates the antioxidant activities of methanolic extracts from *R. chalepensis*, collected in Centre of Tunisia, by DPPH and 2,2'-azinobis-3-ethylbenzothiazoline-6-sulphonate (ABTS) and 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH) assays. This study shows that *R. chalepensis* extracts possess antioxidant activity using different *in vitro* assays (Khlifi et al., 2013). Overall, through the use of different methodologies it was shown that *R. chalepensis* extracts have antioxidant activity by different mechanisms (Kataki et al., 2014). The composition of plant extract can act through various mechanisms like binding of transition metal ion catalysts, reduce capacity and radical scavenging, prevention of chain initiation, decomposition of peroxides and prevention of continued hydrogen abstraction, so it is important to use methodologies with distinct mechanisms to evaluate the antioxidant activity (Gali and Bedjou, 2019).

Also, essential oils have been studied and presented promising results. When considering the influence of the harvest region of the plants, DPPH results showed that the EO obtained from *R. chalepensis* leaves collected in Jerusalem region showed the highest antioxidant potential (% inhibition = 69.56%; IC_{50} = 6.9 ± 0.94 μg/mL) followed by the EO obtained from Hebron (% inhibition = 61.53%; IC_{50} = 7.8 ± 1.05 μg/mL) and Jenin (% inhibition = 24.12%; IC_{50} = 19.9 ± 0.68 μg/mL) regions (Jaradat et al., 2017). The vegetative and the flowering stages of the plants and the plant organs also influenced chelating activity, which were affected by the plant organs. The lowest chelating activity of *R. chalepensis* was measured in the leaves EO and the highest was found in the stems and flowers EO, when the parts were collected at the flowering stage (Krayni et al., 2015).

Regarding to antioxidant activity of *R. graveolens*, ethanolic extracts showed higher DPPH scavenging activity (% inhibition = 59.3%) than the hexane extract (% inhibition = 16.8%) at the same concentration (250 μg/mL) (Molnar et al., 2017). Furthermore, methanolic and ethanolic extracts of wild growing and cultivated rue at the beginning and the end of flowering season exhibited significant antioxidant potential. The methanolic extract of wild *R. graveolens* collected at the end of flowering season exhibiting the strongest antioxidant activity in DPPH and *β*-carotene-linoleic acid tests (IC_{50} = 36.36 ± 1.20 μg/mL and IC_{50} = 31.06 ± 2.30 μg/mL, respectively). The antioxidant effects in the *β*-carotene-linoleic acid test system of this extract are similar to the antioxidant activity of rutin (IC_{50} = 33.29 ± 1.49 μg/mL) (Pavlović et al., 2014).

Also, for *R. montana* EO, DPPH assay showed antioxidant activity in a concentration-dependent manner and producing 50% of inhibition at extract concentration of 16.7 μL/mL (Kambouche et al., 2008). Mohammedi et al. (2019) studied samples of *R. montana* collected from different locations in Algeria that exhibited significant free radical reducing capacity and ferric reducing power (Mohammedi et al., 2019). In this work it was possible to observe the influence of the geographical location of the harvest of plants in their bioactive activity, as already described above.

In sum, the extracts and EO of *Ruta* species display antioxidant activity through different mechanisms, in a concentration-dependent manner, with a positive correlation between total phenolic content and values of antioxidant activity (Ouerghemmi et al., 2017).

6.6 Neuroprotective activity

Neurodegeneration is a characteristic of many incurable and debilitating diseases that are rising in prevalence very quickly (Gitler et al., 2017). Neurodegenerative diseases cause progressive loss of cognitive and/or motor function (Gan et al., 2018) and are often caused by the accumulation of oxidative damage, inflammation, and protein aggregation (Heidari

et al., 2020). Therefore, the develop of new and more effective therapeutic strategies is an urgent need to combat these diseases (Gitler et al., 2017).

Alzheimer's disease (AD) is a chronic progressive degenerative disease of the central nervous system and is the most common form of dementia among the elderly (Adsersen et al., 2006; Li et al., 2016). The degeneration of the cerebral cortex causes the loss of its normal function, like judgment, memory, reasoning ability, abstract thinking ability, and spatial relationship (Li et al., 2016). The decreased levels of acetylcholine in the brain areas in AD patients is related to memory and learning problems (Adsersen et al., 2006), with the inhibition of the enzyme acetylcholinesterase (AChE) in early and moderate stages of AD being seen as an approach to treat this condition (Adsersen et al., 2006; Khadhri et al., 2017; Wszelaki et al., 2010). Several works have evaluated *Ruta* species regarding its potential as inhibitors of acetylcholinesterase. *R. graveolens* aqueous and methanolic extracts, exhibited moderate inhibition of the enzyme AChE (22 and 39% with 0.1 mg/mL respectively) (Adsersen et al., 2006). The evaluation of *R. chalepensis* and *R. montana* leaf and stem extracts (water decoctions and ethanol extracts) demonstrated the greatest inhibitory activity for leaf ethanolic extract of *R. chalepensis* (IC_{50} = 12 ± 1.1 μg/mL) (Khadhri et al., 2017). Regarding, isolated compounds and derivates, several presented promising *in vitro* results. Graveoline and a series of new graveoline analogs synthetized taking as basis the structural characteristics of AChE dual-site inhibitors, displayed an inhibitory ability and selectivity against AChE, with the cytotoxicity study showing that the neurocyte viability was unaffected at low compound concentrations (Li et al., 2016). Butyrylcholinesterase (BuChE) inhibition has additionally been a focus of study, since BuChE activity increases in AD with the severity of dementia progresses (Wszelaki et al., 2010). Among several plants used in traditional European medicine, one of the most potent concerning AChE and BuChE inhibition was *R. graveolens* hexane extract at a concentration of 400 μg/mL. This extract had a dose-dependent inhibitory activity and the inhibition was higher than 50 % at the concentration of 100 μg/mL, the lowest concentration used (Wszelaki et al., 2010). So, it can be concluded that the extracts of rue have phytotherapeutic potential in AChE and BuChE inhibition activity and these compounds offer a potential for application and further research on the treatment of AD.

Other compounds that may be useful for AD and also anxiety disorders and neurodegenerative related illnesses such as Parkinson's are MAO-B inhibitors (Stafford et al., 2007). Monoamine oxidase (MAO) is an enzyme that was present in the neuronal and non-neuronal cells outer mitochondrial membrane and presents two isoforms of MAO commonly referred to as MAO-A and MAO-B. These enzymes are responsible for the oxidative deamination of endogenous and xenobiotic amines and the inhibitors of MAO cause an increase in the amount of these amines stored and released from the nerve

terminals, increasing the monoaminergic activity (Yamada and Yasuhara, 2004). In an investigation to screen southern African plants different solvents were used and the results show that the nonpolar extracts (ethyl acetate and petroleum ether leaf extract) of *R. graveolens* material exhibited the best MAO inhibitory activity and specific MAO-B inhibition (Stafford et al., 2007). These findings may lead to the discovery of novel MAO inhibitors based on natural sources such as *Ruta* plants.

The chronic inflammatory multiple sclerosis is a demyelinating disorder of the central nervous system (CNS), for which clinical evidence related to medicinal plants was demonstrated for management of MS symptoms. Among different medicinal plants, the highest level of clinical evidence was observed for *R. graveolens*, encouraging its potential efficacy on MS symptoms (Farzaei et al., 2017).

Epilepsy is another neurological disorder to which medicinal plants have been used especially in rural communities to manage and treat epilepsy. The possible anticonvulsant effect of *R. graveolens* was investigated in mice by studying the effect of the leaf methanolic extract against seizures provoked by pentylenetetrazole (PTZ), picrotoxin, bicuculline or N-Methyl-DL-Aspartic acid. The data obtained, indicate that *R. graveolens* extracts, like phenobarbitone, diazepam and muscimol significantly antagonized induced seizures (Ahmad and Amabeoku, 2013). The ethanolic extract of *R. chalepensis* aerial parts were also tested on the central nervous system by systemic administration and evaluation of its effects on sodium pentobarbital-induced hypnosis, PTZ-induced seizures, anxiety, exploratory activity and nociception. Results showed a delay in the onset of seizures induced by PTZ and a dose-dependent suppression, a significant attenuation in the anxiety-response, the time of sodium pentobarbital-induced hypnosis was prolonged and a reduction in shaking behaviour and the licking time in the formalin-induced nociception test. The anticonvulsant, sedative-hypnotic potentiation, anxiolytic and antinociceptive effects propose that *R. chalepensis* induces a depressant activity on the central nervous system (Gonzalez-Trujano et al., 2006).

So, different *Ruta* extracts can be used as potential drugs in neuroprotective activity since they inhibited some enzymes or change the neurotransmitter levels responsible to cause diverse neurological diseases.

6.7 Anti-inflammatory effect

The inflammation is an essential protective process against physical, chemical and infective insults but sometimes this protective response may lead to damaging consequences, since it may respond to several insults erroneously leading to the damaging normal tissues (Ratheesh et al., 2010). The autoimmune disorders are characterized by noticeable

inflammation and associated failure of the repair process and some inflammatory disorders associated with the oxidative stress generated by the cells, complicating further the pathology (Raghav et al., 2006). The most commonly prescribed agents to the management of inflammation and pain include conventional non-steroidal anti-inflammatory drugs, but there are several toxic manifestations associated with the use of these agents. The search for new approaches in inflammation and pain management with lower side effects and toxic manifestations were enhancing (Kataki et al., 2014).

The anti-inflammatory application of *Ruta* species is described in several countries (Table 3), with some studies displaying a positive anti-inflammatory effect of the plants against different insults on *in vitro* and *in vivo* models. In 2013, a study concerning the biological activities of methanolic extract from *R. chalepensis* plant, collected in Centre of Tunisia, showed that this extract possesses anti-inflammatory activities (Khlifi et al., 2013). Also, the ethanolic extract of leaves of *R. chalepensis* showed immunopharmacological properties that contradict the harmful effects of LPS (lipopolysaccharide) in BALB/c mice with endotoxemia (Iauk et al., 2004). The authors of this study proposed that the anti-inflammatory activity of ethanol extract of *R. chalepensis* is due to flavonoids (Iauk et al., 2004). Also, the ethanol and ethyl acetate extracts of *R. chalepensis* and *R. graveolens* methanolic extract decreased the nitric oxide (NO) production in murine RAW 264.7 and J-774 macrophages stimulated with LPS (Kacem et al., 2015; Raghav et al., 2006). Furthermore, the methanolic extract of *R. graveolens* leaves presented a significant inhibitory effect on the edema formation after carrageenan injection and revealed effective anti-inflammatory activity in peritoneal inflammation induced by acetic acid in a carrageenan–induced paw edema model (Kataki et al., 2014). The secretion of interleukin-8 (IL-8) was also tested to examine the anti-inflammatory and cytoprotective effects of aqueous ethanol extract of *R. graveolens* at 100 μg/mL, in gastric epithelial cells infected with *Helicobacter pylori*, demonstrating a strong inhibition of IL-8 secretion, partially validating the use of these plants in traditional medicine in gastrointestinal disorders associated with *Helicobacter pylori* (Zaidi et al., 2012).

In India, *R. graveolens* is used in folk medicine for the treatment of rheumatism, arthritis and other inflammatory conditions (Ratheesh et al., 2010). The traditional use was validated through the use of rat models with carrageenan induced edema and adjuvant induced arthritis. Methanolic extract of *R. graveolens* led to an inhibition of edema at a dose of 20 mg/kg at day 21 of adjuvant arthritis, with this effect being higher than that of the standard drug, indomethacin. The histopathology analysis of tissue showed decreased cellular infiltration and oedema formation on supplementation with *R. graveolens* extract (Ratheesh et al., 2009). The same group of investigation, in another work, studied the isolated polyphenolic and alkaloid fraction from *R. graveolens* to evaluate its anti-

inflammatory activity in carrageenan induced acute model. The alkaloid fraction of *R. graveolens* with a dose of 10 mg/kg showed higher anti-inflammatory activity than polyphenols and the used standard drug, diclofenac (Ratheesh et al., 2010). Then, *R. graveolens* extract traditional use and the laboratory test results point to its potential to be used as an anti-arthritic agent.

