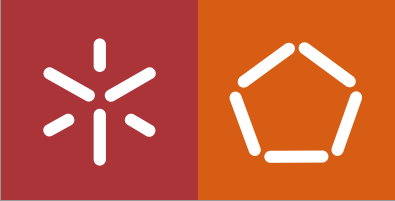




Liliana de Sousa Fernandes

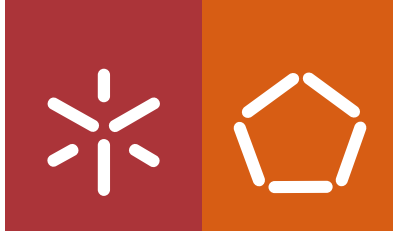
**Essential oils as an alternative treatment
for vaginal candidiasis**

Universidade do Minho
Escola de Engenharia



This study was supported by the Portuguese Foundation for Science and Technology (FCT) under the scope of the grant 2020.05720.BD, with DOI 10.54499/2020.05720.BD and strategic funding of UIDB/04469/2020 unit, and by LABBELS – Associate Laboratory in Biotechnology, Bioengineering and Microelectromechanical Systems, LA/P/0029/2020.





Universidade do Minho
Escola de Engenharia

Liliana de Sousa Fernandes

Essential oils as an alternative treatment for vaginal candidiasis

Tese de Doutoramento
Doutoramento em Engenharia Química e Biológica

Trabalho efetuado sob a orientação da
Doutora Mariana Contente Rangel Henriques
da
Doutora Maria Elisa da Costa Rodrigues
e da
Doutora Ana Sofia Quinta e Costa Neves de Oliveira

DIREITOS DE AUTOR E CONDIÇÕES DE UTILIZAÇÃO DO TRABALHO POR TERCEIROS

Este é um trabalho académico que pode ser utilizado por terceiros desde que respeitadas as regras e boas práticas internacionalmente aceites, no que concerne aos direitos de autor e direitos conexos.

Assim, o presente trabalho pode ser utilizado nos termos previstos na licença abaixo indicada.

Caso o utilizador necessite de permissão para poder fazer um uso do trabalho em condições não previstas no licenciamento indicado, deverá contactar o autor, através do RepositóriUM da Universidade do Minho.

Licença concedida aos utilizadores deste trabalho



Atribuição-NãoComercial-SemDerivações CC BY-NC-ND

<https://creativecommons.org/licenses/by-nc-nd/4.0/>

AGRADECIMENTOS

Essência por essência, a jornada acadêmica que culmina nesta tese foi enriquecida pela influência e apoio daqueles que sempre me acompanharam e apoiaram. Cada um de vocês, as orientadoras que guiaram o caminho, aos amigos que deram cor à minha vida, a família que forneceram alicerce e apoio e as colegas que deram suporte, é como um óleo essencial distinto que deixou sua marca significativa nesta jornada acadêmica. Por isso, queria expressar os meus sinceros agradecimentos:

Professora Mariana, agradeço com imenso carinho e gratidão pela orientação, aconselhamento, sugestões sempre muito relevantes e pelos excelentes ensinamentos que foram indispensáveis para o desenvolvimento deste trabalho. Não tenho palavras para agradecer por todas as oportunidades que me proporcionou e pelo incentivo ao longo deste caminho. O constante apoio ao longo destes anos foram verdadeiramente inspiradores e motivadores, impulsionando-me a superar obstáculos e a alcançar objetivos que não julgava ser capaz. **Elisa**, quero agradecer do fundo do meu coração todo o apoio, amizade, total disponibilidade, dedicação, a imensa paciência manifestada e motivação por encorajares sempre as minhas ideias por mais ambiciosas que fossem. Ao longo destes anos (e já lá vão 9 anos), com a tua orientação fui desenvolvendo diversas ferramentas que foram cruciais não só para a realização desta tese mas para o meu desenvolvimento profissional que será bastante útil no futuro. **Professora Sofia**, agradeço pela orientação, simpatia, dedicação e disponibilidade demonstrada, por me ter recebido no seu laboratório e por todos os ensinamentos, e ideias ao longo deste percurso, foram cruciais para o desenvolvimento deste projeto. Sou muito grata por toda a orientação que recebi durante a realização desta tese.

Gostaria de deixar uma palavra de agradecimento a todos os elementos atuais e os que passaram no laboratório **LMAs**, é incrível a camaradagem deste grupo. Agradeço pelas sugestões e ideias que me deram ao longo do trabalho, foram um grande contributo. Um agradecimento especial à **Sónia, Isabel, Fernanda, Bruna e Daniela** pela simpatia, amizade e ajuda que me disponibilizaram desde do meu primeiro dia no laboratório.

Às **colaborações** que foram feitas, Doutora Raquel Costa, Doutora Lorena Cussó e a sua equipa, Doutor Artur Ribeiro e a equipa do INIAV agradeço por enriquecerem não só o trabalho desenvolvido, como também a minha formação profissional.

Aos meus amigos, sei que posso contar com vocês para partilhar risadas, oferecer ombros amigos e caminhar ao meu lado em todas as jornadas que a vida nos reserva. Em especial, à **Ana Barbosa**, umas das melhores pessoas que o LMAs me colocou na vida, obrigada pelo apoio, encorajamento e amizade incondicional. **Ana Luísa, Jéssica e Alice** agradeço do fundo do meu coração a vossa amizade, é muito importante ter-vos na minha vida. E às minhas meninas da Covilhã, **Sofia e Inês** com quem partilhei e irei sempre partilhar os melhores momentos da minha vida, agradeço pela amizade, incentivo e apoio constante, e por acreditarem sempre no meu trabalho e alegrarem os meus dias.

E por último, mas não menos importante, um grande obrigada a minha **família** pelo apoio absoluto, motivação, por toda a confiança depositada em mim, pelo enorme carinho e compreensão não só agora mas ao longo de toda a minha vida. Um obrigada muito especial a ti **Fábio** pelo amor e carinho, paciência e ajuda que me deste ao longo destes anos. Estiveste lá nas conquistas e nas derrotas, sempre com um abraço reconfortante, proporcionando-me um porto seguro, que sempre me incentivou a continuar e a dar o melhor de mim. Sem vocês esta conquista não seria possível.

A Todos, Muito Obrigada!

“Somos do tamanho dos nossos sonhos” Fernando Pessoa

STATEMENT OF INTEGRITY

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

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

Universidade do Minho, abril de 2024

A crescente prevalência de infecções fúngicas, especialmente causadas por *Candida*, destaca a necessidade de terapias mais eficazes e menos tóxicas. Os antifúngicos convencionais apresentam limitações, tais como efeitos colaterais notáveis e eficácia fungicida inadequada, o que contribui para o aparecimento de espécies resistentes a medicamentos. Nesta perspectiva, surgem os óleos essenciais (OE), como alternativas promissoras. Este estudo apresenta dois métodos inovadores de aplicação de OEs: fase vapor (FV-OE) e encapsulamento em microcápsulas de queratina (OE-MCQ). Métodos que visam maximizar o potencial efeito terapêutico dos óleos. Foram avaliados diferentes OEs, incluindo árvore de chá, niaouli, tomilhos, cajeput e orégão, em espécies de *Candida* resistentes aos antifúngicos convencionais. Os resultados evidenciaram a eficácia dos OEs na inibição do crescimento e na prevenção de biofilmes, confirmando o seu potencial como terapias alternativas. Outro parâmetro avaliado foi a variabilidade na eficácia de diferentes OEs que pertencem ao mesmo gênero, o que destaca a importância de considerar as características químicas específicas de cada OE. No contexto do tratamento da candidíase vulvovaginal (CVV), o foco deste trabalho, os OEs mostraram atividade antifúngica significativa, especialmente na prevenção de biofilmes formados por espécies resistentes relacionadas à CVV. De modo a mimetizar o ambiente vaginal *in vitro*, foram otimizadas as condições experimentais através da utilização de fluido vaginal sintético. Adicionalmente, foi explorado o impacto da VF-OE na microbiota vaginal, evidenciando a sua eficácia contra *Candida* sem perturbações significativas na restante microflora, nomeadamente na espécie de *Lactobacillus*.

O óleo essencial de orégão (OEO) destacou-se pela sua notável eficácia antifúngica contra espécies de *Candida* resistentes a antifúngicos, interferindo com a integridade da membrana e com a atividade metabólica. No entanto, a sua aplicação clínica pode ser desencorajada, devido à possível toxicidade. Nesta perspectiva, foram apresentadas abordagens seguras para a sua aplicação no tratamento da CVV, como a FV-OE e o OE-MCQ. A aplicação intravaginal, *in vivo*, de OEO-QMC num modelo animal resultou na diminuição do crescimento de *Candida albicans*, não afetando o crescimento da espécie *Lactobacillus* presente. Em conclusão, esta tese destaca o potencial dos OEs, particularmente o óleo essencial de orégão, como terapias alternativas para infecções provocadas pela espécie *Candida*. Os métodos de aplicação propostos, VF-OE e OE-MCQ, oferecem possibilidades promissoras para um tratamento seguro e eficaz. As características naturais dos OE apresentam vantagens como menor custo, fácil acesso e menor impacto negativo na saúde da mulher, preservando a saúde vaginal.

Palavras-chave: Aplicações fitoterapêuticas; Biofilmes de *Candida*; Espécies resistentes de *Candida*; Fase de vapor dos óleos essenciais; Microencapsulação.

The increasing prevalence of fungal infections, especially those caused by *Candida* species, underscores the need for more effective and less toxic therapies. Conventional antifungals have limitations such as notable side effects and inadequate fungicidal efficacy, contributing to the emergence of drug-resistant species. In this perspective, essential oils (EOs) emerge as promising alternatives. This study introduces two innovative methods of EOs application: vapor-phase (VP-EO) and encapsulation in keratin microcapsules (EO-KMP). These methods aim to overcome limitations of EOs, maximizing their therapeutic potential. Several EOs, including tea tree, niaouli, thyme, cajeput and oregano, were evaluated against drug-resistant *Candida* species. The results demonstrate the effectiveness of these EOs in inhibiting growth and preventing biofilms, confirming their potential as alternative therapies. Another investigated parameter was the variability in EO effectiveness within the same genus, emphasizing the importance of considering the specific chemical characteristics of each EO.

In the context of vulvovaginal candidiasis (VVC) treatment, the focus of this work, EOs exhibited significant antifungal activity, especially in preventing biofilms formed by drug-resistant species related to VVC. In order to mimic the *in vitro* vaginal environment, experimental conditions were optimized using synthetic vaginal fluid. Additionally, the impact of VP-EO on the vaginal microbiota was explored, demonstrating its efficacy against *Candida* without significant disturbances to the remaining microflora, such as *Lactobacillus* species.

Oregano essential oil (OEO) stood out for its remarkable antifungal efficacy against drug-resistant *Candida* species, interfering with membrane integrity and metabolic activity. However, its clinical application may be discouraged due to potential toxicity. In this perspective, safe approaches for its application in VVC treatment were presented, VP-EO and EO-KMP. Importantly, *in vivo* intravaginal application of OEO-KMP resulted in a decrease in the growth of *Candida albicans* while maintaining the presence of *Lactobacillus* species.

In conclusion, this thesis underscores the potential of EOs, particularly OEO, as alternative therapies for *Candida*-induced infections. The proposed application methods, VP-EO and EO-KMP, offer promising possibilities for safe and effective treatment. The natural characteristics of EOs present advantages such as lower cost, easy accessibility, and fewer negative impacts on women's health, preserving vaginal health.

KEYWORDS: *Candida* biofilms; Microencapsulation; Phytotherapeutic applications; Resistant *Candida* species Vapor-phase of essential oil.