Ruta species extracts have been studied regarding their possible application for treatment of other diseases associated with inflammatory processes, as atherosclerosis, asthma or skin inflammation. Considering the potential use as therapeutic treatment of clinical conditions associated atherosclerosis, the *R. graveolens* methanolic extract in hypercholesteremic rats suggested that feed supplementation with methanolic extract reduces inflammation, oxidative stress, and so the aortic pathology in these rats (Ratheesh et al., 2011).

Moreover, when considering alternatives to the used antihistamine drugs for asthma treatment, the active components from *R. chalepensis* methanolic extract of leaves were separated to evaluate the anti-asthma activities. Quinoline and its derivatives showed a strong relaxant effect (IC_{50} = 0.42 mM) on the contractility induced by histamine, of isolated guinea pig trachea. Due to the low dose required to produce a high relaxant effect, *R. chalepensis* extract and quinoline derivatives (quinoxaline, quinolone, quinazoline and 8-hydroxyquinoline) could have the potential for development as commercial anti-asthma agents (Lee and Lee, 2011).

Extracts of *R. graveolens* were used to test the inhibition and induction of enzymes associated with skin inflammation. The cyclooxygenase (COX) enzyme has two main isoforms that are accountable for the initiation of eicosanoids production and at sites of inflammation are responsible for the progression of inflammation. The results exhibit inhibition activity against COX-1 and -2 and can have a potential use to serve as non-selective inhibitors of COX-1 and -2 enzyme activity (Thibane et al., 2019).

Thus, *R. chalepensis* and *R. graveolens* extracts have been traditionally used for the treatment of several diseases where inflammation needs to be controlled (Table 3).

6.8 Anti-neoplastic activity and anti-cancer activity

Anti-neoplastic efficacy is dependent on the ability to induce cell death, to stop proliferation of malignant cells, to upregulate any tumour suppressor pathways and to suppress oncogenicity (Law et al., 2018).

The inhibition of tumour cell proliferation while simultaneously promoting apoptosis is pointed as the main characteristic of cancer treatment (Law et al., 2018). However, the side effects on normal cells from conventional chemotherapies are a concern (Law et al., 2018;

Pathak et al., 2003). So, it is important to find agents that destroy cancer cells but have no effects on normal cells (Pathak et al., 2003), and preferably drugs with both apoptotic and autophagic abilities, since the cancer cells can become resistant and escape of apoptosis signals, but still be killed by autophagy (Ghosh et al., 2014). Therefore, any agent that may suppress the proliferation of malignant cells and at the same time do not affect normal cells may act as a valuable anticancer source and cytotoxicity of a drug is always considered as the first thing to do in the path to understanding its potential as an anticancer drug (Arora et al., 2013).

Richardson et al. (2016) tested the cytotoxicity of methanolic and fractionated extracts (hexane, chloroform, ethyl acetate and water) of *R. angustifolia* against human colon carcinoma (HCT-116), human lung carcinoma (A549), human cervical carcinoma (CaSki) cells and human normal lung fibroblast (MRC5) cell lines. The chloroform extract (without chlorophyll) exhibited the highest cytotoxic activity against A549 cell line, so a bioguided research was directed to this fraction that led to the isolation of 12 pure compounds. Amongst the pure compounds, chalepin displayed excellent cytotoxicity against A549 cell line. The expression of antioncogenes and apoptosis regulators was observed when A549 cells were treated with chalepin, while anti-apoptotic proteins decreased in a time-dependent manner. This data indicated that chalepin-induced cell death might involve the upregulation of pro-apoptotic proteins and downregulation of anti-apoptotic proteins (Richardson et al., 2016). In another study, chalepin demonstrated notable cytotoxic activity against MCF7 cells (human hormone-dependent breast cancer) and moderate cytotoxic activity against MDA-MB231 cells (human non-hormone-dependent breast cancer), while not presenting toxicity against MRC5 cell line (Fakai et al., 2019).

Rutamarin (Figure 1), is one of the chemical components of *R. angustifolia*, which also exhibited remarkable cytotoxicity against colon cancer cells with no damage against the normal human colon cell line. This compound possessed the ability to kill cancer cells at a dose comparable to cisplatin, a currently used agent for the treatment of several human cancers. Moreover, the induction of apoptosis in HT29 cells (human colon carcinoma cell line) triggered by rutamarin occurred in a dose-dependent manner, taking into account the morphological and biochemical evidence of apoptosis (Suhaimi et al., 2017). These findings proved that chalepin and rutamarin are excellent candidates for the development of an anticancer agent.

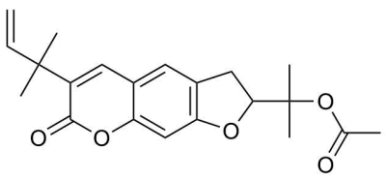

Figure 1. Chemical structure of Rutamarin.

The methanolic extracts from *R. chalepensis* collected in Tunisia have shown to be active against human bladder carcinoma (RT112), human myelogenous leukemia (K562) and human laryngeal carcinoma (Hep2) cell lines (Khlifi et al., 2013) and the ethanolic extract of Italian *R. chalepensis* against colon cancer cell line (Acquaviva et al., 2011).

The comparative study about the cytotoxic activity of the methanolic and ethanolic extracts of wild growing and cultured *R. graveolens* collected at the beginning of the flowering season was made on adherent epithelial cells (HeLa S3 cells, a human cervical adenocarcinoma cell line) (Pavlović et al., 2014). The treatment with extracts had considerable effects on cell proliferation as well as cell morphology. All the tested samples induced inhibition of HeLa cells growth and proliferation (up to 71.81%) and the ethanolic extract of wild growing plants at the beginning of the flowering season showed maximal cytotoxic effect in this assay. These effects were time-dependent since longer exposure to extracts led to stronger inhibition of HeLa cells. These results indicate an important *in vitro* antiproliferative activity of *R. graveolens* extracts in HeLa cells (Pavlović et al., 2014).

Gentile et al. (2015) tested the methanolic extract of *R. graveolens* on colon (HCT116 and RKO), breast (MCF7) and prostate (PC3 and DU-145) cancer cells show to potently inhibit cancer cell proliferation and survival through multiple targets. This study showed that the shrub *R. graveolens* inhibit cell proliferation, activate specific molecular signaling pathways, reduce cell viability and induce DNA damage response and apoptosis that interfere with the survival and proliferation of cancer cells in a dose dependent manner (Fadlalla et al., 2011). A hydroethanolic extract *R. graveolens* was studied to evaluate *in vitro* cytotoxicity activity on solid tumor cell lines: MDA-MB-453, MCF-7, HeLa, SK-OV-3, LNCap-FGC-10, 5637, A549 and Mehr-80; hematopoietic cell lines: U937, RAMOS, RPMI 8866, Jurkat and RAJI and also peripheral blood mononuclear cells (PBMC). The extract showed high cytotoxic activity against RAJI and RAMOS, two Burkitt's lymphoma cell lines, and LNCap-FGC-10, a prostate adenocarcinoma cell line as well as Mehr-80, a large cell lung carcinoma. No significant anti-proliferative activity was observed on other cells. The inhibitory effect of the rue extract was higher against haematopoietic cell lines than solid tumor cells, except for LNCap-FGC-10. The adverse cytotoxic effect of *R. graveolens* on PBMCs was significantly lower compared with RAJI and RAMOS. These results indicate that *R. graveolens* probably has apoptosis inducing capacity with low cytotoxicity on normal mononuclear cells (Varamini et al., 2009). The effects of the aqueous extract of *R. graveolens* on the proliferation of human glioma cells and of neural progenitors from mouse CNS was also evaluated demonstrating that *R. graveolens* induces death in different glioblastoma cell lines (U87MG and U138 human cells, C6 rat cells) while discriminating between proliferating (tumor cells) and non-proliferating neural cells. A major component of the *R. graveolens* water extract, rutin, did not cause cell death, suggesting that rutin by

itself is not the responsible compound for the rue effects. However, the crude extract revealed a potential therapeutic tool in glioblastoma multiforme cancer (Gentile et al., 2015).

A study conducted by Preethi et al. (2006) revealed that *R. graveolens* methanolic extract also possess antitumour activity against ascites DLA (Dalton's lymphoma ascites) and EAC (Ehrlich ascites carcinoma) tumour-bearing animals and reduce the solid tumour in animal models. Nevertheless, it was found that the activity was not concentration dependent as happened in previous works. At higher concentration the activity was reduced compared to what happens with a lower concentration in both ascites and solid tumour model, which may be due to the toxicity of *R. graveolens* extract (Preethi et al., 2006). The anti-neoplastic activity of *R. graveolens* on ascitic Sarcoma-180 bearing mice as a model of human malignant peritoneal ascites was assessed. The study revealed a series of anti-neoplastic events exerted by *R. graveolens* including the boosting of anti-tumour immunity and disruption of cellular energetics. These events led to the induction of apoptosis and impairment of cell division in tumour cells. Also, *R. graveolens* decreased the expression of oncogenic regulator c-Myc supporting the hypothesis that this plant could be an effective chemotherapeutic in the treatment of sarcoma (Law et al., 2018).

Considering isolated compounds, graveoline (Figure 2) also revealed to initiate programmed cell death of human skin melanoma cells (A375 cells) by both apoptosis and autophagy and reduce cell viability in a dose dependent manner. Taking into account that anticancer drugs have to have as a characteristic initiating programmed cell death to the cancer cells, this compound isolated from the ethanolic extract of *R. graveolens* could be a good option to explore as an anticancer drug (Ghosh et al., 2014).

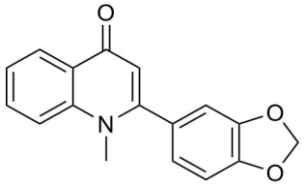

Figure 2. Chemical structure of Graveoline.

The water extract of *R. graveolens* was shown to be able to interfere with the intracellular signaling pathway mediated by ERK1/2 in endothelial cells and effectively inhibit human endothelial cells network formation, in a dose dependent manner and not affecting cell viability. These results point to the role of the extract as a therapeutic strategy to inhibit angiogenesis, which is a relevant bioactivity when considering that the tumour progression and metastasis angiogenic processes are imbalanced and excessive (Gentile et al., 2018).

In sum, the *Ruta* extracts and isolated compounds from these extracts owned anti-neoplastic activity, inhibited the tumour cell proliferation, promoting apoptosis and

inhibiting angiogenesis. These results show the interest of *Ruta* species in search of new chemo-preventive agents against cancer progression or might be useful to prevent carcinogenesis and/or tumour progression, depending on cancer type.

6.9 Anti-diabetic activity

Diabetes is a major health problem that is characterized by hyperglycemia caused by defects in either insulin secretion by the pancreas or insulin action in target tissues (Farid et al., 2017; Loizzo et al., 2018). The drugs used in diabetes treatments are associated with side effects, thus it is important to research and identify drugs that can be used in the management of diabetes with low side effects (Loizzo et al., 2018).

R. chalepensis methanolic extract supplementation was a potent factor in reducing the oxidative severity of zinc deficiency in experimental diabetes through its hypoglycemic and antioxidant actions (Hamdiken et al., 2018). This is important because due the hyperglycaemia, there is an excessive generation of ROS that play a role in the development of complications in diabetes (Loizzo et al., 2018). Therefore, the administration of methanolic extract of *R. chalepensis* reduced the severity of diabetes complications via its antioxidant benefits (Hamdiken et al., 2018). In another study, *R. chalepensis* methanolic extract was investigated for its hypoglycaemic properties, using carbohydrate-hydrolysing enzymes inhibition assays for testing. The extract inhibited both α-amylase and α-glucosidase enzymes in a concentration-dependent manner. The highest activity was found against α-amylase being superior to the positive control, acarbose. These results indicate the potential of this extract in lowering the post-prandial enhancement of blood glucose levels (Loizzo et al., 2018).

In the case of *R. graveolens,* results showed non-dose-dependent effects because the administration of *R. graveolens* methanolic extract at low doses showed hypoglycaemic activity, however when the dose has increased this effect decreased significantly (Figueroa-Valverde et al., 2009). In another study, the authors use streptozotocin (STZ)-diabetic rats to examine the reduction of blood glucose, cholesterol, triglycerides and urea with total, flavonoids and non-flavonoids extracts of *R. graveolens*. *R. graveolens* total extract (70 % ethanol) used to treat diabetic rats lowered blood cholesterol, urea and glucose levels. This extract revealed also protection of renal damage in the STZ-diabetic rats. With this work, the authors concluded that *R. graveolens* extracts contained antihyperglycemia and protective properties (Noori et al., 2019).

The aqueous extract of *R. montana* revealed significant reductions in the blood glucose levels in normal and streptozotocin-induced rats, so exhibiting a potent hypoglycemic effect in normal rats and an antidiabetic effect in streptozotocin induced rats (Farid et al., 2017).

These results demonstrate that different *Ruta* species and different extracts can have a positive effect on decreasing glucose levels, thus can be important in the search for new drugs to fight diabetes.

6.10 Reproductive activity

Contraception received growth interest and various chemotherapeutics have been made to resolve this problem, but like in other disorders, the side effects have been a major concern (Forsatkar et al., 2016). The potential use of plants for fertility regulation has been tested for a large number of species taking into account the history of traditional uses (Forsatkar et al., 2016, 2018). Beyond side effects also the compounds and/or metabolites excreted via urine and faeces that will be released into aquatic ecosystems and that can affect the aquatic organisms and also may have an impact on human health must be considered (Forsatkar et al., 2016, 2018).

6.10.1 Contraceptive effect

Ruta species have been vastly used in folk medicine by its diverse reproductive activities (Table 3). The mentioned information about the contraceptive effects of *R. graveolens* in traditional folk medicine, have caused an increase in the biological activities studies about this subject (Harat et al., 2008). Using a Siamese fighting fish, *Betta splendens,* as model, Forsatkar et al. (2016) demonstrated the effect of *R. graveolens* ethanol extract on key aggressive/reproductive behaviours. The results show that this extract reduced the reproductive behaviours (boldness, activity and aggression) and disrupted testis function causing a decrease in the number of spermatozoa but increased the number of spermatocytes on these fishes (Forsatkar et al., 2016). Furthermore, the toxic and the sublethal effects of *R. graveolens* ethanolic extract on reproductive behaviour, fertility, and steroid and thyroid hormone levels in zebrafish was also evaluated (Forsatkar et al., 2018). The sensitivity of zebrafish to discarded elements can be used for environmental monitoring and physiological and behavioural screening of active compounds that can have a negative impact on long-term health. The results show that ethanolic extract of rue disrupts gonadal and thyroidal functioning, affecting the sexual and reproductive performance of zebrafish. The extract decreases the steroid (testosterone and estradiol) and thyroid (triiodothyronine and thyroxine) hormone levels and also reduce the spawning attempts and lowers the number of eggs spawned and fertilized (Forsatkar et al., 2018). These findings revealed the negative effects on the levels of key hormones involved in reproduction (gonadal and thyroid hormones) and the disruption of egg production and fertilization in zebrafish caused by *R. graveolens* extract. Although the endocrine disrupting properties of *R.*

graveolens could be an advantage for pharmaceuticals endocrine drugs used as contraceptives (Forsatkar et al., 2018).

Motility and viability of cells, mitochondrial activity, DNA status and sperm revival tests in human fresh semen have been used to understand the effects of aqueous *R. graveolens* extract. The extract showed to affect cell viability, mitochondrial function and chromatin structure, with sperm cells still been alive after 2 h and with a motility return after washing. The effect was dose dependent and immobilization of sperm cells was not due to cell death or either ATP depletion or chromatin damage (Harat et al., 2008). A similar effect was observed when evaluating the effect of *R. graveolens* aqueous extract on sperm motility in the spermatozoa of male rats. A significant decrease in sperm motility was seen 1 h after administration of the extract, none the less the motility gradually increased, and after 6 hours, it was identical to the control group, indicating the reversion of the extract' effect. Despite the effects on motility, changes in morphology, viability, DNA structure of spermatozoa or testosterone levels were not observed (Halvaei et al., 2012). However, in another study, the same plant extract (*R. graveolens* aqueous extract) was studied to evaluate the effects on human spermatozoa motility. The analysis of fractions compositions showing that fractions with coumarin could impair sperm motility. The studies of four different coumarins, revealed only the reduction of sperm motility by xanthotoxin (Figure 3). With membrane integrity and sperm viability tests, it was concluded that hexane and ethanolic fractions could impair sperm vitality significantly. So, this means that part of immobilizing effect on human spermatozoa of *R. graveolens* could be due to its coumarins (Harat et al., 2015).

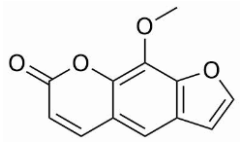

Figure 3. Chemical structure of Xanthotoxin.

The temporary immobility of spermatozoa without any adverse effects on other sperm characteristics can be a good feature for a potential use of *Ruta* extracts for male contraception (Halvaei et al., 2012; Harat et al., 2008).

6.10.2 Abortifacient agent

In traditional folk medicine the abortifacient uses of *R. graveolens* are vastly mentioned. The possible embryotoxicity of *R. graveolens* from Peru was evaluated and based on the *in vivo* administration of rue aqueous extract during the preimplantation phase in mice. An abnormal blastocyst formation in mice was observed, explained in part by diminished embryo cell number and retarded embryo development. Thus, *R. graveolens* extract can

interfere with preimplantation development and embryo transport (Gutiérrez-Pajares et al., 2003). On the other hand, the lyophilized hydroalcoholic extract of *R. graveolens* from Brazil, also administered orally to mice, was found to have maternal nontoxicity, due to mice body weight gain and placenta weight, that did not caused preimplantation embryonic loss and did not affected implantation (De Freitas et al., 2005), so, contradicting the previous conclusions. The simple fact that the route of administration, despite being oral, one was by syringe and the other the mice drank at will, may have influenced the results (De Freitas et al., 2005). Also as mentioned earlier for other biological activities, the composition of plants may vary and in these studies the harvest place and extraction method where different and so may influence the obtained results.

6.11 Other activities

Apart from the described biological activities, *Ruta* species extracts, EO and isolated compounds are also involved in other bioactivities, including anticoagulant activity, cardiovascular effect or as potential treatment of hyperuricemia and gout, through the inhibition of the enzyme xanthine oxidase.

The anticoagulant activity of ethanolic and successively collected extracts (ether/chloroform or ethyl acetate/*n*-butanol) of *R. chalepensis* was evaluated concerning the determination of the required time for clotting. The ethanol, and ethyl acetate/*n*-butanol successive extracts of *R. chalepensis* showed anticoagulant activity at higher concentrations (10 mg/mL) with prolonged clotting time 6 min and 30 s and 4 min and 30 s, respectively, and there was no anticoagulant activity for ether/chloroform extract (Alotaibi et al., 2018).

Regarding cardiovascular effects, *R. graveolens* ethanolic extract and its alkaloid fraction showed to depress the basic and functional properties of isolated rat heart in a dose-dependent manner, similarly to the observed after the addition of verapamil (positive control). This work demonstrated the possible beneficial effect in the treatment of proximal supraventricular tachyarrhythmia, by the modification of electrophysiological properties, indicating a potential antiarrhythmic effect of *R. graveolens* (Khori et al., 2008).

Additionally, methanolic extract of *R. chalepensis* leaves from Albaha region, Kingdom of Saudi Arabia, was screened for xanthine oxidase inhibition. The results showed that *R. chalepensis* extract exhibited high activity with inhibition of 76,2 %, however, the IC_{50} values proved to be higher than the control used (Ali et al., 2019). The findings obtained from this study revealed that an active compound from plant extracts can be promising for new xanthine oxidase inhibitors.

7. Toxicity

Although having good biological activities and vast use in folk medicine, the use of *Ruta* plants have been associated with toxicity.

Phytophototoxicity is an irritant/toxic type of photocontact dermatitis that causes cutaneous phototoxic inflammatory eruption resulting from contact with plant-derived (phyto), photosensitizing compounds, followed by exposure to sunlight (particularly ultraviolet A, between 320–400 nm of the spectrum) (Eickhorst et al., 2007; Morais et al., 2008). The reaction does not require the previous contact with to the provoking agents and occurs in individuals when exposed to both light and the plant chemical. Furanocoumarins, which naturally occurs in plants, are responsible for photoreactive properties (Milesi et al., 2001). The xanthotoxin (Figure 3) and bergapten (Figure 4), which naturally occur in *Ruta*, are furanocoumarins responsible for photosensitivity of human skin (Koblovská et al., 2008; Milesi et al., 2001).

Figure 4. Chemical structure of two furanocoumarins associated with phytotoxicity by *Ruta*. 1. Bergapten; 2. Psoralene.

Psoralenes (Figure 4), other furanocoumarins that are among the main constituents of rue, are also known for their photosensitization effects, which can produce a very strong undesirable syndrome in the form of photodermatitis (Mancuso et al., 2015). In fact, there are several case reports about phototoxic reactions caused by *Ruta* species. In the reports, it was described the development of hyperpigmentation after sun exposure in a girl treated with *R. graveolens* ointment (Morais et al., 2008) and also burn injury (Furniss and Adams, 2007) with skin loss (Radotra et al., 2018). These symptoms are accompanied by increasing pain and a reduced range of movement (Radotra et al., 2018). Behind, the phototoxic reactions also a case of bradycardia, acute renal failure with hyperkalemia, and coagulopathy was reported. This report showed that the use of *Ruta* might even cause multi-organ toxicity (here associated with cardiotoxicity, nephrotoxicity, hepatotoxicity, and coagulopathy) and so recognizing the systemic toxicity of *R. graveolens* (Seak and Lin, 2007).

The dried leaf infusions of *R. chalepensis,* from Argentina, were found to cause perinatal changes in mice. The administration of an aqueous infusion during an arganogenic period in pregnant mice caused significant differences in weight gain of pups, physical signs, development of reflexes, and produced histological changes in fetuses and placenta. The

authors found out that the results tend to confirm the embryotoxic effect of the plant and its harmful use (De Sa et al., 2000). In another study, Preethi and collaborators (2008), report evidences on the genotoxic and clastogenic potential of an extract of *R. graveolens* in mouse. Various types of chromosomal aberrations were noted in bone marrow cells after treatment and the administration of the extract for 30 days also resulted in damage to cellular DNA. Despite inducing genotoxicity in animals, these changes only appeared after 30 days of treatment, so a long-time exposure was needed to cause DNA damage (Preethi et al., 2008).

Considering these case reports, it can be verified that the *Ruta* species have several adverse effects when used. This toxicity is responsible for some of the bioactives described above, such as the abortive effect, the reproductive activity, or anti-cancer when considering its cytotoxicity. Therefore, it is extremely important to carry out complete studies on its safety and its toxicity in order to be able to use these plants for possible therapies and/or drugs to treat different diseases.