TABLE OF CONTENTS

List of figures.....	xiv
List of tables.....	xxii
List of abbreviations, acronyms and symbols.....	xxv
Thesis overview.....	xxviii
Scope of thesis.....	xxix
Aim of the thesis.....	xxx
Outline of the thesis.....	xxxi
Outputs.....	xxxiii
 Chapter I. General Introduction.....	 1
Chapter I.1 Essential oils as alternative treatment to <i>Candida</i> infection: review.....	2
1. Introduction	3
2. Candidiasis, <i>Candida</i> species and its characteristics.....	4
3. Conventional antifungal agents and Mechanism of resistance	9
4. Brief overview of alternative therapies for <i>Candida</i> infection.....	12
4.1 Alternatives treatments based on natural products.....	13
4.1.1 Essential oil antifungal effect	15
4.1.1.1 <i>Melaleuca alternifolia</i> (Tea Tree Essential Oil (TTEO)).....	16
4.1.1.2 <i>Melaleuca quinquenervia</i> (Niaouli Essential Oil).....	17
4.1.1.3 <i>Melaleuca leucadendra</i> (Cajeput Essential Oil)	18
4.1.1.4 <i>Thymus species</i> (Thyme Essential Oil).....	19
4.1.1.5 <i>Origanum species</i> (Oregano Essential Oil)	19
4.1.2 Encapsulated essential oil on <i>Candida</i> species.....	20
5. Conclusion.....	25
 General Introduction: References.....	 27
 Chapter II. Effect of essential oils on drug-resistant <i>Candida</i> strains.....	 36
Chapter II.1 Essential oils as a good weapon against drug-resistant <i>Candida auris</i>	37

1. Introduction.....	38
2. Materials and Methods	39
2.1 Essential oils	39
2.2 Microorganisms and culture conditions in this study	39
2.3 Antifungal susceptibility testing.....	40
2.3.1 Fluconazole	40
2.3.2 Essential oils	40
2.3.2.1 <i>Planktonic antimicrobial susceptibilities</i>	40
2.3.2.2 <i>Biofilms antimicrobial susceptibilities</i>	41
2.4 Statistical analysis	42
3. Results and Discussion	42
4. Conclusions.....	47

Chapter II.2 Comparing the effect of *Thymus* species essential oils no *Candida auris*.....48

1. Introduction	49
2. Materials and Methods	49
2.1 Microorganisms and culture conditions	49
2.2 Essential oils	50
2.2.1 Chromatographic analysis of essential oils	50
2.3 Agar disk diffusion assay	51
2.4 Antifungal activity of essential oil from <i>Thymus</i> species.....	51
2.5 Statistical analysis	52
3. Results and discussion	52
4. Conclusions	56

Chapter II.3 Vapor-phase of essential oils as promising solution to prevent *Candida* vaginal biofilms caused by antifungal resistant strains.....57

1. Introduction	58
2. Materials and Methods	59
2.1 Essential oils	59

2.1.1	Cytotoxicity of vapor-phase of essential oils	59
2.2	Microorganisms and initial culture conditions	60
2.3	Evaluation of the antifungal activity of essential oils	63
2.3.1	Growth inhibition analysis	63
2.3.2	Effect of the vapor-phase of essential oils on biofilms	63
2.3.2.1	<i>Quantification of Candida biofilm cultivable cells</i>	63
2.3.2.2	<i>Quantification of Candida biofilm biomass</i>	64
2.3.2.3	<i>Quantification of metabolic activity of Candida biofilms cells</i>	64
2.3.2.4	<i>Scanning electron microscopy (SEM)</i>	64
2.4	Statistical analysis	65
3.	Results and Discussion	65
4.	Conclusions	71
	Effect of essential oils on drug-resistant <i>Candida</i> strains: References	72
	Chapter III. Vaginal microbiota conditions	78
	Chapter III.1 Simulated Vaginal Fluid: <i>Candida</i> resistant strains' biofilm characterization and vapor-phase of essential oil effect	79
1.	Introduction	80
2.	Materials and Methods	81
2.1	Microorganisms and initial culture conditions	81
2.2	Simulated Vaginal Fluid	81
2.3	Effect of Simulated Vaginal Fluid and Sabouraud Dextrose Broth on vaginal <i>Candida</i> isolates	82
2.3.1	Planktonic growth	82
2.3.2	Biofilm formation	82
2.4	Effect of the vapor-phase of essential oils on biofilms	83
2.5	Statistical analysis	83
3.	Results and Discussion	83
4.	Conclusions	89

Chapter III.2 Vapor-phase of white thyme essential oil effect on *Candida albicans* and *Lactobacillus gasseri* colonization in a reconstituted human vaginal epithelium.....90

1. Introduction.....	91
2. Materials and Methods.....	92
2.1 Microorganisms and culture conditions.....	92
2.2 Essential oils.....	92
2.2.1 Essential oil cytotoxicity.....	93
2.3 Susceptibility of <i>Candida albicans</i> to white thyme essential oil.....	93
2.4 Antifungal activity of vapor-phase of white thyme essential oil on biofilms.....	93
2.4.1 Quantification of <i>Candida</i> biomass.....	94
2.4.2 Quantification of metabolic activity of <i>Candida</i> cells.....	94
2.5 Effect of vapor-phase of white thyme essential oil in colonization of <i>Candida albicans</i> and <i>Lactobacillus gasseri</i> of a reconstituted human vaginal epithelium.....	94
2.5.1 Histological analysis and microscopic observation.....	95
2.5.2 Quantification of <i>Candida</i> and <i>Lactobacillus</i> cells in RHVE.....	95
2.5.3 Lactate dehydrogenase (LDH) assay.....	96
2.6 Statistical analysis.....	96
3. Results and Discussion.....	97
4. Conclusions.....	103

Vaginal microbiota conditions: References.....104

Chapter IV. Effect of oregano essential oil in *Candida* infection109

Chapter IV.1 Effect of vapor-phase of oregano essential oil on resistant *Candida* species biofilms: mechanisms of action.....110

1. Introduction	111
2. Materials and Methods	112
2.1 Oregano essential oil	112
2.1.1 Oregano essential oil cytotoxicity	112
2.2 Microorganisms and culture conditions	113
2.3 Planktonic antimicrobial susceptibilities to oregano essential oil.....	113

2.4	Effect of the vapor-phase of oregano essential oils on biofilms	114
2.4.1	Determination of the mechanism of action	114
2.4.1.1	<i>Biofilm time-dependent killing assay</i>	114
2.4.1.2	<i>Flow cytometry</i>	115
2.4.1.3	<i>Confocal laser scanning microscopy analysis</i>	115
2.5	Effect of oregano essential oil on <i>Candida</i> species infection of a reconstituted human vaginal epithelium	116
2.5.1	Microscopic observation.....	116
2.5.2	Quantification of <i>Candida</i> cells.....	117
2.5.3	Lactate dehydrogenase assay.....	117
2.6	Statistical analysis	118
3.	Results and Discussion	118
4.	Conclusions.....	129

Chapter IV.2 Design and evaluation of microencapsulated oregano essential oil as an alternative treatment to *Candida albicans* infection130

1.	Introduction.....	131
2.	Materials and Methods.....	132
2.1	Oregano essential oil encapsulated into microparticles of keratin.....	132
2.1.1	Oregano essential oil and conditions.....	132
2.1.2	Keratin extraction and purification.....	132
2.1.3	Preparation of the keratin microparticles for encapsulation of oregano essential oil.....	133
2.1.4	Determination of oregano essential oil encapsulated into microparticles of keratin encapsulation efficiency.....	133
2.1.5	Physicochemical properties of the oregano essential oil encapsulated into microparticles of keratin.....	134
2.1.6	Evaluate the oregano essential oil encapsulated into microparticles of keratin release, <i>in vitro</i>	134
2.1.7	Oregano essential oil encapsulated into microparticles of keratin cytotoxicity.....	135
2.2	Evaluate the oregano essential oil encapsulated into microparticles of keratin effect on the <i>Candida</i> infection <i>in vitro</i> assays.....	135
2.2.1	Microorganisms and culture conditions.....	135

2.2.2	Oregano essential oil encapsulated into microparticles of keratin effect on planktonic <i>Candida albicans</i> cells.....	136
2.2.3	Oregano essential oil encapsulated into microparticles of keratin effect on single and mixed <i>Candida albicans</i> and <i>Lactobacillus gasseri</i> biofilms <i>in vitro</i>	136
2.3	Evaluation of the effect of the oregano essential oil encapsulated into microparticles of keratin in a mouse model.....	137
2.3.1	Microorganism culture.....	137
2.3.2	<i>Candida albicans</i> infection and the treatment with oregano essential oil encapsulated into microparticles of keratin.....	137
2.3.2.1	Quantification of <i>Candida albicans</i> and <i>Lactobacillus</i> species.....	138
2.3.2.2	Histopathological analysis of mucosal tissues.....	138
2.4	Statistical analysis.....	139
3.	Results and Discussion.....	139
3.1	Oregano essential oil encapsulated into microparticles of keratin characterization.....	139
3.2	Impact of oregano essential oil encapsulated into microparticles of keratin on <i>Candida albicans</i> growth <i>in vitro</i>	143
3.3	<i>In vivo</i> effect of oregano essential oil encapsulated into microparticles of keratin on <i>Candida albicans</i> infection.....	145
4.	Conclusions.....	150
	Effect of oregano essential oil in <i>Candida</i> infection: References.....	151
	Chapter V. General discussion, final remarks and future work.....	158
	General Discussion and Final Remark.....	159
	Future Work.....	165
	Annex I. Material supplementary.....	167
	Annex I.....	167
I.1	Chemical and physical analysis of tea tree essential oil (Lot 903025) report provided by the company florame® (Saint-Rémy-de-Provence, France).....	167
I.2	Chemical and physical analysis of cajeput essential oil (Lot 801736) report provided by the company florame® (Saint-Rémy-de-Provence, France).....	170

I.3 Chemical and physical analysis of niaouli essential oil (Lot 100378) report provided by the company florame® (Saint-Rémy-de-Provence, France).....	173
I.4 Chemical and physical analysis of white thyme essential oil (Lot 800180) report provided by the company florame® (Saint-Rémy-de-Provence, France).....	175
I.5 Chemical and physical analysis of moroccan oregano essential oil (Lot 10468) report provided by the company florame® (Saint-Rémy-de-Provence, France).....	178
 Annex II.....	180
II.1 Complete chromatography analysis of the composition of essential oils from <i>Thymus vulgaris</i> , <i>Thymus zygis</i> , <i>Thymus satureioides</i> and <i>Thymus mastichina</i>	180
 Annex III.....	182
III.1 Histological images of vaginal tissue of mice infected with <i>Candida albicans</i> without any vaginal treatment.....	182
 Annex IV.....	183
IV.1 Confirmation of publication and licensing rights of Biorender (Figure 1)	183
IV.2 Confirmation of publication and licensing rights of Biorender (Figure 35)	184

Figure 1. Workflow of the thesis (Created with BioRender.com (KW26ABVIPV))	xxviii
---	--------

Chapter I. General Introduction

Chapter I.1 Essential oils as alternative treatment to *Candida* infection: review

Figure 2. Subchapter I.1 workflow.....	2
---	---

Figure 3. Biofilm formation stages. (A) Initial adhesion; (B) Proliferation and formation of an extracellular matrix; (C) Mature biofilm with continuous growth and (D) Propagation through the release of fungal cells (adapted from Araújo <i>et al.</i> [35]).....	6
--	---

Figure 4. Biofilm Structure of <i>Candida</i> species. <i>Candida albicans</i> (A) Blastospores shaped cells and (B) Filamentous forms (hyphae or pseudo hyphae); (C) <i>Candida glabrata</i> ; (D) <i>Candida tropicalis</i> ; (E) <i>Candida guilliermondii</i> ; (F) <i>Pichia kudriavzevii</i> ; (G) <i>Candida parapsilosis</i> ; (H) <i>Candida auris</i> . Images obtained with scanning electron microscopy and adapted from Fernandes <i>et al.</i> [39], Araújo <i>et al.</i> [35] and Kean <i>et al.</i> [40].....	7
--	---

Figure 5. Mechanism of action of antifungals against <i>Candida</i> species and main mechanisms drug resistance (Adapted from Gómez-Gaviria <i>et al.</i> [42]). Green squares indicate mechanism of action of antifungals and red squares resistance mechanisms.....	9
--	---

Figure 6. Images of plants of the genus <i>Myrtaceae</i>: (A) <i>Melaleuca alternifolia</i> , (B) <i>Melaleuca quinquenervia</i> and (C) <i>Melaleuca leucadendra</i>	16
--	----

Figure 7. Images of plants of the <i>Lamiaceae</i> family, (A) <i>Thymus</i> species and (B) <i>Origanum</i> species.....	18
--	----

Chapter II. Effect of essential oils on drug-resistant *Candida* strains

Chapter II.1 Essential oils as a good weapon against drug-resistant *Candida auris*

Figure 8. Subchapter II.1 workflow.....	37
--	----

Figure 9. Glass system: (A) well, (B) well and petri dishes and (C) petri dishes and glass well inside glass petri dishes.....41

Figure 10. Antifungal activity of essential oils on *Candida auris* NCPF 8971 evaluated using the disk-diffusion assay. **** $p < 0.0001$ indicates a statistically different reduction in comparison with the control (Tukey`s multiple comparison test).....44

Figure 11. Antifungal effect of four essential oils (tea tree, niaouli, white thyme and cajeput) in (A) biofilm formation and (B) 24 h-old biofilms of *Candida auris* NCPF 8971 by quantification of CFUs per mL. Error bars indicate the respective standard derivation (SD). *** $p < 0.001$, **** $p < 0.0001$ indicates a statistically different reduction in comparison with the control (Tukey`s multiple comparison test).....45

Chapter II.2 Comparing the effect of *Thymus* species essential oils no *Candida auris*

Figure 12. Subchapter II.2 workflow.....48

Figure 13. Assessment of the susceptibility of *Candida auris* NCPF 8971 24 h old biofilms to different essential oils from *Thymus* genus by quantification of CFUs per mL. Each essential oil was tested at 100 % and at 50 % with both (A) direct and (B) indirect application. Error bars indicate the respective standard derivation (SD). Differences in mean Log_{10} CFU mL^{-1} of the different treatments were compared to the control using one-way ANOVA with a post hoc Tukey test (significance at $P < 0.05$), with ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$54

Chapter II.3 Vapor-phase of essential oils as promising solution to prevent *Candida* vaginal biofilms caused by antifungal resistant strains