8. Conclusion

The genus *Ruta* presents ten species and all of them were described to be used in folk medicine. The study of biological activities and therapeutic applications of *Ruta* extracts, essential oils and its isolated compounds demonstrate the possible uses in treatment of a wide range of diverse diseases and the benefits of these plants, as well as, confirmed the traditional usage of *Ruta* species as folk medicine. In short, *Ruta* species represents a potential source of natural compounds for the treatment of many diseases involved in public health problems, such as infections, neurological diseases, cardiovascular and reproductive systems disorders and cancer. However, more *in vitro*, *in vivo* and clinical studies are strongly needed to confirm the mode of action and the efficacy of these reported uses and to discover potential uses that are not yet known. All taking into consideration that toxicity is associated with the use of these plants, so requiring an extensive safety assessment.

References

Abbad, A., Kasrati, A., Jamali, C.A., Zeroual, S., Ba M'Hamed, T., Spooner-Hart, R., Leach, D., 2014. Insecticidal properties and chemical composition of essential oils of some aromatic herbs from Morocco. Nat. Prod. Res. 28, 2338–2341. https://doi.org/10.1080/14786419.2014.936015

Abdel-Sattar, E., Zaitoon, A., El Sayed, A.M., Bakhashwain, A.A., 2014. Evaluation of some medicinal plants in controlling *Culex Pipiens*. J. Egypt. Soc. Parasitol. 44, 771–778. https://doi.org/10.12816/0007880

Acquaviva, R., Iauk, L., Sorrenti, V., Lanteri, R., Santangelo, R., Licata, A., Licata, F., Vanella, A., Malaguarnera, M., Ragusa, S., Di Giacomo, C., 2011. Oxidative profile in patients with colon cancer: Effects of *Ruta chalepensis* L. Eur. Rev. Med. Pharmacol. Sci. 15, 181–191.

Adamska-Szewczyk, A., Glowniak, K., Baj, T., 2016. Furochinoline alkaloids in plants from Rutaceae family - A review. Curr. Issues Pharm. Med. Sci. 29, 33–38. https://doi.org/10.1515/cipms-2016-0008

Adsersen, A., Gauguin, B., Gudiksen, L., Jäger, A.K., 2006. Screening of plants used in Danish folk medicine to treat memory dysfunction for acetylcholinesterase inhibitory activity. J. Ethnopharmacol. 104, 418–422. https://doi.org/10.1016/j.jep.2005.09.032

Ahmad, A.H., Amabeoku, G.J., 2013. Involvement of gamma aminobutyric acid in the anticonvulsant effect of the leaf methanol extract of *Ruta graveolens* L. (Rutaceae) in mice. Int. J. Pharmacol. 9, 134–142. https://doi.org/10.3923/ijp.2013.134.142

Ahmed, S.B.H., Sghaier, R.M., Guesmi, F., Kaabi, B., Mejri, M., Attia, H., Laouini, D., Smaali, I., 2011. Evaluation of antileishmanial, cytotoxic and antioxidant activities of essential oils extracted from plants issued from the leishmaniasis-endemic region of Sned (Tunisia). Nat. Prod. Res. 25, 1195–1201. https://doi.org/10.1080/14786419.2010.534097

Akkari, H., Ezzine, O., Dhahri, S., B'chir, F., Rekik, M., Hajaji, S., Darghouth, M.A., Jamâa, M.L. Ben, Gharbi, M., 2015. Chemical composition, insecticidal and *in vitro* anthelmintic activities of *Ruta chalepensis* (Rutaceae) essential oil. Ind. Crop. Prod. 74, 745–751. https://doi.org/10.1016/j.indcrop.2015.06.008

Ali, A., Demirci, B., Kiyan, H.T., Bernier, U.R., Tsikolia, M., Wedge, D.E., Khan, I.A., Başer, K.H.C., Tabanca, N., 2013. Biting deterrence, repellency, and larvicidal activity of *Ruta chalepensis* (Sapindales: Rutaceae) essential oil and its major individual constituents against mosquitoes. J. Med. Entomol. 50, 1267–1274. https://doi.org/ 10.1603/ME12177

Ali, N.A.A., Mansi, I., Ahmed, N., Alghamdi, S., Alhalawah, R.A., Al-Khulaidi, A., Anwar, S., 2019. Ultra High Performance Liquid Chromatography-Electrospray Ionization Mass Spectroscopy quantification, xanthine oxidase inhibitory, and antioxidant activity profile of some medicinal plants from Albaha region. Phcog. Mag. 15, 162–167. https://doi.org/ 10.4103/pm.pm_514_18

Al-Massarani, S., El-Shaibany, A., Tabanca, N., Ali, A., Estep, A.S., Becnel, J.J., Goger, F., Demirci, B., El-Gamal, A., Baser, K.H.C., 2019. Assessment of selected Saudi and Yemeni plants for mosquitocidal activities against the yellow fever mosquito *Aedes aegypti*. Saudi Pharmaceut. J. 27, 930–938. https://doi.org/10.1016/j.jsps.2019.07.001

Al-mazra'awi, M.S., Ateyyat, M., 2009. Insecticidal and repellent activities of medicinal plant extracts against the sweet potato whitefly, *Bemisia tabaci* (Hom.: Aleyrodidae) and its parasitoid *Eretmocerus mundus* (Hym.: Aphelinidae). J. Pest. Sci. 82, 149–154. https://doi.org/10.1007/s10340-008-0233-x

Alonso-Castro, A.J., Maldonado-Miranda, J.J., Zarate-Martinez, A., Jacobo-Salcedo, M.D.R., Fernández-Galicia, C., Figueroa-Zuñiga, L.A., Rios-Reyes, N.A., De LeónRubio, M.A., Medellín-Castillo, N.A., Reyes-Munguia, A., Méndez-Martínez, R., Carranza-Alvarez, C., 2012. Medicinal plants used in the Huasteca Potosina, México. J. Ethnopharmacol. 143, 292–298. https://doi.org/10.1016/j.jep.2012.06.035

Alotaibi, S.M., Saleem, M.S., Al-humaidi, J.G., 2018. Phytochemical contents and biological evaluation of *Ruta chalepensis* L. growing in Saudi Arabia. Saudi Pharmaceut. J. 26, 504–508. https://doi.org/10.1016/j.jsps.2018.02.008

Alzoreky, N.S., Nakahara, K., 2003. Antibacterial activity of extracts from some edible plants commonly consumed in Asia. Int. J. Food Microbiol. 80, 223–230. https://doi.org/10.1016/S0168-1605(02)00169-1

Amdouni, T., Ben Abdallah, S., Msilini, N., Merck, F., Chebbi, M., Lachâal, M., KarrayBouraoui, N., Ouerghi, Z., Fernandez, X., 2016. Effect of salt stress on the antimicrobial activity of *Ruta chalepensis* essential oils. Acta Physiol. Plant. 38. https://doi.org/10.1007/s11738-016-2167-x

Appelhans, M.S., Krohm, S., Manafzadeh, S., Wen, J., 2016. Phylogenetic placement of *Psilopeganum*, a rare monotypic genus of Rutaceae (the citrus family) endemic to China. J. Systemat. Evol. 54, 535–544. https://doi.org/10.1111/jse.12208

Arora, S., Aggarwal, A., Singla, P., Jyoti, S., Tandon, S., 2013. Anti-proliferative effects of homeopathic medicines on human kidney, colon and breast cancer cells. Homeopathy. 102, 274–282. https://doi.org/10.1016/j.homp.2013.06.001

Bagchi, G.D., Dwivedi, P.D., Singh, A., Haider, F., Naqvi, A.A., 2003. Variations in essential oil constituents at different growth stages of *Ruta chalepensis* on cultivation at north indian plains. J. Essent. Oil Res. 15, 263–264. https://doi.org/10.1080/10412905.2003.9712137

Bedini, S., Flamini, G., Ascrizzi, R., Venturi, F., Ferroni, G., Bader, A., Girardi, J., Conti, B., 2018. Essential oils sensory quality and their bioactivity against the mosquito *Aedes albopictus*. Sci. Rep. 8, 1–10. https://doi.org/10.1038/s41598-018-36158-w

Bejaoui, I., Karmous, T., 2012. Tunisian *Ruta graveolens* essential oil: Influence of factors on its yield and composition. J. Essent. Oil-Bear. Plant. 15, 276–282. https://doi.org/10.1080/0972060X.2012.10644047

Bennaoum, Z., Benhassaini, H., Falconieri, D., Piras, A., Porcedda, S., 2017. Chemical variability in essential oils from *Ruta* species among seasons, and its taxonomic and ecological significance. Nat. Prod. Res. 31, 2329–2334. https://doi.org/10.1080/14786419.2017.1303692

Berhane, K., van der Maesen, L.J.G., Van Andel, T., Zemede, A., 2014. Ethnoveterinary medicinal plants used by the Maale and Ari ethnic communities in southern Ethiopia. J. Ethnopharmacol. 10, 46. https://doi.org/10.1186/1746-4269-10-46

Bertrand, C., Fabre, N., Moulis, C., Bessiere, J.-M., 2003. Composition of the essentials oil of *Ruta corsica* DC. J. Essent. Oil Res. 15, 98–99. https://doi.org/10.1080/10412905.2003.9712079

Bouabidi, W., Hanana, M., Gargouri, S., Amri, I., Fezzani, T., Ksontini, M., Jamoussi, B., Hamrouni, L., 2015. Chemical composition, phytotoxic and antifungal properties of *Ruta chalepensis* L. essential oils. Nat. Prod. Res. 29, 864–868. https://doi.org/10.1080/14786419.2014.980246

Bouajaj, S., Romane, A., Benyamna, A., Amri, I., Hanana, M., Hamrouni, L., Romdhane, M., 2014. Essential oil composition, phytotoxic and antifungal activities of *Ruta chalepensis* L. leaves from High Atlas Mountains (Morocco). Nat. Prod. Res. 28, 1910–1914. https://doi.org/10.1080/14786419.2014.945085

Boutoumi, H., Moulay, S., Khodja, M., 2009. Essential oil from *Ruta montana* L. (Rutaceae) chemical composition, insecticidal and larvicidal activities. J. Essent. Oil-Bear. Plant. 12, 714–721. https://doi.org/10.1080/0972060X.2009.10643780

Bouzeraa, H., Bessila-Bouzeraa, M., Labed, N., 2019. Repellent and fumigant toxic potential of three essential oils against *Ephestia kuehniella*. Biosyst. Divers. 27, 349–353. https://doi.org/10.15421/011946

Calzada, F., Yépez-Mulia, L., Aguilar, A., 2006. *In vitro* susceptibility of *Entamoeba histolytica* and *Giardia lamblia* to plants used in Mexican traditional medicine for the treatment of gastrointestinal disorders. J. Ethnopharmacol. 108, 367–370. https://doi.org/10.1016/j.jep.2006.05.025

Cartaxo, S.L., Souza, M.M. de A., de Albuquerque, U.P., 2010. Medicinal plants with bioprospecting potential used in semi-arid northeastern Brazil. J. Ethnopharmacol. 131, 326–342. https://doi.org/10.1016/j.jep.2010.07.003

Chaaban, S. Ben, Hamdi, S.H., Mahjoubi, K., Jemâa, J.M. Ben, 2019. Composition and insecticidal activity of essential oil from *Ruta graveolens*, *Mentha pulegium* and *Ocimum basilicum* against *Ectomyelois ceratoniae* Zeller and *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae). J. Plant Dis. Prot. 126, 237–246. https://doi.org/10.1007/s41348-019-00218-8

Chaftar, N., Girardot, M., Labanowski, J., Ghrairi, T., Hani, K., Frère, J., Imbert, C., 2016. Comparative evaluation of the antimicrobial activity of 19 essential oils. Adv. Exp. Med. Biol. 901, 1–15. https://doi.org/10.1007/5584_2015_5011

Chaftar, N., Girardot, M., Quellard, N., Labanowski, J., Ghrairi, T., Hani, K., Frère, J., Imbert, C., 2015. Activity of six essential oils extracted from Tunisian plants against *Legionella pneumophila*. Chem. Biodivers. 12, 1565–1574. https://doi.org/10.1002/cbdv.201400343

Ciganda, C., Laborde, A., 2003. Herbal infusions used for induced abortion. J. Toxicol. Clin. Toxicol. 41, 235–239. https://doi.org/10.1081/CLT-120021104

Conti, B., Leonardi, M., Pistelli, L., Profeti, R., Ouerghemmi, I., Benelli, G., 2013. Larvicidal and repellent activity of essential oils from wild and cultivated *Ruta chalepensis* L. (Rutaceae) against *Aedes albopictus* Skuse (Diptera: Culicidae), an arbovirus vector. Parasitol. Res. 112, 991–999. https://doi.org/10.1007/s00436-012-3221-2

Da Silva, F.G.E., Francisco, F.R., João, J.C., Santiago, G.M.P., Bezerra, M.A.X., Barbosa, F.G., Mafezoli, J., Rocha, R.R., 2014. Seasonal variation, larvicidal and nematicidal activities of the leaf essential oil of *Ruta graveolens* L. J. Essent. Oil Res. 26, 204–209. https://doi.org/10.1080/10412905.2014.882276

De Carvalho, L.S.A., Queiroz, L.S., Alves Junior, I.J., Almeida, A.D.C., Coimbra, E.S., De Faria Pinto, P., Da Silva, M.P.N., De Moraes, J., Da Silva Filho, A.A., 2019. *In vitro* schistosomicidal activity of the alkaloid-rich fraction from *Ruta graveolens* L. (Rutaceae) and its characterization by UPLC-QTOF-MS. Evid. base Compl. Alternative Med. 1–8, 2019. https://doi.org/10.1155/2019/7909137

De Freitas, T.G., Augusto, P.M., Montanari, T., 2005. Effect of *Ruta graveolens* L. on pregnant mice. Contraception. 71, 74–77. https://doi.org/10.1016/j.contraception.2004.07.014

De La Torre Rodriguez, Y.C., Martínez Estrada, F.R., Flores Suarez, A.E., De Torres, N.W., Salazar Aranda, R., 2013. Larvicidal and cytotoxic activities of extracts from 11 native plants from northeastern Mexico. J. Med. Entomol. 50, 310–313. https://doi.org/10.1603/ME12056

De Medeiros, P.M., Ladio, A.H., Albuquerque, U.P., 2013. Patterns of medicinal plant use by inhabitants of Brazilian urban and rural areas: A macroscale investigation based on available literature. J. Ethnopharmacol. 150, 729–746. https://doi.org/10.1016/j.jep.2013.09.026

De Queiroz, A.C., Dias, T.L.M.F., Da Matta, C.B.B., Cavalcante Silva, L.H.A., de AraújoJúnior, J.X., de Araújo, G.B., Moura, F.B.P., Alexandre-Moreira, M.S., 2014. Antileishmanial activity of medicinal plants used in endemic areas in northeastern Brazil. Evid. base Compl. Alternative Med. 2014, 1–9. https://doi.org/10.1155/2014/478290

De Sa, R. Zeichen, Rey, A., Argañaraz, E., Bindstein, E., 2000. Perinatal toxicology of *Ruta chalepensis* (Rutaceae) in mice. J. Ethnopharmacol. 69, 93–98. https://doi.org/10.1016/S0378-8741(98)00232-3

Eickhorst, K., DeLeo, V., Csaposs, J., 2007. Rue the herb: *Ruta graveolens*-associated phytophototoxicity. Dermatitis. 18, 52–55. https://doi.org/10.2310/6620.2007.06033

El Sayed, K., Al-Said, M.S., El-Feraly, F.S., Ross, S.A., 2000. New quinoline alkaloids from *Ruta chalepensis*. J. Nat. Prod. 63, 995–997. https://doi.org/10.1021/np000012y

Emam, A., Eweis, M., Elbadry, M., 2010. A new furoquinoline alkaloid with antifungal activity from the leaves of *Ruta chalepensis* L. Drug Discov. Ther. 4, 399–404.

Emam, A., Swelam, E., Megally, N., 2009. Furocoumarin and quinolone alkaloid with larvicidal and antifeedant activities isolated from *Ruta chalepensis* leaves. J. Nat. Prod. 2, 10–22.

Fadlalla, K., Watson, A., Yehualaeshet, T., Turner, T., Samuel, T., 2011. *Ruta graveolens* extract induces DNA damage pathways and blocks Akt activation to inhibit cancer cell proliferation and survival. Anticancer Res. 31, 233–241.

Fakai, M.I., Abd Malek, S.N., Karsani, S.A., 2019. Induction of apoptosis by chalepin through phosphatidylserine externalisations and DNA fragmentation in breast cancer cells (MCF-7). Life Sci. 220, 186–193. https://doi.org/10.1016/j.lfs.2019.01.029

Fakhfakh, N., Zouari, S., Zouari, M., Loussayef, C., Zouari, N., 2012. Chemical composition of volatile compounds and antioxidant activities of essential oil, aqueous and ethanol

extracts of wild Tunisian *Ruta chalepensis* L. (Rutacea). J. Med. Plants Res. 6, 593–600. https://doi.org/10.5897/JMPR11.1121

Faria, J.M.S., Barbosa, P., Bennett, R.N., Mota, M., Figueiredo, A.C., 2013. Bioactivity against *Bursaphelenchus xylophilus*: Nematotoxics from essential oils, essential oils fractions and decoction waters. Phytochemistry. 94, 220–228. https://doi.org/10.1016/j.phytochem.2013.06.005

Faria, J.M.S., Rodrigues, A.M., Sena, I., Moiteiro, C., Bennett, R.N., Mota, M., Figueiredo, A.C., 2016a. Bioactivity of *Ruta graveolens* and *Satureja montana* essential oils on *Solanum tuberosum* hairy roots and *Solanum tuberosum* hairy roots with *Meloidogyne chitwoodi* co-cultures. J. Agric. Food Chem. 64, 7452–7458. https://doi.org/10.1021/acs.jafc.6b03279

Faria, J.M.S., Sena, I., Ribeiro, B., Rodrigues, A.M., Maleita, C.M.N., Abrantes, I., Bennett, R., Mota, M., Figueiredo, A.C. da S., 2016b. First report on *Meloidogyne chitwoodi* hatching inhibition activity of essential oils and essential oils fractions. J. Pest. Sci. 89, 207–217. https://doi.org/10.1007/s10340-015-0664-0

Farid, O., Hebi, M., Ajebli, M., Hidani, A. El, Eddouks, M., 2017. Antidiabetic effect of *Ruta montana* L. in streptozotocin-induced diabetic rats. J. Basic Clin. Physiol. Pharmacol. 28, 275–282. https://doi.org/10.1515/jbcpp-2016-0030

Farzaei, M.H., Shahpiri, Z., Bahramsoltani, R., nia, M.M., Najafi, F., Rahimi, R., 2017. Efficacy and tolerability of phytomedicines in Multiple Sclerosis patients: A review. CNS Drugs. 31, 867–889. https://doi.org/10.1007/s40263-017-0466-4

Fekhar, N., Moulay, S., Asma, D., Krea, M., Boutoumi, H., Benmaamar, Z., 2017. Thionation of essential oils from Algerian *Artemisia herba-alba* L. and *Ruta montana* L.: Impact on their antimicrobial and insecticidal activities. Chem. J. Mold. 12, 50–57. https://doi.org/10.19261/cjm.2017.410

Figueroa-Valverde, L., Díaz-Cedillo, F., Camacho-Luis, A., López Ramos, M., 2009. Efectos inducidos por *Ruta graveolens* L., *Cnidoscolus chayamansa* McVaugh y *Citrus aurantium* L. sobre los niveles de glucosa, colesterol y triacilglicéridos en un modelo de rata diabética. Braz. J. Pharmacogn. 19, 898–907. https://doi.org/10.1590/S0102-695X2009000600019

Forsatkar, M.N., HedayatiRad, M., Luchiari, A.C., 2018. "Not tonight zebrafish": The effects of *Ruta graveolens* on reproduction. Pharm. Biol. 56, 60–66. https://doi.org/10.1080/13880209.2017.1421234

Forsatkar, M.N., Nematollahi, M.A., Brown, C., 2016. The toxicological effect of *Ruta graveolens* extract in Siamese fighting fish: A behavioral and histopathological approach. Ecotoxicology. 25, 824–834. https://doi.org/10.1007/s10646-016-1639-5

Furniss, D., Adams, T., 2007. Herb of Grace: An unusual cause of phytophotodermatitis mimicking burn injury. J. Burn Care Res. 28, 767–769. https://doi.org/10.1097/BCR.0B013E318148CB82

Gali, L., Bedjou, F., 2019. Antioxidant and anticholinesterase effects of the ethanol extract, ethanol extract fractions and total alkaloids from the cultivated *Ruta chalepensis*. South Afr. J. Bot. 120, 163–169. https://doi.org/10.1016/j.sajb.2018.04.011

Gan, L., Cookson, M.R., Petrucelli, L., La Spada, A.R., 2018. Converging pathways in neurodegeneration, from genetics to mechanisms. Nat. Neurosci. 21, 1300–1309. https://doi.org/10.1038/s41593-018-0237-7

Gentile, M.T., Ciniglia, C., Reccia, M.G., Volpicelli, F., Gatti, M., Thellung, S., Florio, T., Melone, M.A.B., Colucci-D'Amato, L., 2015. *Ruta graveolens* L. induces death of glioblastoma cells and neural progenitors, but not of neurons, via ERK 1/2 and AKT activation. PloS One. 10, e0118864. https://doi.org/10.1371/journal.pone.0118864

Gentile, M.T., Russo, R., Pastorino, O., Cioffi, S., Barbieri, F., Illingworth, E.A., Grieco, M., Chambery, A., Colucci-D'Amato, L., 2018. *Ruta graveolens* water extract inhibits cell-cell network formation in human umbilical endothelial cells via MEK-ERK1/2 pathway. Exp. Cell Res. 364, 50–58. https://doi.org/10.1016/j.yexcr.2018.01.025

Ghabbari, M., Guarino, S., Caleca, V., Saiano, F., Sinacori, M., Baser, N., 2018. Behavior modifying and insecticidal effects of plant extracts on adults of *Ceratitis capitata* (Wiedemann) (Diptera Tephritidae). J. Pest. Sci. 91, 907–917. https://doi.org/10.1007/s10340-018-0952-6

Ghazghazi, H., Aouadhi, C., Weslati, M., Trakhna, F., Maaroufi, A., Hasnaoui, B., 2015. Chemical composition of *Ruta chalepensis* leaves essential oil and variation in biological activities between leaves, stems and roots methanolic extracts. J. Essent. Oil Bear. Plant. 18, 570–581. https://doi.org/10.1080/0972060X.2014.905757

Ghosh, S., Bishayee, K., Khuda-Bukhsh, A.R., 2014. Graveoline isolated from ethanolic extract of *Ruta graveolens* triggers apoptosis and autophagy in skin melanoma cells: A novel apoptosis-independent autophagic signaling pathway. Phytother Res. 28, 1153–1162. https://doi.org/10.1002/ptr.5107

Gitler, A.D., Dhillon, P., Shorter, J., 2017. Neurodegenerative disease: Models, mechanisms, and a new hope. Dis. Models Mech. 10, 499–502. https://doi.org/10.1242/dmm.030205

Gonzalez-Trujano, M.E., Carrera, D., Ventura-Martinez, R., Cedillo-Portugal, E., Navarrete, A., 2006. Neuropharmacological profile of an ethanol extract of *Ruta chalepensis* L. in mice. J. Ethnopharmacol. 106, 129–135. https://doi.org/10.1016/j.jep.2005.12.014

Günaydin, K., Savci, S., 2005. Phytochemical studies on *Ruta chalepensis* (Lam.) Lamarck. Nat. Prod. Res. 19, 203–210. https://doi.org/10.1080/14786410310001630546

Gutiérrez-Pajares, J.L., Zúñiga, L., Pino, J., 2003. *Ruta graveolens* aqueous extract retards mouse preimplantation embryo development. Reprod. Toxicol. 17, 667–672. https://doi.org/10.1016/j.reprotox.2003.07.002

Haddouchi, F., Chaouche, T.M., Zaouali, Y., Ksouri, R., Attou, A., Benmansour, A., 2013. Chemical composition and antimicrobial activity of the essential oils from four *Ruta* species growing in Algeria. Food Chem. 141, 253–258. https://doi.org/10.1016/j.foodchem.2013.03.007

Hadis, M., Lulu, M., Mekonnen, Y., Asfaw, T., 2003. Field trials on the repellent activity of four plant products against mainly *Mansonia* population in western Ethiopia. Phytother. Res. 17, 202–205. https://doi.org/10.1002/ptr.1051

Hale, A.L., Meepagala, K.M., Oliva, A., Aliotta, G., Duke, S.O., 2004. Phytotoxins from the leaves of *Ruta graveolens*. J. Agric. Food Chem. 52, 3345–3349. https://doi.org/10.1021/jf0497298

Halvaei, I., Roodsari, H.R.S., Harat, Z.N., 2012. Acute effects of *Ruta graveolens* L. on sperm parameters and DNA integrity in rats. J. Reproduction Infertil. 13, 33–38.