Figure 14. Subchapter II.3 workflow57

Figure 15. Vapor-phase of essential oils (VP-EOs) cytotoxicity, expressed as the percentage of viable cells in relation to the absorbance values (OD 490 nm) of cells cultured without VP-EOs (100 % cell viability). The dashed line stands for the normative limit of 70 % metabolic activity (ISO 10993-5:2009).....66

Figure 16. Effect of the vapor-phase of tea tree essential oil (VP-TTEO) on the biofilm formation of antifungal-resistant *Candida* isolates. Biofilms of *Candida albicans* Ca2, *Candida guilliermondii* Cgj1,

Candida glabrata Cg7 and *Candida krusei* Ck1 were developed in the absence (control) and presence of the VP-TTEO. **(A)** Number of cultivable cells (Log CFUs mL⁻¹); **(B)** Absorbance values of Crystal Violet solutions (Abs CV) and **(C)** Absorbance values of XTT solutions (Abs XTT). * indicate statistical reduction of biofilms cell cultivability in comparison with the respective control (* p< 0.1, ** p< 0.01, *** p< 0.001, **** p< 0.0001). **(D)** Scanning electron microscope (SEM) images. The bar represents 30 µm (lower magnification image) or 10 µm (higher magnification image).....69

Figure 17. Effect of the vapor-phase of tea tree essential oil (VP-TTEO) on pre-formed biofilms of antifungal-resistant *Candida* isolates. Biofilms of *Candida albicans* Ca2, *Candida guilliermondii* Cgi1, *Candida glabrata* Cg7 and *Candida krusei* Ck1 were developed in the absence (control) and presence of the VP-TTEO. **(A)** Number of cultivable cells (Log CFUs mL⁻¹); **(B)** Absorbance values of Crystal Violet solutions (Abs CV) and **(C)** Absorbance values of XTT solutions (Abs XTT). * indicate statistical reduction of biofilms cell cultivability in comparison with the respective control (* p< 0.1, ** p< 0.01, *** p< 0.001, **** p< 0.0001). **(D)** Scanning electron microscope (SEM) images. The bar represents 30 µm (lower magnification image) or 10 µm (higher magnification image).....70

Chapter III. Vaginal microbiota conditions

Chapter III.1 Simulated Vaginal Fluid: *Candida* resistant strains' biofilm characterization and vapor-phase of essential oil effect

Figure 18. Subchapter III.1 workflow.....79

Figure 19. Simulated Vaginal Fluid (SVF) and Sabouraud Dextrose Broth (SDB) on vaginal *Candida* isolates planktonic cells. Planktonic growth curves of **(A)** *Candida albicans* Ca2, **(B)** *Candida guilliermondii* Cgi1, **(C)** *Candida glabrata* Cg7 and **(D)** *Candida krusei* Ck1 cells cultivated in SVF and SDB over 24 h.....85

Figure 20. Biofilm formation of antifungal-resistant *Candida* isolates in Simulated Vaginal Fluid (SVF) and Sabouraud Dextrose Broth (SDB). Number of cultivable cells (Log CFUs cm⁻²) for **(A)** 24 h and **(B)** 48 h. * indicate statistical reduction of biofilms cell cultivability in comparison with the respective control (* p< 0.1, ** p< 0.01, *** p< 0.001, **** p< 0.0001) (Dunnett's multiple comparisons test).....86

Figure 21. Scanning Electron Microscope (SEM) observations of biofilms formation structure (24 h) of antifungal-resistant *Candida* isolates in **(A)** Sabouraud Dextrose Broth (SDB) and **(B)** Simulated Vaginal Fluid (SVF). Magnification: 5,000×. Scale bar=30 μm.....86

Figure 22. Effect of the vapor-phase of essential oils (oregano (VP-OEO) and white thyme (VP-WTEO)) on **(A)** biofilm formation and **(B)** pre-formed biofilms of antifungal-resistant *Candida* isolates. Biofilms of *Candida albicans* Ca2, *Candida guilliermondii* Cgi1, *Candida glabrata* Cg7 and *Candida krusei* Ck1 were developed in the absence (control) and presence of the VP-EOs. * indicate statistical reduction of biofilms cell cultivability in comparison with the respective control (* $p < 0.1$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).....88

Chapter III.2 Vapor-phase of white thyme essential oil effect on *Candida albicans* and *Lactobacillus gasseri* colonization in a reconstituted human vaginal epithelium

Figure 23. Subchapter III.2 workflow.....90

Figure 24. The standard calibration curve of threshold cycle (CT) values vs Log number of cells.....96

Figure 25. Vapor-phase of white thyme essential oil (VP-WTEO) toxicity measured in *in vivo* *Galleria mellonella* model. Survival curves of *G. mellonella* larvae exposed to VP-WTEO and respective control (without exposure to VP-WTEO). There was no significant difference between control and VP-WTEO exposure.....97

Figure 26. Effect of the vapor-phase of white thyme essential oils (VP-WTEO) on the biofilm formation and pre-formed biofilm of antifungal-resistant *Candida albicans* Ca2. **(A)** Biofilm biomass (Abs CV) and **(B)** metabolic activity (Abs XTT). * indicate statistical reduction of biofilms cell cultivability in comparison with the respective control (* $p < 0.1$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).....99

Figure 27. Effect of the vapor-phase of white thyme essential oil (VP-WTEO) on single and co-colonization of *Candida albicans* with *Lactobacillus gasseri* of the reconstituted human vaginal epithelium (RHVE) after 24 h. **(A)** Number of cells (Log cells/ tissue) on single and mixed colonization in relation to the treated RHVE (VP-WTEO). * indicate statistical reduction of number of cells in comparison with the respective control (*** $p < 0.001$, **** $p < 0.0001$). **(B)** Relative lactate dehydrogenase (LDH) activity measured in the culture supernatants after 24 h of single and co-colonization in relation to simulated vaginal fluid (&) and VP-WTEO effect (*). */& indicate statistical reduction of LDH activity in comparison with the respective

control ($^{\wedge}/8 p < 0.1$, $^{***} p < 0.001$).....	100
---	-----

Figure 28. Effect of the vapor-phase of white thyme essential oils (VP-WTEO) on single and mixed colonization of <i>Candida albicans</i> and <i>Lactobacillus gasseri</i> in reconstituted human vaginal epithelium (RHVE) after 24 h. Image acquired by epifluorescence microscopy. The bar represents 50 μm and 20 μm	101
---	-----

Chapter IV. Effect of oregano essential oil in *Candida* infection

Chapter IV.1 Effect of vapor-phase of oregano essential oil on resistant *Candida* species biofilms: mechanisms of action

Figure 29. Subchapter IV.1 workflow	110
---	-----

Figure 30. Toxicity of the vapor-phase of oregano essential oil (VP-OEO) measured in a <i>Galleria mellonella</i> model. (A) Survival curves of <i>G. mellonella</i> larvae exposed to VP-OEO and the respective controls (without exposure to VP-OEO). (B) Total numbers of hemocytes counted in <i>G. mellonella</i> larvae after 4 and 72 h of exposure and those unexposed to VP-OEO.....	118
---	-----

Figure 31. Effect of the vapor-phase of oregano essential oil (VP-OEO) on biofilm formation and preformed biofilms of the antifungal-resistant <i>Candida</i> isolates (A) <i>Candida albicans</i> Ca2 and (B) <i>Candida glabrata</i> Cg8. * indicate a statistically significant reduction in biofilm cell cultivability in comparison with the respective controls (*, $P < 0.1$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).....	123
---	-----

Figure 32. Effects of the vapor-phase of oregano essential oil (VP-OEO) on (A) biofilm formation and (B) preformed biofilms of <i>Candida albicans</i> Ca2. Evaluated by a (1) time-killing assay, (2) cell viability, and (3) metabolic activity (an SI of <1 indicates metabolic activity). (C) Confocal laser scanning microscopy images of <i>C. albicans</i> Ca2 biofilms (biofilm formation and preformed biofilms) after 12 h of exposure to VP-OEO. CLSM images show the staining patterns for live cells (SYTO-9 [green]) and dead cells (propidium iodide [red]). * indicate a statistically significant reduction in biofilm cell cultivability compared to that at the previous time point (* $p < 0.1$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).....	125
--	-----

Figure 33. Effects of the vapor-phase of oregano essential oil (VP-OEO) on (A) biofilm formation and (B) preformed biofilms of <i>Candida glabrata</i> Cg8. Evaluated by a (1) time-killing assay, (2) cell viability, and (3) metabolic activity (an SI of <1 indicates metabolic activity). (C) Confocal laser scanning microscopy	
--	--

images of *C. glabrata* Cg8 biofilms (biofilm formation and preformed biofilms) after 12 h of exposure to VP-OEO. CLSM images show the staining patterns for live cells (SYTO-9 [green]) and dead cells (propidium iodide [red]). * indicate a statistically significant reduction in biofilm cell cultivability compared to that at the previous time point (* $p < 0.1$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).....125

Figure 34. Effect of the vapor-phase of oregano essential oil (VP-OEO) on *Candida* species infection of the reconstituted human vaginal epithelium (RHVE). (A) *Candida albicans* Ca2 and *Candida glabrata* Cg8 infection of the RHVE and effect of VP-OEO after 24 h. (B) *C. albicans* Ca2 and *C. glabrata* Cg8 infection of the RHVE after 24 h (Log CFU per milliliter). (C) Relative lactate dehydrogenase (LDH) activity measured in the RHVE culture supernatant after 24 h of incubation with the *C. albicans* Ca2 and *C. glabrata* Cg8 strains compared to the untreated epithelium (without VP-OEO) and effect of VP-OEO compared to simulated vaginal fluid. * indicate a statistically significant reduction in biofilm cell cultivability in comparison with that of the respective control (* $p < 0.001$; **** $p < 0.0001$).....128**

Chapter IV.2 Design and evaluation of microencapsulated oregano essential oil as an alternative treatment to *Candida albicans* infection

Figure 35. Subchapter IV.2 workflow (Created with BioRender.com (BT26ABXE9C)).....130

Figure 36. The standard calibration curve of oregano essential oil (OEO) in ethyl acetate.....134

Figure 37. The standard calibration curve of oregano essential oil (OEO) in simulated fluid vaginal (SFV).....134

Figure 38. Characterization of keratin microparticles prepared in Phosphate-buffered saline (PBS) encapsulating oregano oil (OEO-KMP's) for 5 months (155 days). (A) particle size (Z-average) and polydispersity (PDI); (B) surface charge (zeta-potential). *indicate statistical difference in particle size when compared to the results obtained at time 0 (initial conditions) (* $p < 0.1$, ** $p < 0.01$, ** $p < 0.0001$)....140**

Figure 39. *In vitro* release profiles of oregano essential oil from keratin-based particles in simulated vaginal and sweat fluids, over 72 h. * indicate statistical difference compared to the results obtained at time 0 h (* $p < 0.1$, ** $p < 0.01$, * $p < 0.001$, **** $p < 0.0001$).....141**

Figure 40. Keratin microparticles encapsulating oregano oil (OEO-KMP's) cytotoxicity measured in *Galleria mellonella* model. (A) Survival curves of *G. mellonella* larvae in contact with OEO-KMP's and

respective controls (without exposure to OEO-KMP's and KMP's) during 72 h; and **(B)** *G. mellonella* larvae health index after 24, 48 and 72 h in contact with OEO-KMP's and respective controls.....143

Figure 41. Effect of the keratin microparticles encapsulating oregano oil (OEO-KMP's) on single and mixed infection of *Candida albicans* with *Lactobacillus gasseri*. **(A)** Single infection of *C. albicans*; **(B)** Mixed infection (*C. albicans*); **(C)** Mixed infection (*L. gasseri*). *indicate statistical reduction of biofilms cell cultivability in comparison with the respective control (**p< 0.01, *** p< 0.001, **** p< 0.0001).....144

Figure 42. Effect of OEO-KMP's on mice model of vaginal candidiasis. **(A)** Timeline of *Candida albicans* infection and OEO-KMP's treatment of mice; **(B)** Mice weight according to the timeline of infection; **(C)** *C. albicans* and *Lactobacillus* cells detected in mice vaginal lavage fluid (Log CFU mL⁻¹) represented is the median with 95 % confidence interval (*p < 0.05); **(D)** Histological images of vaginal tissue stained with periodic acid-Schiff (PAS), increasing magnification along the column: **I.** Mice uninfected (negative control); **II.** Mice infected with *C. albicans*, untreated (positive control); **III.** Mice infected with *C. albicans*, treated with OEO-KMP's for 24 h after infection. (a) layer of superficial squamous epithelial cells, (b) basal membrane, (c) lamina propria (d) vaginal lumen, (e) and (f) *C. albicans* cells and (g) neutrophils. The results were registered under 10x and 20x magnification. The data presented in this study are representative of at least two independent experiments.....146

Annex I. Material supplementary

Annex I

Figure 43. Chromatographic profile (polar column) obtained for the tea tree essential oil (Lot 903025).168

Figure 44. Chromatographic profile (non-polar column) obtained for the tea tree essential oil (Lot 903025).....169

Figure 45. Chromatographic profile (polar column) obtained for the cajeput essential oil (Lot 801736).171

Figure 46. Chromatographic profile (non-polar column) obtained for the cajeput essential oil (Lot 801736).....172