Hamdiken, M., Bouhalit, S., Kechrid, Z., 2018. Effect of *Ruta chalepensis* on zinc, lipid profile and antioxidant levels in the blood and tissue of streptozotocin-induced diabetes in rats fed zinc-deficient diets. Can. J. Diabetes. 42, 356–364. https://doi.org/10.1016/j.jcjd.2017.08.239

Hammami, I., Smaoui, S., Hsouna, A. Ben, Hamdi, N., Triki, M.A., 2015. *Ruta montana* L. leaf essential oil and extracts: Characterization of bioactive compounds and supression of Crown Gall disease. Excli J. 14, 83–94. https://doi.org/10.17179/excli2014-655

Hammiche, V., Maiza, K., 2006. Traditional medicine in central Sahara: Pharmacopoeia of Tassili N'ajjer. J. Ethnopharmacol. 105, 358–367. https://doi.org/10.1016/j.jep.2005.11.028

Harat, Z.N., Lakpour, N., Sadeghipoor, H.R., Kamalinejad, M., Eshraghian, M.R., Naghibi, B., Akhondi, M.M., Binaafar, S., Sadeghi, M.R., 2015. Immobilising effect of *Ruta graveolens* L. on human spermatozoa: Coumarin compounds are involved. Andrologia. 47, 1183–1189. https://doi.org/10.1111/and.12401

Harat, Z.N., Sadeghi, M.R., Sadeghipour, H.R., Kamalinejad, M., Eshraghian, M.R., 2008. Immobilization effect of *Ruta graveolens* L. on human sperm: A new hope for male contraception. J. Ethnopharmacol. 115, 36–41. https://doi.org/10.1016/j.jep.2007.09.004

Heidari, S., Mahdiani, S., Hashemi, M., Kalalinia, F., 2020. Recent advances in neurogenic and neuroprotective effects of curcumin through the induction of neural stem cells. Biotechnol. Appl. Biochem. 67, 430-441. https://doi.org/10.1002/bab.1891

Hmamouchi, M., Lahlou, M., Agoumi, A., 2000. Molluscicidal activity of some medicinal plants. Fitoterapia. 71, 308–314. https://doi.org/10.1016/S0367-326X(99)00152-5

Hyldgaard, M., Mygind, T., Meyer, R.L., 2012. Essential oils in food preservation: Mode of action, synergies, and interactions with food matrix components. Front. Microbiol. 3, 1–24. https://doi.org/10.3389/fmicb.2012.00012

Iauk, L., Mangano, K., Rapisarda, A., Ragusa, S., Maiolino, L., Musumeci, R., Costanzo, R., Serra, A., Speciale, A., 2004. Protection against murine endotoxemia by treatment with *Ruta Chalepensis* L., a plant with anti-inflammatory properties. J. Ethnopharmacol. 90, 267–272. https://doi.org/10.1016/j.jep.2003.10.004

Ivanova, A., Mikhova, B., Najdenski, H., Tsvetkova, I., Kostova, I., 2005. Antimicrobial and cytotoxic activity of *Ruta graveolens*. Fitoterapia. 76, 344–347. https://doi.org/10.1016/j.fitote.2005.02.008

Jaradat, N., Adwan, L., K'aibni, S., Zaid, A.N., Shtaya, M.J.Y., Shraim, N., Assali, M., 2017. Variability of chemical compositions and antimicrobial and antioxidant activities of *Ruta chalepensis* leaf essential oils from three Palestinian regions. BioMed Res. Int. 2017, 1-9. https://doi.org/10.1155/2017/2672689

Jasso Díaz, G., Hernández, G.T., Zamilpa, A., Becerril Pérez, C.M., Ramírez Bribiesca, J.E., Hernández Mendo, O., Sánchez Arroyo, H., González Cortazar, M., Mendoza de Gives, P., 2017. *In vitro* assessment of *Argemone mexicana*, *Taraxacum officinale*, *Ruta chalepensis* and *Tagetes filifolia* against *Haemonchus contortus* nematode eggs and infective (L3) larvae. Microb. Pathog. 109, 162–168. https://doi.org/10.1016/j.micpath.2017.05.048

Jeon, J.H., Kim, M.G., Lee, H.S., 2013. Insecticidal activities of *Ruta chalepensis* leaves isolated constituent and structure-relationships of its analogues against *Sitophilus oryzae*. J. Kor. Soc. Appl. Biol. Chem. 56, 591–596. https://doi.org/10.1007/s13765-013-3215-5

Kabouche, Z., Benkiki, N., Seguin, E., Bruneau, C., 2003. A new dicoumarinyl ether and two rare furocoumarins from *Ruta montana*. Fitoterapia. 74, 194–196. https://doi.org/10.1016/S0367-326X(02)00313-1

Kacem, M., Elfeki, A., Chaabouni, S., Bouaziz, M., Simon, G., Ben Mansour, A., Kacem, I., 2015. Phytochemicals and biological activities of *Ruta chalepensis* L. growing in Tunisia. Food Biosci. 12, 73–83. https://doi.org/10.1016/j.fbio.2015.08.001

Kacem, M., Simon, G., Leschiera, R., Misery, L., ElFeki, A., Lebonvallet, N., 2014. Antioxidant and anti-inflammatory effects of *Ruta chalepensis* L. extracts on LPS-stimulated RAW 264.7 cells. In Vitro Cell. Dev. Biol. Anim. 51, 128–141. https://doi.org/10.1007/s11626-014-9813-7

Kamal, L.Z.M., Hassan, N.M., Taib, N.M., Soe, M.K., 2018. Graveoline from *Ruta angustifolia* (L.) Pers. and its antimicrobial synergistic potential in erythromycin or vancomycin combinations. Sains Malays. 47, 2429–2435. https://doi.org/10.17576/jsm-2018-4710-19

Kambouche, N., Merah, B., Bellahouel, S., Bouayed, J., Dicko, A., Derdour, A., Younos, C., Soulimani, R., 2008. Chemical composition and antioxidant potential of *Ruta montana* L. essential oil from Algeria. J. Med. Food. 11, 593–595. https://doi.org/10.1089/jmf.2007.0515

Kataki, M.S., Kakoti, B.B., Bhuyan, B., Rajkumari, A., Rajak, P., 2014. Garden rue inhibits the arachidonic acid pathway, scavenges free radicals, and elevates FRAP: Role in inflammation. Chin. J. Nat. Med. 12, 172–179. https://doi.org/10.1016/S1875-5364(14)60029-7

Khadhri, A., Bouali, I., Belkhir, S., Mokded, R., Smiti, S., Falé, P., Araújo, M.E.M., Serralheiro, M.L.M., 2017. *In vitro* digestion, antioxidant and antiacetylcholinesterase activities of two species of *Ruta*: *Ruta chalepensis* and *Ruta montana*. Pharm. Biol. 55, 101–107. https://doi.org/10.1080/13880209.2016.1230634

Khadhri, A., Bouali, I., Belkhir, S., Mokni, R. El, Smiti, S., Almeida, C., Nogueira, J.M.F., Araújo, M.E.M., 2014. Chemical variability of two essential oils of Tunisian rue: *Ruta montana* and *Ruta chalepensis*. J. Essent. Oil-Bear. Plants. 17, 445–451. https://doi.org/10.1080/0972060X.2014.914001

Khlifi, D., Sghaier, R.M., Amouri, S., Laouini, D., Hamdi, M., Bouajila, J., 2013. Composition and anti-oxidant, anti-cancer and anti-inflammatory activities of *Artemisia herba-alba*, *Ruta chalpensis* L. and *Peganum harmala* L. Food Chem. Toxicol. 55, 202–208. https://doi.org/10.1016/j.fct.2013.01.004

Khori, V., Nayebpour, M., Semnani, S., Golalipour, M.J., Marjani, A., 2008. Prolongation of AV nodal refractoriness by *Ruta graveolens* in isolated rat hearts. Potential role as an anti-arrhythmic agent. Saudi Med. J. 29, 357–363.

Khoury, M., Stien, D., Ouaini, N., Eparvier, V., Apostolides, N.A., El Beyrouthy, M., 2014. Chemical composition and antimicrobial activity of the essential oil of *Ruta chalepensis* L. growing wild in Lebanon. Chem. Biodivers. 11, 1990–1997. https://doi.org/10.1016/j.fct.2013.01.004

Kim, M.K., Jang, Y.S., Ahn, Y.J., Lee, D.K., Lee, H.S., 2002. Larvicidal activity of Australian and Mexican plant extracts against *Aedes aegypti* and *Culex pipiens pallens* (Diptera: Culicidae). J. Asia Pac. Entomol. 5, 227–231. https://doi.org/10.1016/S1226-8615(08)60157-0

Knaak, N., Da Silva, L.D., Andreis, T.F., Fiuza, L.M., 2013. Chemical characterization and anti-fungal activity of plant extracts and essential oils on the *Bipolaris oryzae* and *Gerlachia oryzae* phytopathogens. Australas. Plant Pathol. 42, 469–475. https://doi.org/10.1007/s13313-013-0220-4

Koblovská, R., Macková, Z., Vítková, M., Kokoška, L., Klejdus, B., Lapčík, O., 2008. Isoflavones in the Rutaceae family: Twenty selected representatives of the genera *Citrus*, *Fortunella*, *Poncirus*, *Ruta* and *Severinia*. Phytochem. Anal. 19, 64–70. https://doi.org/10.1002/pca.1016

Kostova, I., Ivanova, A., Mikhova, B., Klaiber, I., 1999. Alkaloids and coumarins from *Ruta graveolens*. Monatsh. Chem. 130, 703–707. https://doi.org/10.1007/PL00010251

Krayni, H., Fakhfakh, N., Aloui, L., Zouari, N., Kossentini, M., Zouari, S., 2015. Chemical composition and chelating activity of *Ruta chalepensis* L. (Rutaceae) essential oil as influenced by phenological stages and plant organs. J. Essent. Oil Res. 27, 514–520. https://doi.org/10.1080/10412905.2015.1023906

Lans, C., Turner, N., Khan, T., Brauer, G., 2007. Ethnoveterinary medicines used to treat endoparasites and stomach problems in pigs and pets in British Columbia, Canada. Vet. Parasitol. 148, 325–340. https://doi.org/10.1016/j.vetpar.2007.06.014

Laquale, S., Candido, V., Avato, P., Argentieri, M.P., D'Addabbo, T., 2015. Essential oils as soil biofumigants for the control of the root-knot nematode *Meloidogyne incognita* on tomato. Ann. Appl. Biol. 167, 217–224. https://doi.org/10.1111/aab.12221

Law, S., Sanyal, S., Chatterjee, R., Law, Atrayo, Law, Aditya, Chattopadhyay, S., 2018. Therapeutic management of peritoneal ascitic sarcomatosis by *Ruta graveolens*: A study in experimental mice. Pathol. Res. Pract. 214, 1282–1290. https://doi.org/10.1016/j.prp.2018.07.006

Lee, C.H., Lee, H.S., 2011. Relaxant effect of quinoline derivatives on histamine-induced contraction of the isolated Guinea pig trachea. J. Appl. Biol. Chem. 54, 118–123. https://doi.org/10.3839/jksabc.2011.017

Li, Z., Mu, C., Wang, B., Jin, J., 2016. Graveoline analogs exhibiting selective acetylcholinesterase inhibitory activity as potential lead compounds for the treatment of Alzheimer's disease. Molecules. 21, 132. https://doi.org/10.3390/molecules21020132

Loizzo, M.R., Falco, T., Bonesi, M., Sicari, V., Tundis, R., Bruno, M., 2018. *Ruta chalepensis* L. (Rutaceae) leaf extract: Chemical composition, antioxidant and hypoglicaemic activities. Nat. Prod. Res. 32, 521–528. https://doi.org/ 10.1080/14786419.2017.1326491

López, A.M., Muñoz, M.C., Molina, J.M., Hermosilla, C., Taubert, A., Zárate, R., Hildebrandt, I., Mcnaughton-smith, G., Eiroa, J.L., Ruiz, A., 2018. Anticoccidial efficacy of Canary rue (*Ruta pinnata*) extracts against the caprine apicomplexan *Eimeria ninakohlyakimovae*. J. Anim. Sci. 97, 101–110. https://doi.org/ 10.1093/jas/sky389

Madari, H., Jacobs, R.S., 2004. An analysis of cytotoxic botanical formulations used in the traditional medicine of ancient Persia as abortifacients. J. Nat. Prod. 67, 1204–1210. https://doi.org/10.1021/np049953r

Mancuso, G., Borgonovo, G., Scaglioni, L., Bassoli, A., 2015. Phytochemicals from *Ruta graveolens* activate TAS2R bitter taste receptors and TRP channels involved in gustation and nociception. Molecules. 20, 18907–18922. https://doi.org/ 10.3390/molecules201018907

Mejri, J., Abderrabba, M., Mejri, M., 2010. Chemical composition of the essential oil of *Ruta chalepensis* L.: Influence of drying, hydro-distillation duration and plant parts. Ind. Crop. Prod. 32, 671–673. https://doi.org/10.1016/j.indcrop.2010.05.002

Mejri, J., Abderrabba, M., Mejri, M., 2012. Investigation and modeling of extraction parameters of *Ruta chalepensis* L. essential oil. J. Essent. Oil-Bear. Plant. 15, 516–525. https://doi.org/10.1080/0972060X.2012.10644081

Meloni, M., Reid, A., Caujapé-Castells, J., Marrero, Á., Fernández-Palacios, J.M., Mesa-Coelo, R.A., Conti, E., 2013a. Effects of clonality on the genetic variability of rare, insular species: The case of *Ruta microcarpa* from the Canary Islands. Ecol. Evol. 3, 1569–1579. https://doi.org/10.1002/ece3.571

Meloni, M., Reid, A., Caujapé-Castells, J., Soto, M., Fernández-Palacios, J.M., Conti, E., 2015. High genetic diversity and population structure in the endangered Canarian endemic *Ruta oreojasme* (Rutaceae). Genetica. 143, 571–580. https://doi.org/10.1007/s10709-015-9855-0

Meloni, Marilena, Reid, A., Conti, E., 2013b. Characterization of microsatellites for the endangered *Ruta oreojasme* (Rutaceae) and cross-amplification in related species. Appl. Plant Sci. 1, 1200347. https://doi.org/10.3732/apps.1200347

Milesi, S., Massot, B., Gontier, E., Bourgaud, F., Guckert, A., 2001. *Ruta graveolens* L.: A promising species for the production of furanocoumarins. Plant Sci. 161, 189–199. https://doi.org/10.1016/S0168-9452(01)00413-7

Mohammedi, H., Mecherara-Idjeri, S., Hassani, A., 2019. Variability in essential oil composition, antioxidant and antimicrobial activities of *Ruta montana* L. collected from different geographical regions in Algeria. J. Essent. Oil Res. 32, 23–36. https://doi.org/10.1080/10412905.2019.1660238

Molina-Garza, Z.J., Bazaldúa-Rodríguez, A.F., Quintanilla-Licea, R., Galaviz-Silva, L., 2014. Anti-*Trypanosoma cruzi* activity of 10 medicinal plants used in northeast Mexico. Acta Trop. 136, 14–18. https://doi.org/10.1016/j.actatropica.2014.04.006

Molnar, M., Jerković, I., Suknović, D., Bilić Rajs, B., Aladić, K., Šubarić, D., Jokić, S., Rajs, B.B., Aladić, K., Šubarić, D., Jokić, S., 2017. Screening of six medicinal plant extracts obtained by two conventional methods and supercritical CO_2 extraction targeted on coumarin content, 2,2-diphenyl-1-picrylhydrazyl radical scavenging capacity and total phenols content. Molecules. 22, 1–10. https://doi.org/10.3390/molecules22030348

Morais, P., Mota, A., Cunha, A.P., Peralta, L., Azevedo, F., 2008. Phytophotodermatitis due to homemade ointment for *Pediculosis capitis*. Contact Dermatitis. 59, 373–374. https://doi.org/10.1111/j.1600-0536.2008.01451.x

Morton, C., Telmer, C., 2014. New subfamily classification for the Rutaceae. Ann. Mo. Bot. Gard. 99, 620–641. https://doi.org/10.3417/2010034

Nega, S.S., Bekele, H.M., Meles, G.G., Nordeng, H., 2019. Medicinal plants and concomitant use with pharmaceutical drugs among pregnant women. J. Alternative Compl. Med. 25, 427–434. https://doi.org/10.1089/acm.2018.0062

Nogueira, J.C.R., Diniz, M. de F.M., Lima, E.O., 2008. *In vitro* antimicrobial activity of plants in Acute Otitis Externa. Braz. J. Otorhinolar. 74, 118–124. https://doi.org/10.1016/S1808-8694(15)30761-8

Noori, M., Jafari, M., Azimi, H., Node-Farahani, M., 2019. Effects of *Ruta graveolens* total and flavonoids extracts on rat blood glucose, cholesterol, triglycerides and urea comparing synthetic drugs. Nus. Biosci. 11, 23–29. https://doi.org/10.13057/nusbiosci/n110105

Ntalli, N.G., Manconi, F., Leonti, M., Maxia, A., Caboni, P., 2011. Aliphatic ketones from *Ruta chalepensis* (Rutaceae) induce paralysis on root knot nematodes. J. Agric. Food Chem. 59, 7098–7103. https://doi.org/10.1021/jf2013474

Oliva, A., Meepagala, K.M., Wedge, D.E., Harries, D., Hale, A.L., Aliotta, G., Duke, S.O., 2003. Natural fungicides from *Ruta graveolens* L. leaves, including a new quinolone alkaloid. J. Agric. Food Chem. 51, 890–896. https://doi.org/10.1021/jf0259361

Orlanda, J.F.F., Nascimento, A.R., 2015. Chemical composition and antibacterial activity of *Ruta graveolens* L. (Rutaceae) volatile oils, from São Luís, Maranhão, Brazil. South Afr. J. Bot. 99, 103–106. https://doi.org/10.1016/j.sajb.2015.03.198

Ortu, E., Sanna, G., Scala, A., Pulina, G., Caboni, P., Battacone, G., 2017. *In vitro* anthelmintic activity of active compounds of the fringed rue *Ruta chalepensis* against dairy ewe gastrointestinal nematodes. J. Helminthol. 91, 447–453. https://doi.org/10.1017/S0022149X16000419

Ouerghemmi, I., Rebey, I.B., Rahali, F.Z., Bourgou, S., Pistelli, L., Ksouri, R., Marzouk, B., Tounsi, M.S., 2017. Antioxidant and antimicrobial phenolic compounds from extracts of cultivated and wild-grown Tunisian *Ruta chalepensis*. J. Food Drug Anal. 25, 350–359. https://doi.org/10.1016/j.jfda.2016.04.001

Owlia, P., Saderi, H., Rasooli, I., Sefidkon, F., 2009. Antimicrobial characteristics of some herbal oils on *Pseudomonas aeruginosa* with special reference to their chemical compositions. Iran. J. Pharm. Res. 8, 107–114.

Pacifico, S., Piccolella, S., Galasso, S., Fiorentino, A., Kretschmer, N., Pan, S.P., Bauer, R., Monaco, P., 2016. Influence of harvest season on chemical composition and bioactivity

of wild rue plant hydroalcoholic extracts. Food Chem. Toxicol. 90, 102–111. https://doi.org/10.1016/j.fct.2016.02.009

Pathak, S.E.N., Multani, A.S., Banerji, Pratip, Banerji, Prasanta, 2003. Ruta 6 selectively induces cell death in brain cancer cells but proliferation in normal peripheral blood lymphocytes: A novel treatment for human brain cancer. Int. J. Oncol. 23, 975–982. https://doi.org/10.3892/ijo.23.4.975

Patil, A.G., Jobanputra, A.H., 2015. Rutin-chitosan nanoparticles: Fabrication, characterization and application in dental disorders. Polym. Plast. Technol. Eng. 54, 202–208. https://doi.org/10.1080/03602559.2014.935425

Pavlović, D.R., Vukelić, M., Najman, S., Kostić, M., Zlatković, B., Mihajilov-krstev, T., Kitić, D., 2014. Assessment of polyphenol content, *in vitro* antioxidant, antimicrobial and toxic potentials of wild growing and cultured rue. J. Appl. Bot. Food Qual. 181, 175–181. https://doi.org/10.5073/JABFQ.2014.087.025

Pollio, A., De Natale, A., Appetiti, E., Aliotta, G., Touwaide, A., 2008. Continuity and change in the Mediterranean medical tradition: *Ruta* spp. (Rutaceae) in Hippocratic medicine and present practices. J. Ethnopharmacol. 116, 469–482. https://doi.org/10.1016/j.jep.2007.12.013

Preethi, K.C., Kuttan, G., Kuttan, R., 2006. Anti-tumour activity of *Ruta graveolens* extract. Asian Pac. J. Cancer Prev. 7, 439–443.

Preethi, K.C., Nair, C.K.K., Kuttan, R., 2008. Clastogenic potential of *Ruta graveolens* extract and a homeopathic preparation in mouse bone marrow cells. Asian Pac. J. Cancer Prev. 9, 763–769.