Figure 47. Chromatographic profile obtained for the niaouli essential oil (Lot 100378).....174

Figure 48. Chromatographic profile (polar column) obtained for the white thyme essential oil (Lot

800180).....	176
--------------	-----

Figure 49. Chromatographic profile (non-polar column) obtained for the white thyme essential oil (Lot 800180).....	177
---	-----

Figure 50. Chromatographic profile obtained for the oregano essential oil (Lot 10468).....	179
---	-----

Annex III

Figure 51. Histological images of vaginal tissue of mice infected with <i>C. albicans</i> without any vaginal treatment or lavage, stained with periodic acid-Schiff (PAS), increasing magnification along the column. The results were registered under 10x and 20x magnification. The data presented in this study are representative of at least two independent experiments.....	182
---	-----

Chapter I. General Introduction**Chapter I.1 Essential oils as alternative treatment to *Candida* infection: review**

Table 1. Essential oil encapsulation against *Candida* species, information about several factors that can influence the antifungal effect of the formulations. Encapsulation structure, particle size (nm), polydispersity (PDI), zeta potential (mV), encapsulation efficiency (% EE) an cytotoxicity.....21

Chapter II. Effect of essential oils on drug-resistant *Candida* strains**Chapter II.1 Essential oils as a good weapon against drug-resistant *Candida auris***

Table 2. Identification of the component of tea tree, cajeput, niaouli and white thyme essential oils expressed as a percentage (%) of individual compound in the total essential oil sample.....43

Chapter II.2 Comparing the effect of *Thymus* species essential oils no *Candida auris*

Table 3. Chromatography analysis of the composition of essential oils from *Thymus vulgaris*, *Thymus zygis*, *Thymus satureioides* and *Thymus mastichina*, here shown in percentage (%).....52

Table 4. Diameter of the inhibition zone, in mm, of the essential oils from different plants of *Thymus* genus (10 µL) for *Candida auris* NCPF 8971.....53

Chapter II.3 Vapor-phase of essential oils as promising solution to prevent *Candida* vaginal biofilms caused by antifungal resistant strains

Table 5. List of vaginal isolates used in this study and respective data, including species, features of the women at the moment of sample collection and minimum inhibitory concentration (MIC) of fluconazole, ketoconazole and caspofungin for each isolate.....62

Table 6. Anti-*Candida* activity of essential oils on drug-resistant isolates evaluated through the disk-diffusion method.....67

Chapter III. Vaginal microbiota conditions

Chapter III.2 Vapor-phase of white thyme essential oil effect on *Candida albicans* and *Lactobacillus gasseri* colonization in a reconstituted human vaginal epithelium

Table 7. Primers for real time-PCR assay for quantification of *Candida albicans* and *Lactobacillus gasseri* cells in reconstituted human vaginal epithelium.....95

Table 8. Anti-*Candida* activity of white thyme essential oil on drug-resistant vaginal isolates. Results obtained from the agar disk diffusion method and zones of inhibition measured through the diameter of the halo (D_{halo}) in millimeters (mm).....98

Chapter IV. Effect of oregano essential oil in *Candida* infection

Chapter IV.1 Effect of vapor-phase of oregano essential oil on resistant *Candida* species biofilms: mechanisms of action

Table 9. Primers for real time-PCR assay for quantification of *Candida albicans* and *Candida glabrata* cells.....117

Table 10. Anti-*Candida* activity of oregano essential oil on drug-resistant isolates evaluated through the disk-diffusion method by contact direct (DC-OEO) and vapor-phase (VP-OEO).....119

Table 11. Compounds identified (%) in oregano essential oil, and their characteristics, such as vapor pressure (mm Hg), water solubility (mg L^{-1}) and Log K_{ow}120

Chapter IV.2 Design and evaluation of microencapsulated oregano essential oil as an alternative treatment to *Candida albicans* infection

Table 12. Characterization of keratin microparticles prepared in Phosphate-buffered saline (PBS) encapsulating oregano oil (OEO-KMP's) when in contact with simulated vaginal fluid and simulated sweat fluid for 72 h. * indicate statistical difference in particle size when compared to the results obtained at time 0 (Figure 38) (**** $p < 0.0001$).....142

Table 13. Histopathological evaluation of vaginal irritation after infection and 24 h of rectovaginal application of PBS (positive group) or OEO-KMP's. Score interpretation: minimal (1–4), mild (5–8),

moderate (9–11), and severe (12–16).....	149
--	-----

Annex I. Material supplementary

Annex I

Table 14. Polar column analysis of tea tree essential oil (Lot 903025).....	168
Table 15. Non-polar column analysis of tea tree essential oil (Lot 903025).....	169
Table 16. Polar column analysis of cajeput essential oil (Lot 801736).....	171
Table 17. Polar noncolumn analysis of cajeput essential oil (Lot 801736).....	172
Table 18. Column analysis of niaouli essential oil (Lot 100378).....	174
Table 19. Polar column analysis of white thyme essential oil (Lot 800180).....	176
Table 20. Non-polar column analysis of white thyme essential oil (Lot 800180).....	177
Table 21. Column analysis of oregano essential oil (Lot 10468).....	179

Annex II

Table 22. Complete chromatography analysis of the composition of essential oils from <i>Thymus vulgaris</i> , <i>Thymus zygis</i> , <i>Thymus satureioides</i> and <i>Thymus mastichina</i> , here shown in percentage (%).....	180
--	-----

LIST OF ABBREVIATIONS AND SYMBOLS

Abbreviations

Abs Absorbance

AmB Amphotericin B

ANOVA Analysis of variance

ATCC American Type Culture Collection

CDC Centers for Disease Control and Prevention

CFU Colony-forming Units

CLSI Clinical & Laboratory Standards Institute

CLSM Confocal Laser Scanning Microscopy

CT Threshold Cycle

CV Crystal Violet

DC Direct contact

DC-OEO Direct contact of oregano essential oil

DMEM Dulbecco's modified eagle medium

DNA Deoxyribonucleic acid

ECM Polymeric Extracellular Matrix

EE Encapsulation Efficiency

EO Essential oil

EUCAST European Committee for Antimicrobial Susceptibility Testing

E2 17 β -Estradiol

FBS Fetal Bovine Serum

FDA Food and Drug Administration

FEMS Federation of European Microbiological Societies

FID Flame-Ionization Detection

FISH Fluorescence *in situ* hybridization

FLC Fluconazole

FUN-1 [2-chloro-4-(2,3-dihydro-3-methyl-(benzo-1,3-thiazol-2-yl)-methylidene)-1-phenylquinolinium iodide]

GC Gas Chromatography

GC-FID Gas Chromatography with Flame-Ionization Detection

GC-MS Gas Chromatography–Mass Spectrometry

HGUGM Hospital General Universitario Gregorio Marañón

HIV Human Immunodeficiency Virus

ICP-MS Inductively coupled plasma mass spectrometry

ICU Intensive care Units

IUD Intrauterine Devices

KMP Microparticles of Keratin

LDH Lactate Dehydrogenase

MFC Minimum Fungicidal Concentrations

MIC Minimum Inhibitory Concentrations

MRS Mann Rogosa and Sharpe

MRSA Mann Rogosa and Sharpe Agar

MRSB Mann Rogosa and Sharpe Broth

MTS (3-[4,5-carboxymethoxyphenyl]-2-(4-sulfophenyl)-2H-tetrazolium)

NCAC Non-*Candida albicans Candida*

NCPF National Collection of Pathogenic Fungi

NO Nitric oxide

OD Optical density

OEO Oregano essential oil

OEO-KMP Microparticles of Keratin loaded with oregano essential oil

PAS Periodic acid-Schiff

PBS Phosphate-buffered saline

PCR Polymerase chain reaction

PDI Polydispersity index

PDT Photodynamic therapy

PI Propidium Iodide

PMS Phenazine Methosulphate

qRT-PCR Quantitative real-time PCR

RHVE Reconstituted Human Vaginal Epithelium

RNA Ribonucleic acid

ROS Reactive Oxygen Species

RPMI Roswell Park Memorial Institute

RVVC Recurrent Vulvovaginal Candidiasis

SDA Sabouraud Dextrose Agar

SDB Sabouraud Dextrose Broth

SDS Sodium Dodecyl Sulfate

SEM Scanning Electron Microscope

SGLT2 Sodium glucose cotransporter 2

SI Staining Index

SSF Simulated Sweat Fluid

SVF Simulated Vaginal Fluid

TTEO Tea Tree Essential Oil

UPLC/ PDA Ultra-performance liquid chromatography coupled to photodiode array detection

VP Vapor- phase

VP-EO Vapor- phase of Essential Oil

VP-OEO Vapor- phase of Oregano Essential Oil

VP-TTEO Vapor-phase of Tea Tree Essential Oil

VP-WTEO Vapor-phase of White Thyme Essential Oil

WVC Vulvovaginal Candidiasis

WHO World Health Organization

WTEO White Thyme Essential Oil

XTT (2,3-(2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide)

YNB Yeast Nitrogen Base

ZI Inhibition zone

Symbols

p Significance value

% Percent

°C Degrees

Pka Negative base-10 logarithm of the acid dissociation constant

W/I without information

rpm Revolutions per minute

g Gravitational force

Log K_{ow} Octanol/water partition coefficient

THESIS OUTLINE

THESIS OVERVIEW, AIM & OUTPUTS

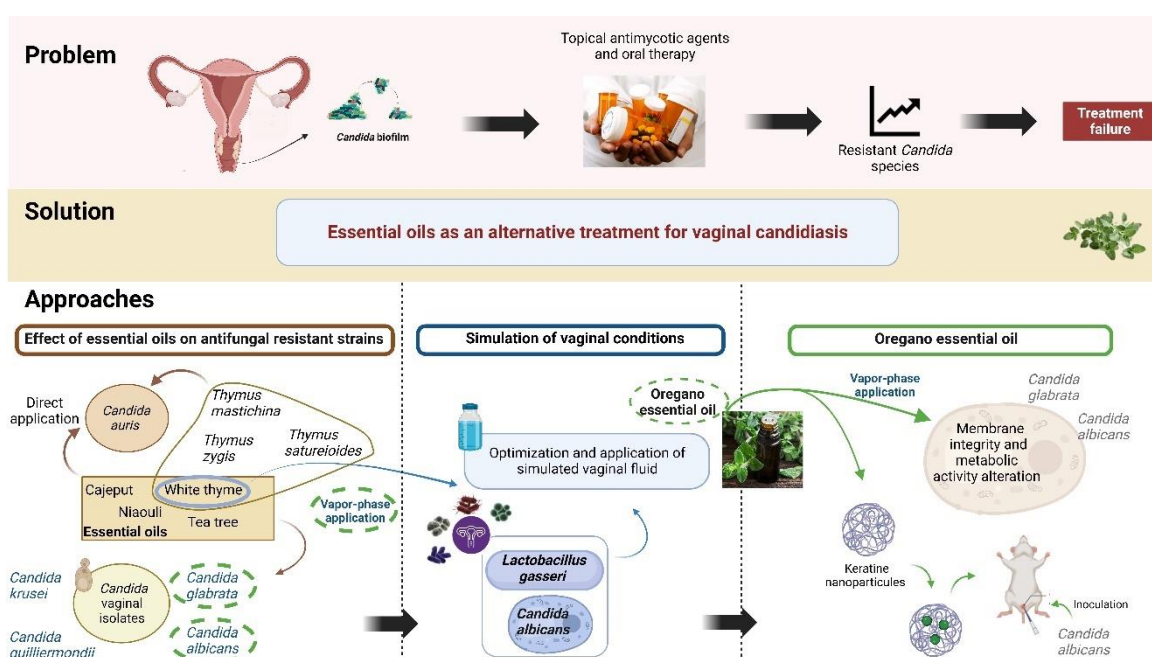


Figure 1. Workflow of the thesis (Created with BioRender.com (KW26ABVIPV)).

A significant infection, Vulvovaginal Candidiasis (VVC), caused by *Candida* species, is frequently accompanied by biofilm formation. The equilibrium between *Candida* organisms and the natural defense in the vaginal environment normally allows *Candida* species to persist as commensals. However, alterations in the host's vaginal/vulvar environment can disrupt this equilibrium, leading to the development of infections. VVC is a global concern due to the associated morbidity, interfering with patients' physical and mental health. Changes in self-confidence often occur, as the condition may evoke sensations of embarrassment or frustration. In addition, interference in sexual and affective relationships can cause emotional distress. From an economic point of view, the direct and indirect costs associated with the management and treatment of VVC can be considerable. The repercussions of untreated VVC are particularly alarming, potentially leading to serious complications, such as pelvic inflammatory disease, which can have implications for fertility. Ectopic pregnancy, pelvic abscess and spontaneous abortion are additional risks that emphasise the potential seriousness of untreated VVC. Furthermore, systemic candidiasis is related to high mortality. Despite remarkable progress in therapeutic approaches, VVC still to affect millions of women every year. Although topical antimycotic agents and oral therapies represent available options for the VVC treatment, their use has several disadvantages. For instance, the development of drug-resistant species and the presence of certain risk factors can compromise the effectiveness of these therapies. The persistence of VVC cases, along with the associated negative consequences and therapeutic limitations (e.g. toxicity), emphasises the urgency of developing alternative treatments that are not only effective, but also economical and devoid of toxic side effects.

Numerous natural substances, including essential oils (EOs), have demonstrated antifungal properties. EOs exhibit a range of biological properties, including anti-leishmanial, anti-inflammatory, antioxidant, hepatoprotective, anti-tumoral, and antimicrobial activities. Therefore, EOs are considered a promising alternative for VVC treatment, due their low toxicity and potent activity against drug-resistant *Candida* strains, including in the biofilm form, offering a viable alternative to antibiotics. However, the specific spectrum of activity and mechanisms of action of many EOs remain largely unknown.

Therefore, this work aims to study the potential of EOs and their method of application as an alternative treatment for VVC, without undesirable effects on the skin or the beneficial vaginal microbiota.

The increasing incidence of Candidiasis, along with the associated negative consequences and the growing inefficacy of conventional antifungal drugs, make it crucial to increase knowledge about therapeutic alternatives for the treatment of vaginal *Candida* infections. This research aims to respond to the urgent need for a more effective and non-toxic treatment for Vulvovaginal Candidiasis (VVC), considering the limitations of current therapeutic approaches. The key hypothesis guiding this research is that essential oils (EOs) have broad-spectrum activity against drug-resistant *Candida* species, including when these species form more complex structures, such as biofilms. In addition, it is suggested that these EOs are safe and more effective in relieving the symptoms of candidiasis compared to common antifungal drugs.

Therefore, the main goal of this thesis is the development and validation of a new, effective, and non-toxic treatment for VVC, using essential oils (EOs) in their vapor-phase or in their encapsulated form, overcoming the limitations of current antifungal medications. So, this research aligns with the current health research priorities and has significant implications for women's reproductive health and overall well-being.

The present thesis reports the works performed at the Centre of Biological Engineering of University of Minho integrated in the biofilm research group under the coordinated of Professor Doctor Mariana Henriques and Doctor Maria Elisa Rodrigues, with part of research carried out at the Pathology Department of the Faculty of Medicine of the University of Porto under the coordination of Doctor Ana Sofia Quinta e Costa Neves de Oliveira.

This work was also guided by the suggestions and clinical evidence provided by Doctor Raquel Costa from the Costa Raquel, Aromas Aqua Spa–Health Clinic. One of the tasks developed during the thesis was carried out in collaboration with the Bioprocesses and Bionanotechnology group from the Centre of Biological Engineering, under the supervision of Doctor Artur Ribeiro. Another part of the work was developed at the National Institute of Agricultural and Veterinary Research in Vila do Conde, under the supervision of Doctor Sónia Silva. A scientific collaboration was established with Doctor Lorena Cussó from the *Instituto de Investigación Sanitaria Gregorio Marañón* (Madrid, Spain).

The present thesis is divided into five chapters, briefly:

- **Chapter I: General Introduction**

In the first chapter is provide an overview of the latest information concerning novel alternative strategies being explored for the treatment of *Candida* infections. Special attention is given to natural therapies, plant extracts, and essential oils, as well as their incorporation into controlled delivery systems, such as micro and nanocapsules.

- **Chapter II: Effect of Essential oils on Drug-resistant *Candida* Strains**

The second chapter reports the research carried out to evaluate the effect of different essential oils against *Candida auris*, an emerging *Candida* species, and drug-resistant *Candida* vaginal isolates, in both planktonic and biofilm states. For this, an *in vitro* screening of EOs was carried out and the most effective essential oils were identified.

- **Chapter II.1: Impact of Essential Oils on *Candida auris*.** The first part of this chapter outlines the experiments performed to determine the effect of four essential oils, namely tea tree, niaouli, white thyme and cajeput against *Candida auris*.
- **Chapter II.2: Variations in the Effects of Essential Oils from the Same Genus on *Candida auris*.** Continuing with the exploration of *Candida auris*, the second part of this chapter investigates how essential oils derived from the same genus, such as *Thymus vulgaris*,

Thymus zygis, *Thymus satureioides* and *Thymus mastichina*, can exhibit diverse effects against *Candida auris* species.

- **Chapter II.3: Antifungal Activity of Essential Oils on Drug-Resistant *Candida* Vaginal Isolates.** The third part of this chapter presents the work carried out to evaluate the antifungal properties of essential oils (tea tree, niaouli, white thyme, and cajeput) on drug-resistant *Candida* vaginal isolates.

- **Chapter III: Optimization of the methodology to mimic the conditions of the vaginal microbiota.**

The third chapter consisted of optimizing the conditions in order to mimic the vaginal conditions as much as possible, providing a better understanding of the antifungal behavior of essential oils.

- **Chapter III.1: Simulated Vaginal Fluid.** In the first part of this chapter, the most appropriate culture medium that is similar to vaginal conditions was defined and optimized.
- **Chapter III.2: Essential oil effect induced in *Lactobacillus* species.** In the second part of this chapter was observed how the chosen therapeutic approach, essential oils, impacts the beneficial species within the vaginal microflora, such as *Lactobacillus* species.

- **Chapter IV: Effect of oregano essential oil in *Candida* infection**

The fourth chapter is dedicated entirely to the research conducted with oregano essential oil, as it emerged as the most promising among the several essential oils examined in this thesis.

- **Chapter IV.1: Impact of Oregano Essential Oil and Its Vapor-Phase on Antifungal-Resistant *Candida* Species.** The initial section of this chapter focuses on evaluating the effect of oregano essential oil and its vapor-phase on antifungal-resistant *Candida* species (*Candida albicans* and *Candida glabrata*), aiming to elucidate its mode of action.
- **Chapter IV.2: Keratin-Loaded Nanoparticles with Oregano Essential Oil.** The second part of this chapter involves the design, production and characterization of microparticles of keratin loaded with oregano essential oil as another alternative application of oregano essential oil for the treatment of *Candida albicans* infection.
- **Chapter V: General discussion, final remarks and future work.** This chapter presents a general discussion and the main conclusions of this thesis and future perspectives for possible future research work.

The present dissertation resulted in the publication of six scientific papers, one manuscript is under review and two others are submitted, as described below:

- Fernandes L, Ribeiro R, Costa R, Henriques M, Rodrigues ME. Essential Oils as a Good Weapon against Drug-Resistant *Candida auris*. Antibiotics 2022, Vol 11, Page 977 2022;11:977.
- Ribeiro R, Fernandes L, Costa R, Cavaleiro C, Salgueiro L, Henriques M, Rodrigues M.E. Comparing the effect of *Thymus* spp. essential oils on *Candida auris*. Ind Crops Prod 2022; 178:114667.
- Fernandes L, Ribeiro R, Henriques M, Rodrigues ME. *Candida auris*, a singular emergent pathogenic yeast: its resistance and new therapeutic alternatives. European Journal of Clinical Microbiology & Infectious Diseases 2022,41(12):1371-1385
- Fernandes L, Gonçalves B, Costa R, Fernandes Â, Gomes A, Nogueira-Silva C, Silva S, Rodrigues ME, Henriques M. Vapor-Phase of Essential Oils as a Promising Solution to Prevent *Candida* Vaginal Biofilms Caused by Antifungal Resistant Strains. Healthcare 2022, Vol 10, Page 1649 2022;10:1649.
- Fernandes L, Costa R, Henriques M, Rodrigues ME. Simulated Vaginal Fluid: *Candida* resistant strains' biofilm characterization and vapor phase of essential oil effect. Journal of Medical Mycology 2022:101329.
- Fernandes L, Costa R, Silva S, Henriques M, Costa-de-Oliveira S, Rodrigues ME. Effect of Vapor-Phase Oregano Essential Oil on Resistant *Candida* Species Biofilms: Mechanisms of Action. Microbiol Spectr 2023;11.
- Fernandes L, Costa-de-Oliveira S, Henriques M, Rodrigues ME. Nanoencapsulated essential oil as an alternative treatment for *Candida* infection: review (under review in the Fungal Biology Reviews)
- Fernandes L, Silva I, Costa R, Silva S, Mira Pereira N, Costa-de-Oliveira S, Henriques M, Rodrigues ME. Vapor-phase of white thyme essential oil effect on *Candida albicans* and *Lactobacillus gasseri* colonization in a reconstituted human vaginal epithelium (In submission)
- Fernandes L, Blázquez E, Tejada A, Ribeiro A, Silva S, Cussó L, Costa-de-Oliveira S, Rodrigues ME, Henriques M. Design and evaluation of microencapsulated oregano essential oil as alternative treatment to *Candida albicans* infection. (In submission)

During the development of this thesis, the following works were presented in congresses:

- Fernandes L, Costa-de-Oliveira S, Costa R, Silva S, Henriques M, Rodrigues ME. Effect of vapor-phase essential oil on resistant *Candida* species: Mechanisms of action. Poster presented in FEMS Conference on Microbiology 2022, Belgrade, Serbia.
- Fernandes L, Costa R, Silva S, Henriques M, Rodrigues ME. *Efeito da fase de vapor de óleos essenciais em Candida albicans e Lactobacillus gasseri aderidos a epitélio vaginal humano reconstituído*. Video presented in *Congresso internacional em língua portuguesa Microbiologia* 2022, Online.
- Fernandes L, Costa R, Silva S, Mira Pereira N, Costa-de-Oliveira S, Henriques M, Rodrigues ME. Vapor-phase of white thyme essential oil effect on *Candida albicans* and *Lactobacillus gasseri* colonization in a reconstituted human vaginal epithelium. Oral presentation presented in 5TH Congress of Baltic Microbiologists (FEMs grant), 2023, Vilnius, Lithuania.
- Fernandes L, Blázquez E, Tejada A., Ribeiro,A, Silva S, Mira N, Cussó L, Costa-de-Oliveira S, Rodrigues ME, Henriques M. Oregano Essential Oil: An Effective and Non-Toxic Approach for prevent or treat Resistant *Candida* Species. Poster presented in Congress of Microbiology and Biotechnology (FEMs grant),2023, Covilhã, Portugal.

CHAPTER I

GENERAL INTRODUCTION

In recent decades, the incidence of human fungal infections has increased, affecting millions worldwide, with *Candida* species among the main nosocomial agents. Fungal infections are commonly treated with antifungal drugs, mainly belonging to the azoles, polyenes and echinocandins categories. However, most of these antifungals have several disadvantages, such as the intensity of side effects or a lack of fungicidal efficacy. Therefore, there is a growing need to search for new drugs that are more effective and less toxic compared to those already available. In this regard, therapy based on natural products has emerged, as they have several advantages, including lower costs and adverse reactions, easy access, application in various pathologies and less negative impact on the patient's health. Among these natural products, essential oils (EOs) have been acknowledged as versatile sources for potential therapeutic products, recognized for their multifaceted properties, including antiviral, antileishmanial, anti-inflammatory, antioxidant, antitumor, hepatoprotective, and mainly for its antimicrobial properties.

The antifungal activity of EO in *Candida* species has been the subject of several studies. However, the possible toxicity, strong taste, chemical instability, restricted administration routes and volatility of EOs limit their application. So, nanotechnology is receiving particular attention to overcome these obstacles. The incorporation of a controlled delivery system represents a promising strategy to avoid the problems, making their application safer, protecting EO degradation and prolonging the duration of the agent at the site of action. This approach proves to be a viable choice, particularly in highly complicated environments such as biofilms.

CHAPTER I.1

ESSENTIAL OIL AS ALTERNATIVE TREATMENT TO *CANDIDA* INFECTION: REVIEW

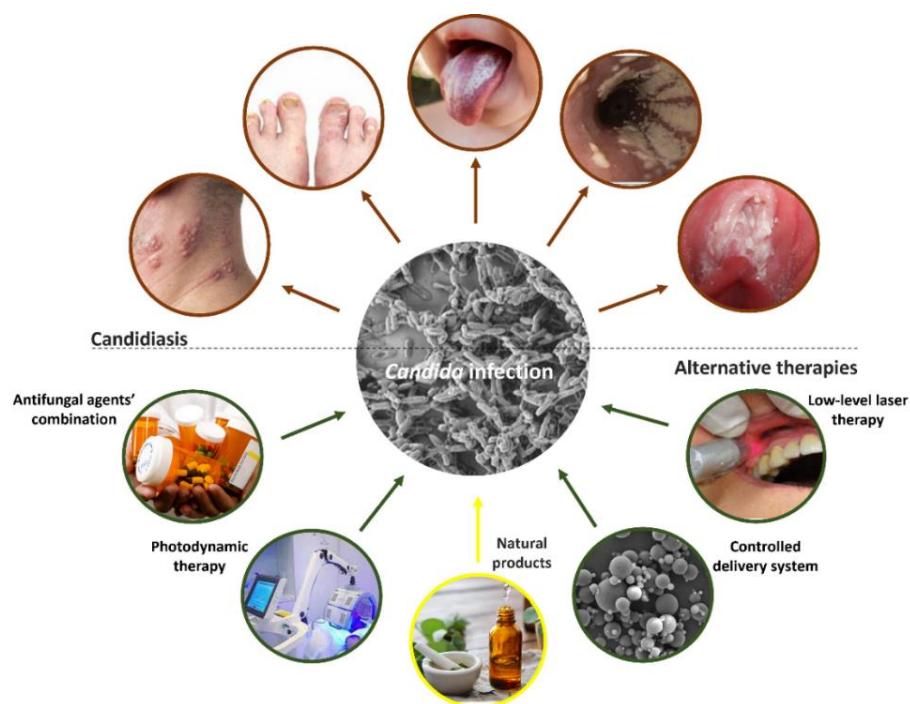


Figure 2. Subchapter I.1 workflow.