Quintanilla-Licea, R., Mata-Cárdenas, B.D., Vargas-Villarreal, J., Bazaldúa-Rodríguez, A.F., Ángeles-Hernández, I.K., Garza-González, J.N., Hernández-García, M.E., 2014. Antiprotozoal activity against *Entamoeba histolytica* of plants used in northeast Mexican traditional medicine. Bioactive compounds from *Lippia graveolens* and *Ruta chalepensis*. Molecules. 19, 21044–21065. https://doi.org/10.3390/molecules191221044

Radotra, I., Gardiner, S., Barnes, D., 2018. A phytophototoxic injury at a Burns Unit: The ungraceful after-effects of the “common rue” plant. J. Burn Care Res. 39, 1064–1066. https://doi.org/10.1093/jbcr/irx040

Raghav, S.K., Gupta, B., Agrawal, C., Goswami, K., Das, H.R., 2006. Anti-inflammatory effect of *Ruta graveolens* L. in murine macrophage cells. J. Ethnopharmacol. 104, 234–239. https://doi.org/10.1016/j.jep.2005.09.008

Ratheesh, M., Shyni, G.L., Helen, A., 2009. Methanolic extract of *Ruta graveolens* L. inhibits inflammation and oxidative stress in adjuvant induced model of arthritis in rats. Inflammopharmacology. 17, 100–105. https://doi.org/10.1007/s10787-009-8044-0

Ratheesh, M., Shyni, G.L., Sindhu, G., Helen, A., 2010. Protective effects of isolated polyphenolic and alkaloid fractions of *Ruta graveolens* L. on acute and chronic models of inflammation. Inflammation. 33, 18–24. https://doi.org/10.1007/s10753-009-9154-y

Ratheesh, M., Shyni, G.L., Sindhu, G., Helen, A., 2011. Inhibitory effect of *Ruta graveolens* L. on oxidative damage, inflammation and aortic pathology in hypercholesteromic rats. Exp. Toxicol. Pathol. 63, 285–290. https://doi.org/10.1016/j.etp.2010.01.007

Redžić, S., 2007. The ecological aspect of ethnobotany and ethnopharmacology of population in Bosnia and Herzegovina. Coll. Antropol. 31, 869–890.

Ren, M.-X., Tang, J.-Y., 2012. Up and down: Stamen movements in *Ruta graveolens* (Rutaceae) enhance both outcrossing and delayed selfing. Ann. Bot. 110, 1017–1025. https://doi.org/10.1093/aob/mcs181

Richardson, J.S.M., Sethi, G., Lee, G.S., Malek, S.N.A., 2016. Chalepin: Isolated from *Ruta angustifolia* L. Pers induces mitochondrial mediated apoptosis in lung carcinoma cells. BMC Compl. Alternative Med. 16, 1–27. https://doi.org/10.1186/s12906-016-1368-6

Rustaiyan, A., Khossravi, M., Sultani-Lotfabadi, F., Yari, M., Masoudi, S., Monfared, A., 2002. Constituents of the essential oil of *Ruta chalepensis* L. from Iran. J. Essent. Oil Res. 14, 378–379. https://doi.org/10.1080/10412905.2002.9699892

Salvo, G., Bacchetta, G., Ghahremaninejad, F., Conti, E., 2008. Phylogenetic relationships of Ruteae (Rutaceae): New evidence from the chloroplast genome and comparisons with non-molecular data. Mol. Phylogenet. Evol. 49, 736–748. https://doi.org/10.1016/j.ympev.2008.09.004

Salvo, G., Ho, S.Y.W., Rosenbaum, G., Ree, R., Conti, E., 2010. Tracing the temporal and spatial origins of island endemics in the Mediterranean region: A case study from the citrus family (*Ruta* L., Rutaceae). Syst. Biol. 59, 705–722. https://doi.org/10.1093/sysbio/syq046

Sampaio, O.M., Vieira, L.C.C., Bellete, B.S., King-Diaz, B., Lotina-Hennsen, B., Da Silva, M.F. das G.F., Veiga, T.A.M., 2018. Evaluation of alkaloids isolated from *Ruta graveolens* as photosynthesis inhibitors. Molecules. 23, 1–12. https://doi.org/10.3390/molecules23102693

Samuel, R., Ehrendorfer, F., Chase, M.W., Greger, H., 2001. Phylogenetic analyses of Aurantioideae (Rutaceae) based on non-coding plastid DNA sequences and phytochemical features. Plant Biol. 3, 77–87. https://doi.org/10.1055/s-2001-11747

Seak, C., Lin, C., 2007. *Ruta graveolens* intoxication. Clin. Toxicol. 45, 173–175. https://doi.org/10.1080/15563650600956667

Shuib, N.A., Iqbal, A., Sulaiman, F.A., Razak, I., Susanti, D., 2015. Antioxidant and antibacterial activities of *Ruta angustifolia* extract. Jurnal Teknologi 77, 101–105. https://doi.org/10.11113/jt.v77.6747

Silva, P.T.M., Silva, M.A.F., Silva, L., Seca, A.M.L., 2019. Ethnobotanical knowledge in Sete Cidades, Azores archipelago: First ethnomedicinal report. Plants. 8, 1–20. https://doi.org/10.3390/plants8080256

Sivakamavalli, J., Deepa, O., Vaseeharan, B., 2014. Discrete nanoparticles of *Ruta graveolens* induces the bacterial and fungal biofilm inhibition. Cell Commun. Adhes. 21, 229–238. https://doi.org/10.3109/15419061.2014.926476

Soares, C., Morales, H., Faria, J., Figueiredo, A.C., Pedro, L.G., Venâncio, A., 2016. Inhibitory effect of essential oils on growth and on aflatoxins production by *Aspergillus parasiticus*. World Mycotoxin J. 9, 525–534. https://doi.org/10.3920/WMJ2015.1987

Soudani, M., Hadj Meliani, M., El-Miloudi, K., Azari, Z., Sorour, A.A., Merah, N., Pluvinage, G., 2018. Reduction of hydrogen embrittlement of API 5L X65 steel pipe using a green inhibitor. Int. J. Hydrogen Energy 43, 11150–11159. https://doi.org/10.1016/j.ijhydene.2018.04.236

Stafford, G.I., Pedersen, M.E., Staden, J. Van, Jäger, A.K., 2008. Review on plants with CNS-effects used in traditional South African medicine against mental diseases. J. Ethnopharmacol. 119, 513–537. https://doi.org/10.1016/j.jep.2008.08.010

Stafford, G.I., Pedersen, P.D., Jäger, A.K., Van Staden, J., 2007. Monoamine oxidase inhibition by southern African traditional medicinal plants. South Afr. J. Bot. 73, 384–390. https://doi.org/10.1016/j.sajb.2007.03.001

Suhaimi, S.A., Hong, S.L., Malek, S.N.A., 2017. Rutamarin, an active constituent from *Ruta angustifolia* Pers., induced apoptotic cell death in the HT29 Colon Adenocarcinoma cell line. Pharmacogn. Mag. 13, 179–188. https://doi.org/10.4103/pm.pm_432_16

Szopa, A., Ekiert, H., Szewczyk, A., Fugas, E., 2012. Production of bioactive phenolic acids and furanocoumarins in *in vitro* cultures of *Ruta graveolens* L. and *Ruta graveolens*

ssp. *divaricata* (Tenore) Gams. under different light conditions. Plant Cell Tissue Organ Cult. 110, 329–336. https://doi.org/10.1007/s11240-012-0154-5

Tagboto, S., Townson, S., 2001. Antiparasitic properties of medicinal plants and other naturally occurring products. Adv. Parasitol. 50, 199–295. https://doi.org/10.1016/s0065-308x(01)50032-9

Tampe, J., Parra, L., Huaiquil, K., Quiroz, A., 2016. Potential repellent activity of the essential oil of *Ruta chalepensis* (Linnaeus) from Chile against *Aegorhinus superciliosus* (Guérin) (Coleoptera: Curculionidae). J. Soil Sci. Plant Nutr. 16, 11–24. https://doi.org/10.4067/s0718-95162016005000004

Teixeira, T.L., Teixeira, S.C., da Silva, C.V., de Souza, M.A., 2014. Potential therapeutic use of herbal extracts in trypanosomiasis. Pathog. Glob. Health. 108, 30–36. https://doi.org/10.1179/2047773213Y.0000000120

Thibane, V.S., Ndhlala, A.R., Finnie, J.F., Van Staden, J., 2019. Modulation of the enzyme activity of secretory phospholipase A2, lipoxygenase and cyclooxygenase involved in inflammation and disease by extracts from some medicinal plants used for skincare and beauty. South Afr. J. Bot. 120, 198–203. https://doi.org/10.1016/j.sajb.2018.06.001

Thring, T.S.A., Weitz, F.M., 2006. Medicinal plant use in the Bredasdorp Elim region of the southern Overberg in the western Cape Province of South Africa. J. Ethnopharmacol. 103, 261–275. https://doi.org/10.1016/j.jep.2005.08.013

Touati, D., Atta-Ur-Rahman, Ulubelen, A., 2000. Alkaloids from *Ruta montana*. Phytochemistry. 53, 277–279. https://doi.org/10.1016/S0031-9422(99)00486-0

Traka, C.K., Petrakis, E.A., Kimbaris, A.C., Polissiou, M.G., Perdikis, D.C., 2018. Effects of *Ocimum basilicum* and *Ruta chalepensis* hydrosols on *Aphis gossypii* and *Tetranychus urticae*. J. Appl. Entomol. 142, 413–420. https://doi.org/10.1111/jen.12486

Tzakou, O., Couladis, M., 2001. Essential oil of *Ruta chalepensis* L. from Greece. J. Essent. Oil Res. 13, 258–259. https://doi.org/ 10.1080/10412905.2001.9699688

Varamini, P., Soltani, M., Ghaderi, A., 2009. Cell cycle analysis and cytotoxic potential of *Ruta graveolens* against human tumor cell lines. Neoplasma. 56, 490–493. https://doi.org/10.4149/neo_2009_06_490

Wahyuni, T.S., Mahfud, H., Permatasari, A.A., Widyawaruyanti, A., Fuad, A., 2019. Synergistic anti-Hepatitis C Virus activity of *Ruta angustifolia* extract with NS3 protein inhibitor. J. Basic Clin. Physiol. Pharmacol. 30, 1–8. https://doi.org/10.1515/jbcpp-2019-0348

Wahyuni, T.S., Widyawaruyanti, A., Lusida, M.I., Fuad, A., Soetjipto, Fuchino, H., Kawahara, N., Hayashi, Y., Aoki, C., Hotta, H., 2014. Inhibition of Hepatitis C Virus replication by chalepin and pseudane IX isolated from *Ruta angustifolia* leaves. Fitoterapia. 99, 276–283. https://doi.org/10.1016/j.fitote.2014.10.011

Wei, L., Wang, Y.Z., Li, Z.Y., 2012. Floral ontogeny of Ruteae (Rutaceae) and its systematic implications. Plant Biol. 14, 190–197. https://doi.org/10.1111/j.1438-8677.2011.00475.x

Wei, L., Xiang, X.G., Wang, Y.Z., Li, Z.Y., 2015. Phylogenetic relationships and evolution of the androecia in Ruteae (Rutaceae). PloS One. 10, 10–14. https://doi.org/ 10.1371/journal.pone.0137190

Wszelaki, N., Kuciun, A., Kiss, A., 2010. Screening of traditional European herbal medicines for acetylcholinesterase and butyrylcholinesterase inhibitory activity. Acta Pharm. 60, 119–128. https://doi.org/10.2478/v10007-010-0006-y

Yamada, M., Yasuhara, H., 2004. Clinical pharmacology of MAO inhibitors: Safety and future. Neurotoxicology. 25, 215–221. https://doi.org/ 10.1016/s0161-813x(03)00097-4

Yazbek, P.B., Tezoto, J., Cassas, F., Rodrigues, E., 2016. Plants used during maternity, menstrual cycle and other women's health conditions among Brazilian cultures. J. Ethnopharmacol. 179, 310–331. https://doi.org/10.1016/j.jep.2015.12.054

Yosra, B., Manef, A., Sameh, A., 2019. Biological study from *Ruta* plants extracts growing in Tunisia. Iran. J. Chem. Chem. Eng. 38, 85–89.

Zaidi, S.F., Muhammad, J.S., Shahryar, S., Usmanghani, K., Gilani, A.H., Jafri, W., Sugiyama, T., 2012. Anti-inflammatory and cytoprotective effects of selected Pakistani medicinal plants in *Helicobacter pylori*-infected gastric epithelial cells. J. Ethnopharmacol. 141, 403–410. https://doi.org/10.1016/j.jep.2012.03.001