This first chapter aims, firstly, to present an overview of the global situation concerning fungal infections, particularly those induced by *Candida* species, along with the current therapeutic options available; and secondly, to provide insights into emerging alternative approaches for the treatment of *Candida* infections, with a specific emphasis on natural therapies, plant extracts and essential oils, as well as their integration into controlled administration systems.

This chapter is based on the following manuscript: Fernandes L, Costa-de-Oliveira S, Henriques M, Rodrigues ME. Nanoencapsulated essential oil as an alternative treatment for *Candida* infection: review (under review in the Fungal Biology Reviews, Manuscript Number: FBR-D-23-00067)

1. Introduction

According to the World Health Organization (WHO), infections continue to be one of the primary causes of death worldwide. Despite considerable advances in science and medicine, it has not yet been possible to control the exponential spread of infectious diseases [1]. The kingdom of fungi has approximately 1.5- 5 million species, of which 500- 600 can cause infections in humans [2–4]. Following the evolution of medicine and surgical procedures, there is a growing concern regarding fungal healthcare-associated infections [5–7]. Recently, a wide variety of biological agents have been approved for the treatment of cancer or autoimmune diseases, some targeting the inhibition of various host immune response pathways [8]; however, these agents also have the potential to disrupt the body's immune response that generally protects against pathogens, conferring greater susceptibility to infectious complications [8]. During the last decade, fungal infections induced 11.5 million severe cases and 1.5 million deaths, worldwide [4]. The global market for invasive fungal infections was valued at \$ 7.13 billion in 2022 and is projected to reach \$ 9.00 billion by 2028 [9]. Among all invasive fungal infections worldwide the genus *Cryptococcus*, *Aspergillus*, *Mucor*, *Pneumocystis*, *Saccharomyces* and *Candida* are responsible for more than 75 % of mortality [4,10]. Most fungi are part of the human microbiome as a commensal organism, however, in immunocompromised hosts, they become opportunistic agents since weakened immune systems cannot fight these pathogens [4]. This change in the microorganism pathogenicity can result from applying more aggressive treatments such as hematopoietic stem cell or organ transplantation, new chemotherapeutic and immunomodulatory agents and invasive devices, especially intravascular central lines [7]. Consequently, the global population of immunocompromised individuals at high risk for fungal infections is rising, accounting for approximately 3 % of the total population [7]. Therefore, the high mortality rates associated with fungal infections are a significant and pressing concern. One of the most recent factors is the COVID-19 pandemic, during which opportunistic fungal infections were documented in COVID-positive hosts [4]. Indeed, coronavirus 2 (SARS-CoV-2) has led to the widespread use of empiric broad-spectrum antimicrobial agents due to the clinical uncertainty that prevailed during the pandemic [11]. This uncontrolled use of antibiotics is a major contributing factor to the increase in microorganism resistance [12].

Fungi can cause several types of illnesses, including asthma or allergies, irritations or infections on the nails and skin, pneumonia (with symptoms resembling tuberculosis or flu), bloodstream infections and meningitis (resulting from outdoor environmental or healthcare exposure) [5,13]. These infections are often underestimated, and their diagnosis is challenging since the symptoms of fungal infections are often resemble those of viral or bacterial infections. This similarity can lead to diagnostic delays and

misdiagnosis, consequently resulting in delayed or incorrect treatment [5]. In fact, Dr. Hanan Balkhy, WHO Assistant Director-General, stated “Emerging from the shadows of the bacterial antimicrobial resistance pandemic, fungal infections are growing, and are ever more resistant to treatments, becoming a public health concern worldwide” [14]. In this regard, estimates indicate that mortality related to fungal infections may be five times higher than reported [5,6]. Due to the limited antifungal therapies available and the consequent emergence of drug resistance species, coupled with the aforementioned increase in the vulnerable population, morbidity and mortality caused by fungal infections are continuously rising and are an emerging problem [15]. Indeed, the current antifungal therapies are based on inhibition targets and are limited to 4 classes: azoles, polyenes, echinocandins and pyrimidine analogs [15].

So, the high incidence of fungal infections, its negative consequences and the increase of antifungal drug failure treatments make it crucial to drive knowledge on this subject and to invest in more effective and safer alternative therapies.

2. Candidiasis, *Candida* species and its characteristics

Infections caused by *Candida* fungus are the most common fungal disease, being able to cause mucocutaneous infection (mouth, throat, gut, and vagina) and, in more complicated situations, deep-seated or bloodstream (invasive candidiasis) infections [2,6,16]. According to the Centers for Disease Control and Prevention (CDC), the incidence of invasive candidiasis in the United States is approximately 9 cases per 100,000 people, totaling around 25,000 cases per year [9,17]. The associated in-hospital mortality is estimated to be about 25 % [9,17]. Candidiasis in the esophagus is known as *Candida* esophagitis or esophageal candidiasis and is one of the most common infections in individuals with HIV/AIDS. Typical symptoms of this type of candidiasis include difficulty and pain while swallowing [12]. Candidiasis affecting the throat and mouth is termed oropharyngeal candidiasis or thrush, presenting with a range of symptoms. These may include the presence of white patches on the inner tongue, cheeks, roof of the mouth, and throat, along with soreness, redness, pain, loss of taste, a cotton-like feeling in the mouth, and cracking at the corners of the mouth [18,19]. In contrast to *Candida* infections in the throat and mouth, invasive candidiasis represents a more severe infection capable of affecting multiple organs and systems, including the brain, bloodstream, heart, bones, and eyes [20]. Candidemia, a bloodstream infection, is a common occurrence in hospitalized patients and is associated with high morbidity and mortality rates in immunosuppressed individuals, HIV, chemotherapy and organ transplant patients [2,6,16]. In such cases, determining associated symptoms can be challenging, as

invasive candidiasis often occurs in patients with other medical conditions. Nevertheless, the most common symptoms include fever and chills that persist despite antibiotic treatment [20].

Compared to invasive candidiasis, vaginal candidiasis is not as dangerous; however, this infection affects millions of women every year. It is estimated that 70- 75 % of women experience an episode in their lifetimes [21]. Commonly known as vulvovaginal candidiasis (VVC), *Candida vaginitis*, or vaginal yeast infection, this condition is primarily caused by *Candida* species, especially *Candida albicans*. Infection occurs when vaginal conditions are altered in a way that favors the growth of these microorganisms. The main contributors to this change include broad-spectrum antibiotics, hormones changes, or alterations in the immune system. Pregnancy is also a critical condition for the development of VVC, as it is considered more challenging to eradicate. This may be supported by factors related to pregnancy itself, such as immunological changes, increased estrogen levels and vaginal glycogen production [22].

VCC can be sporadic or recurrent, depending on episodic frequency. Sporadic VVC includes mild to moderate clinical symptoms, such as vaginal and vulvar pruritus, thick secretion, pain, erythema, burning and/or edema, along with external dysuria and dyspareunia [22,23]. Recurrent VVC (RVVC) may be defined as four or more episodes in a 12- month period, associated with more severe symptoms [22,23]. RVVC affects approximately 138 million women worldwide annually (with a range of 103– 172 million), resulting in a global annual prevalence of 3871 per 100.000 women. Over the course of their lifetime, 372 million women experience RVVC [23]. In a study conducted by our team, it was observed that women with a history of RVVC infections use non-cotton underwear, over-the-counter antifungals and oral contraceptive pills, factors considered to be significantly higher risk for the development of VVC [21].

Understanding and monitoring the epidemiology of clinically relevant species and their antimicrobial susceptibility patterns is critical to interpreting the significance of antifungal susceptibility test results and guiding the most appropriate therapy [24,25]. However, there is no national or international surveillance for *Candida* infections and fungal diseases are likely widely underdiagnosed, making the exact number of cases of candidiasis difficult to determine [5,26].

The genus *Candida* includes about 150 species and 15- 20 of these species are regularly reported from human infections, mainly in immunosuppressed hosts [2,27]. *Candida albicans* is responsible for around 80- 90 % of infections and is the most virulent species in the genus, although *non-Candida albicans Candida* (NCAC) species (such as *Candida tropicalis*, *Candida glabrata*, *Candida guilliermondii*, *Candida parapsilosis*, *Candida dubliniensis*, *Candida auris*, *Candida lusitaniae*, *Candida nivariensis* and *Candida krusei*) are becoming frequently implied [2]. The incidence of *Candida* infection varies according to several

risk factors, including the individual's age and health stage, the specific sites of infection associated with each species and geographic region [28]. For instance, in a study conducted in Portugal, a total of 341 vaginal isolates were recovered from 308 participants. This included 59.2 % of *C. albicans*, 21.7 % of *C. glabrata*, 9.6 % of *C. parapsilosis*, 3.5 % of *C. guilliermondii*, 2.1 % of *C. lusitaniae* and *C. nivariensis*, 0.9 % of *C. krusei* and *C. tropicalis* [21].

Although each *Candida* strain presents specific virulence factors, in general, the virulence of this species is determined by the expression of adhesion proteins and hydrolytic enzymes, the characteristics of the cell wall, the morphological modification of yeast to pseudohyphal/hyphal forms and biofilm formation [29–31]. Biofilms are microbial communities impregnated in a polymeric extracellular matrix (ECM) derived from the cells, which are irreversibly linked to each other, to a substrate or to an interface. Consequently, these cells exhibit a different phenotype, with altered growth rates and gene transcription [32,33]. Biofilm formation is a dynamic process that initially occurs with the microorganism's adhesion to an abiotic or biotic surface, such as mucous membranes or medical devices, followed by subsequent cells co-aggregation and, consequently, colonization. This process constitutes a cycle and can be divided into four distinct phases (Figure 3) [34].

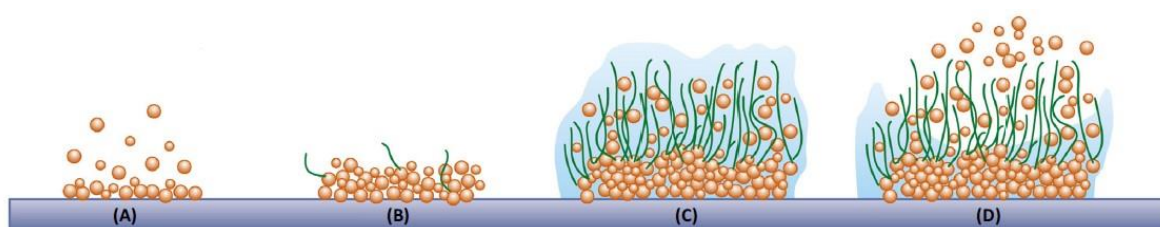


Figure 3. Biofilm formation stages. (A) Initial adhesion; (B) Proliferation and formation of an extracellular matrix; (C) Mature biofilm with continuous growth and (D) Propagation through the release of fungal cells (adapted from Araújo *et al.* [35])

The first phase involves adhesion, mediated by cell wall proteins (Figure 3.A). The intermediate phase corresponds to proliferation, cell growth and multiplication occurs through the development of hyphae (depending on the species), initiating the formation of a matrix consisting of polysaccharides, carbohydrates and proteins, among other components (Figure 3.B) [33,36]. Subsequently, maturation occurs, with increased density and complexity of the biofilm as the cells divide/die. In this stage, extracellular components generated by the cells interact with organic and inorganic molecules from the surrounding environment and the ECM is completely developed, making the biofilms highly hydrated (Figure 3.C). Finally, there is propagation, when the biofilm reaches a critical mass and dynamic

equilibrium is reached. Some fungal cells are released in a planktonic state, dispersing and multiplying, thereby colonizing new surfaces and forming new biofilms (Figure 3.D) [34,36]. The structural complexity of biofilms represents the ideal arrangement to facilitate nutrient flow and waste elimination, establish of micro-niches, enable metabolic cooperation, gene expression regulation and, once established, serves as a persistent reservoir of infection [33].

As previously mentioned, most candidiasis is due to *C. albicans* and its virulence is related to the ability to form dense biofilms that include yeast cells, pseudohyphae and hyphae within the abundant matrix (Figure 4.A and 4.B) [37]. This species colonizes the skin and mucous membranes of the mouth or vagina, as well as the gastrointestinal and genitourinary tracts [6,38]. Persons at risk include those who are immunocompromised due to treatment with immunosuppressive agents, following dysbiosis induced by antibiotics or chemotherapy, and those with HIV/AIDS [7].

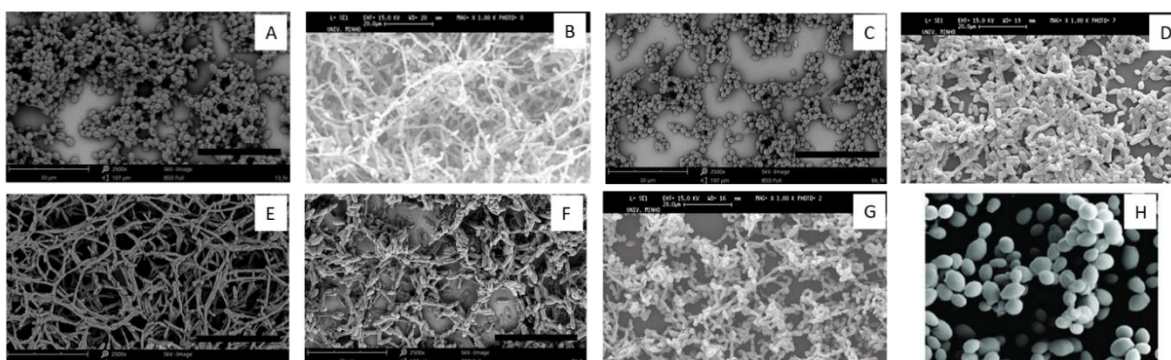


Figure 4. Biofilm Structure of *Candida* species. *Candida albicans* (A) Blastospores shaped cells and (B) Filamentous forms (hyphae or pseudo hyphae); (C) *Candida glabrata*; (D) *Candida tropicalis*; (E) *Candida guilliermondii*; (F) *Pichia kudriavzevii*; (G) *Candida parapsilosis*; (H) *Candida auris*. Images obtained with scanning electron microscopy and adapted from Fernandes *et al.* [39], Araújo *et al.* [35] and Kean *et al.* [40].

C. glabrata, or *Nakaseomyces glabratus* as currently defined [41], is the second most common *Candida* species, accounting for 20- 25 % of cases and 40- 60 % of mortality, particularly in nosocomial infections [6,42]. The primary associated risk factor is the advanced age of patients, with the age group from 70 years and above having a significantly higher rate *C. glabrata* infection [6]. *C. glabrata* is characterized by single biofilm formation, and its pathogenesis is linked to stress and rapid environmental adaptation, a stealth mode and evasion of host defenses (Figure 4.C) [6,42].

C. tropicalis is associated with both superficial and systemic infections [43]. Major risk factors include neutropenic patients and individuals with altered intestinal microbiota after broad-spectrum

antibiotics [6]. *C. tropicalis* is considered a prominent biofilm producer, exhibiting high diversity of adhesins that mediate adhesion to biotic and abiotic surfaces (Figure 4.D) [43]. Environment and culture conditions, phenotypic switching and quorum sensing molecules can influence these abilities [43].

C. guilliermondii (teleomorph *Meyerozyma* (*Pichia*) *guilliermondii* [44]) has become a significant causative agent of candidiasis in recent years [42]. It has been isolated as the etiological agent of VVC and mucocutaneous infections [45]. Commonly found in the environment, human skin, and mucosa as a microflora saprophyte, it can cause invasive infections [46]. Risk factors include pediatric and HIV patients, with most *C. guilliermondii* infections reported among oncology patients [46]. Phospholipase production and biofilm formation are characteristic virulence of this species (Figure 4.E) [45].

C. krusei, (teleomorph *Pichia kudriavzevii* [44]), is a non-conventional yeast ubiquitous in natural environments, occurring in fermented foods and beverages [47]. *P. kudriavzevii* is an emerging in patients with solid organ malignancy, hematological diseases and cancer patients, causing both systemic and superficial infections [6,47]. Similar to *C. albicans*, *C. krusei* exhibits thermomorphism, producing hyphae at 37 °C and blastoconidia and pseudohyphae at lower temperatures, which is one of its main virulence factors, along with adhesion to cervical epithelium cells (Figure 4.F) [6,47].

C. parapsilosis is a common cause of life-threatening candidaemia, mainly in individuals with haematopoietic system cancers, premature neonates, and organ transplant recipients requiring prolonged use of central venous catheter or other indwelling devices [48,49]. The widespread presence of this species is attributed to its capacity to persist in hospital settings for prolonged periods, adhering to abiotic surfaces and constructing biofilms that act as a conduit for systemic colonization (Figure 4.G) [49].

C. auris is a concerning pathogen associated with a high rate of incidence, prevalence, and mortality rate, particularly in the nosocomial environment [50]. Unlike other *Candida* species, *C. auris* is often found on the skin, and can colonize different body sites, such as wounds, nares, urinary tract, and bloodstream [50,51]. Factors such as difficult identification, easy adaptation to stressful conditions, and phenotypic plasticity contribute to its rapid transmission and the challenges in prevention, control and eradication [50]. Invasive infection and colonization are predominantly detected in hospital patients, with varying profiles of resistance to antifungals [51]. The high virulence of *C. auris* is associated with biofilm

development, consequent multidrug resistance, and the ability to overcome the innate immune system response (Figure 4.H) [50].

3. Conventional antifungal agents and Mechanism of resistance

For many years, antifungals with activity against *Candida* species were divided into three main classes: echinocandins, azoles and polyenes (Figure 5). Recently, pyrimidine analogs group has been included (Figure 5). This group constitutes the latest class of systemic antifungals, which includes 5-fluorocytosine [52]. However, due to the high resistance observed with this antifungal, 5-fluorocytosine cannot be used alone, and must always be administered in conjunction with another antifungal, usually an azole or polyene drug [52].

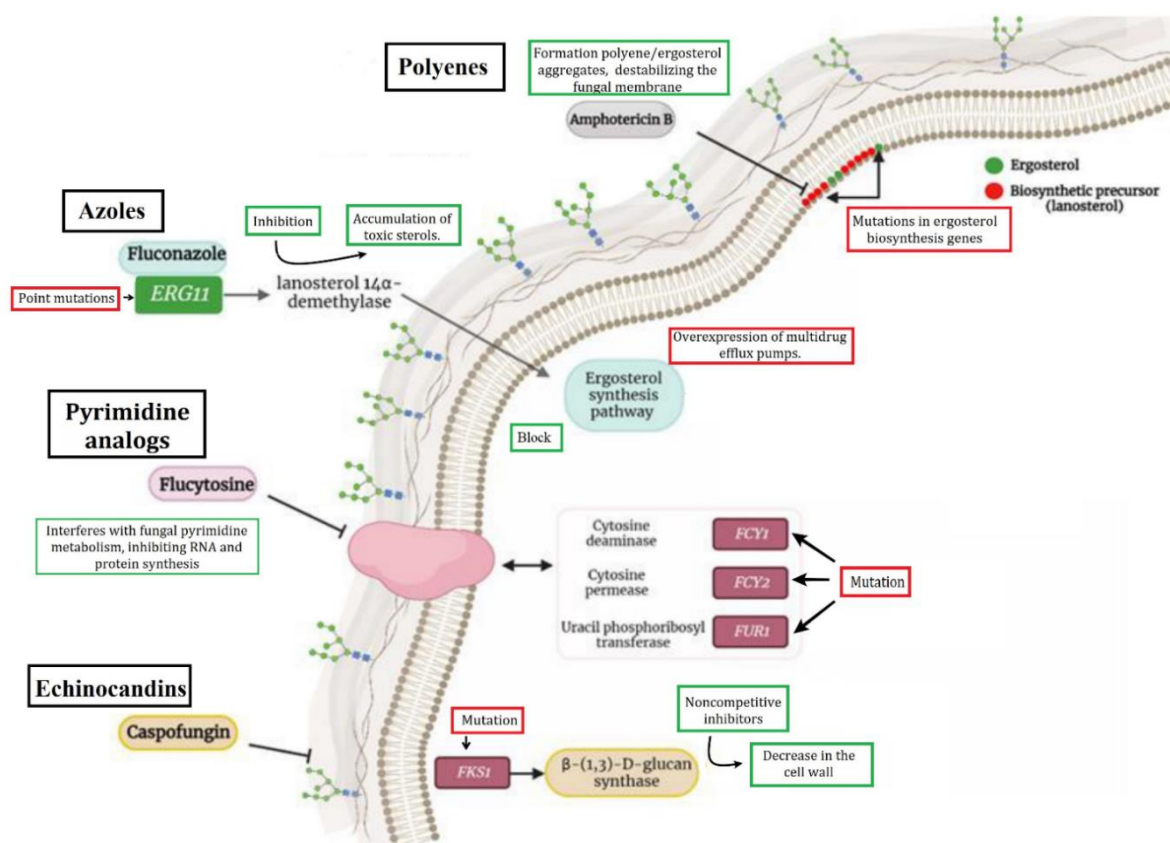


Figure 5. Mechanism of action of antifungals against *Candida* species and main mechanisms drug resistance (Adapted from Gómez-Gaviria *et al.* [42]). Green squares indicate mechanism of action of antifungals and red squares resistance mechanisms.

Antifungal agents are classified based on their mode of action on fungal cells, each class proceeding differently to eliminate or inhibit the growth of pathogenic fungi. The mode of action of polyenes (e.g. nystatin and amphotericin B) is to form polyene/ergosterol aggregates, destabilizing the fungal membrane [49]. This action can be overcome through mutations in the ergosterol biosynthesis genes, causing a decrease in ergosterol content, inducing the replacement of biosynthetic precursors such as lquesterol and lanosterol, and altering the activation of stress response pathways, such as catalase and hsp [42,49]. The azoles (e.g. fluconazole (FLC), miconazole and ketoconazole) act by blocking synthesis of ergosterol in the cell membrane, inhibiting the P450 dependent enzyme lanosterol 14- α -demethylase encoded by the *ERG11* gene. This led to the accumulation of toxic sterols. Resistance to azoles is associated with point mutations in the *ERG11* and *ERG* genes, particularly *ERG3*, involved in the ergosterol biosynthesis pathway, and overexpression of multidrug efflux pumps [42,49]. The class of echinocandins (e.g. caspafungin, micafungin and anidulafungin) acts as non-competitive inhibitors of (1,3)- β -D-glucan synthase, encoded by the *FKS* genes, causing a decrease in 1,3- β -glucan in the cell wall. Echinocandins resistance involves mutations in *FKS* genes and activation of cell wall stress response mediating pathways, thereby increasing chitin content [42,49]. The antifungal action of pyrimidine analogues (flucytosine) involves interfering with fungal pyrimidine metabolism, inhibiting RNA and protein synthesis [52]. Due to conversion in the cytoplasm of fungal cells via uridine phosphoribosyl transferase and cytosine deaminase into 5-fluorouridine triphosphate or 5-fluoro-deoxyuridine monophosphate, which is incorporated into RNA and blocks protein synthesis, and which inhibits thymidylate synthase encoded by *CDC21* (a key enzyme of DNA synthesis), respectively [52]. Resistance to flucytosine is facilitated by mutation in cytosine permease (*FCY2*), cytosine deaminase (*FCY1*), and uracil phosphoribosyltransferase (*FUR1*) [42].

Recently, novel formulations have emerged, including rezafungin, a new echinocandin that targets alterations in FKS hotspots, and ibrexafungerp, which secured Food and Drug Administration (FDA) approval in 2021, marking the first oral non-azole agent for VVC. Ibrexafungerp impedes the biosynthesis of β -1,3-glucan [50,53].

Resistance to antifungals can be intrinsic or innate (primary resistance), or acquired (secondary resistance), related to the intrinsic or acquired characteristics of the fungal pathogen [54]. Intrinsic resistance is related to innate characteristics of the microorganism, is highly stable, and consequently, predictive of therapeutic failure. For example, *C. krusei* and *C. auris* are intrinsically resistant to FLC. *ABC1* under-expression and reduced affinity to *ERG11* lead to azole intrinsic resistance in *C. krusei* [6]. Moreover, it presents variable resistance to other drugs, including amphotericin B, voriconazole,

itraconazole, micafungin and 5-flucytosine, among others [42]. Furthermore, *C. auris* also exhibits intrinsic resistance to other antifungals, including polyenes [54]. The resistance mechanisms of this species, to azoles, is associated with the overexpression and mutations in the *ERG11* gene and alterations in the sterol synthesis pathway [42]. Similarly, double mutations of the ergosterol synthase encoding gene *ERG11* and *ERG3* are important mechanisms of resistance in *C. tropicalis*, especially to azoles and amphotericin B (AmB) [6,42,43]. FLC-resistant *C. parapsilosis* strains are associated with high mortality rates and the resistance mechanism is like that described for *C. tropicalis* [42,49]. In addition, multidrug-resistant and echinocandin-tolerant isolates of *C. parapsilosis* have recently emerged and are of increasing concern [48]. The echinocandin resistance mechanism in *C. parapsilosis* is related a natural polymorphism in *FKS1* [42]. Also, *C. guilliermondii* seems to be intrinsically resistant to the echinocandins and the most common mechanisms of drug resistance include mutations in *FKS1*, increased activity of the efflux pump and alteration of the 14 α -demethylase [42,46]

On the other hand, acquired resistance can be stable or transient and is less common than intrinsic resistance. In this case, resistance develops due to long exposure to antifungals in a previously susceptible isolate. For example, *C. albicans* acquired azole resistance after azole therapy [54]. *C. glabrata* among the non-*Candida albicans* *Candida* (NCAC) species can acquire drug resistance. Indeed, *C. glabrata* drug-resistant strains are great concern and is related to the mutations in *ERG11/CYP51*, over-expression of membrane transporters, altered sterol import, and genome plasticity [42]. *Candida kefyr* and *Candida lusitanae* have also been reported to acquire drug resistance after antifungal exposure [55].

Despite the diverse antifungal agents available, as mentioned above *Candida* species have developed multiple molecular strategies to exert resistance to these agents and consequently the treatment failure rate is considerable [56]. Moreover, most of these antifungals have several disadvantages in terms of toxicity, intensity of side effects, drug interactions, lack of fungicidal efficacy, high costs and constantly increasing numbers of resistant isolates caused by prolonged therapies [56–59].

Therefore, there is a critical need for alternative therapies that are not only more effective but also safer and cost-effective, aiming to improve the overall outcomes for patients suffering with *Candida* infections.

4. Brief overview of alternative therapies for *Candida* infection

Fungal infections, including candidiasis, are recognized as significant risk factors for various pathologies, such as autoimmune diseases, diabetes, cancer, and chronic inflammatory conditions. To combat these challenges, antibiotics and traditional antifungals persist as essential tools in the battle against infectious diseases [60]. However, adverse effects associated with the use of these antifungal agents have been reported on the stability of the microbiome, and especially the increase of resistant pathogens [60]. In recent years, several investigators have worked on the discovery of more efficient therapies against *Candida* species, to overcome these challenges. Following this approach and drawing from the literature review conducted by Rodrigues *et al.* [56], new formulations with potential therapeutic effects against *Candida* species have been developed.

Antifungal agents' combination (e.g. AmB and posaconazole/ caspofungin, micafungin and FLC/ voriconazole or liposomal AmB, flucytosine and voriconazole) might improve the antifungal efficacy, with several predictable advantages, which include a wider spectrum and potency of drug activity, synergy, faster antifungal effect, lower dosing of toxic drug and reduced risk of antifungal resistance [56]. In fact, the first clinical application of this therapy for invasive candidiasis involved the use of AmB and flucytosine, which was found to induce an additive effect, furthermore research suggests that it reduces the onset of flucytosine resistance [61]. Conversely, the combinations may be antagonistic or clinically indifferent with additive side effects and give a false perception of security [56,62].

In the context of combinational therapies, Hodgetts *et al.* [63] demonstrated the combination of a human antibody fragment, efungumab, with caspofungin enhanced the activity of this agent *in vivo*. From this perspective, immunotherapy, consisting of innate and cell-mediated immunity, has been recently investigated and could become a promising tool against disseminated *Candida* infections. The determination of host defense mechanisms against *Candida* infections has prompted the development of new immunotherapeutic methods to combat these infections. These methods include the use of recombinant cytokines (e.g., granulocyte-macrophage colony-stimulating factor or interferon gamma), therapeutic antibodies (e.g. efungumab or monoclonal antibodies), vaccination (e.g., diphtheria toxoid CRM197 conjugated with the algal antigen laminarin, mannan protein conjugates or adhesins Als1p and Als3p) and adoptive transfer of primed immune cells (e.g., antigen primed dendritic cells or adoptive transfer of anti-*Candida* T cells) [56].

Another alternative antifungal strategy is photodynamic therapy (PDT). This technique combines a sensitizing drug and visible light. In this treatment, *Candida* cells are treated with a photosynthetic agent, making them susceptible to death by exposure to an appropriate wavelength of light, promoting a

phototoxic response of the cells, frequently via oxidative damage, which leads to selective destruction of microbial cells through disarrangements in the cell wall and DNA damage [64].

Also, low-level laser therapy, laser light, has been studied as an alternative antimicrobial treatment, using low doses of visible light with adequate wavelength to the generation of reactive oxygen species (ROS) by activating biological chromophores in cells [56]. Although there are few studies using this technique for the treatment of *Candida* species, they report a fungicidal effect [56].

In a very different approach, inhibitors application of early *Candida* yeast adhesion to different surfaces is an important step in the manufacture of innovative medical devices. For this purpose, proteases immobilized on polypropylene, treatment with filastin, the combination of graphene-curcumin-polyethylene glycol oxide and clioquinol have been used [65].

Nanotechnology focused on designing drugs with extended persistence and controlled release have been seen as powerful anti-*Candida* therapeutic alternative. Nitric oxide (NO), Ag–Cu–Co trimetallic, silver, bismuth nanoparticles are some examples applied in the treatment of *Candida* infections [50]. Incorporation of the drug into a controlled delivery system is a promising alternative; liposomes or nanoliposomes, chitosan or sodium alginate are materials used for this purpose. Moreover, nanoemulsions, known as self-nano emulsifying drug delivery systems, have been shown as an alternative with several advantages such as drug targeting ability, protection against hydrolysis and enzymatic actions, increased drug load and provide better drug dissolution and bioavailability [66].

Despite the advances in the aforementioned alternative therapies, there has been no reduction in the incidence of *candida* infections worldwide. This lack of progress can be attributed to the inherent challenges associated with their application, possibly decreasing from the complexities involved. In addition, the high cost associated with these therapeutic options is a barrier, preventing their widespread accessibility and adoption, thus limiting their impact on the general population. Consequently, the expected reduction in *Candida* infections remains elusive, underlining the pressing need for more accessible and practical solutions to address this widespread health problem.

4.1 Alternatives treatments based on natural products

The search for relevant alternatives to conventional antimicrobials based on natural products aimed the control of fungal diseases is a focal point of research due to their low toxicity, costs and adverse reactions, easy access and application to several pathologies. Besides, it reduces drastically the risk of induction of antimicrobial resistance, decreasing the negative impact on individual health and the environment [67]. In this sense, it becomes interesting and important to identify compounds and extracts

from medicinal plants that are active against clinically resistant *Candida* isolates and that present selectivity to the pathogen [56].

Over the years, many plant extracts and compounds have demonstrated antimicrobial activity against *Candida* species. However, the investigation of new compounds from plants, covers new, complex and diverse chemical structures, requiring a more complete study before their use as new antifungal agents [56]. In fact, there are a large number of *in vitro* studies showing the effect of natural compounds on *Candida* species. Nevertheless, the number of studies that include *in vivo* assays are more limited. In most of these *in vivo* studies, the antifungal effect is investigated against *C. albicans*, including the effect of seaweed (*Dictyota dichotoma*) [68]; *Arthrospira platensis*, topical cream application, induced mild lysis and vacuolation of cytoplasmic organelles [69] *Ambrosia maritima* and *Bituminaria bituminosa* [70]; wild shrub *Calotropis procera* [71]; synergistic effects of gypenosides, a class of triterpenoid saponins, combined with FLC [72]; *Acorus tatarinowii* Schott that inhibit biofilm formation by regulation of the *C. albicans* protein kinase C pathway [73]; and *Amukkara Chooram*, preventing the transition from yeast to hyphae [74]. Morin and aqueous extract of *Tinospora cordifolia* showed effect against *C. albicans*-associated systemic candidiasis, morin exhibited strong affinity to the Hwp-1 protein, which regulates biofilm expression and hyphae formation [75,76];

The effect of some natural compounds for oral candidiasis treatment has also been reported. For instance, the encapsulation of *Erodium glaucophyllum* extract in synthesized silver nanoparticles exhibited a strong inhibitory effect on the growth and morphogenesis of *C. albicans* [77]; *Cryptocarya moschata* [78]; and the hydroalcoholic extract derived from the bark of *Anadenanthera colubrina*, demonstrated the ability to reduce colony counts within biofilms while inducing structural changes and cellular damage [79].

In the treatment of VVC, the effect of *Annona muricata* L. and spilanthol, a bioactive alkylamide from *Acmella oleracea*, significantly reduced the proliferation and adhesion of *C. albicans* biofilms and their concentration of carbohydrates, proteins and DNA in the matrix [80,81]; water extract of *Sapindus mukorossi* Gaertn. pericarps decreased interleukin IL-1 β , IL-6, IL-8 and lactate dehydrogenase (LDH) in mice infected with *C. albicans* [82]; a chitosan gel formulated with a methanolic extract from *Mitracarpus frigidus* exhibited significant efficacy in reducing vulvovaginal fungal burden and associated infections (38); and *Anethum graveolens* (dill) [84] was demonstrated.

Moreover, the effect on several *Candida* species, including *C. albicans*, *C. krusei*, *C. parapsilosis*, *C. glabrata*, *C. auris* and *C. tropicalis*, has been reported: *Syzygium samarangense* leaf extracts and

ethanolic and methanolic extracts of *Rosmarinus officinalis* and *Zingiber officinale* [85,86], *Salvia kronenburgii* Rech. f. and *Salvia euphratica* Montbret [87] and *Camellia sinensis* crude extracts [88].

However, apart from this very limited number of studies that include *in vivo* experiments, studies on the application of natural therapies against more resistant structures such as *Candida* biofilms are few, focusing on essential oils (EOs) [89].

4.1.1 Essential oil antifungal effect

From the past until today, aromatic plants and their derivatives have been used in traditional medicine to treat several diseases [90,91]. In this sense, EOs and their main components such as thymol, carvacrol and linalool have been suggested as potential sources of new therapeutic products, with fewer side effects, less toxicity and higher biodegradability when compared to available antimicrobial agents [92]. In addition, EOs have multiple biological properties, such as anti-leishmanial, antiviral, anti-inflammatory, antioxidant, hepatoprotective, anti-tumor and antimicrobial activities [93,94]. Besides that, they are also used as anti-inflammatory, analgesic, spasmolytic and local anesthetic medicine. Plants are believed to emit a wide variety of volatile organic compounds as a defense against pathogens [95]. In this way, some volatile organic compounds can be refined into EOs via steam distillation [95]. EOs are complex natural mixtures of volatile organic compounds of terpenoid or non-terpenoid origin. Indeed, an oil can contain more than 200 components, including monoterpenes, sesquiterpenes, diterpenes, phenylpropanoids, fatty acids and their esters or their decomposition products [96,97]. Each component displays a variety of mechanisms of action or cellular pathways against microorganisms [98]. The EOs composition, even with the same original species, is influenced by several factors, such as geographical and climatic conditions of production, physical-chemical and biological properties of the soil during plant growth, cultivation practices such as stress and use of fertilizers, storage conditions and light spectrum [59,99,100]. In fact, light quality and quantity during cultivation play an important role in the production and synthesis of EOs and have been shown to affect the volatile compounds in herbs [101].

EOs can be extracted from different parts of plants, such as leaves, flowers, fruits, seeds, roots, shoots, stems and wood by many methods, including expression, fermentation, enfleurage or extraction and steam distillation (most widely adopted) [93,96,98]. Caputo *et al.* [102] observed that the choice of drying method to obtain the EO is crucial concerning the highest yield and mainly the percentage presence of components that can direct the EO to a suitable use. Thus, the same plant or plants from the same taxon but with specific genetic characteristics can produce chemically distinct EO [103]. Some botanical species produce EOs with substantial differences in intraspecies composition, which are referred to as

chemotypes of that species. As chemotypes can have different biological activity, it is necessary to specify the chemotype of an EO when applicable [104]. In contrast, EOs shows several physical properties in common, including liquid state, very volatile, limpid (rarely colored), insoluble in water and soluble in organic solvents [97]. Thus, a complete characterization of the composition of EOs before their application is indispensable. Salvo *et al.* [105] propose multi analytical techniques, evaluation of the variation in the EO content and composition realized by GC and GC-MS analyses, the phenolic fraction analyzed by Ultra-performance liquid chromatography (UPLC) coupled to photodiode array detection (PDA) (UPLC/ PDA) and the inorganic elements by Inductively coupled plasma mass spectrometry (ICP-MS).

EOs have been traditionally used for their bioactivity and may represent a valuable therapeutic alternative for *Candida* infections, particularly in the case of mucocutaneous infections [106,107]. The antifungal activity of EOs in *Candida* species has been the subject of several studies that confirmed its effect and described its mode of action. Among the commercially available EOs, tea tree (TTEO) (*Melaleuca alternifolia*) and thyme (*Thymus vulgaris*) are the most studied for their antifungal activity [108]. Rajkowska *et al* [109] demonstrated that although the EOs of thyme, tea tree, peppermint and clove oils have different chemical compositions, they exhibited similar modes of action. Their results indicate that EOs induced the loss of cell membrane integrity in *C. albicans*. The biological activity profiles indicated that the thyme was the most active regarding DNA leakage, ergosterol binding, and cytotoxicity.

The genus *Myrtaceae*, including for example *M. alternifolia*, *Melaleuca quinquenervia* and *Melaleuca leucadendra* (Figure 6), is one of the most important EOs-producing species, whose EOs have several bioactivities, such as insecticide, antioxidant, antiviral, antibacterial and antifungal [110].



Figure 6. Images of plants of the genus *Myrtaceae*: (A) *Melaleuca alternifolia*, (B) *Melaleuca quinquenervia* and (C) *Melaleuca leucadendra*.

4.1.1.1 *Melaleuca alternifolia* (Tea Tree Essential Oil (TTEO))

Native to Australia, *M. alternifolia* (Figure 6.A), commonly known as tea tree, is distinguished by a diverse chemical composition. It demonstrates a wide spectrum of antimicrobial activity, encompassing

antifungal properties [111]. The EO from *M. alternifolia* is known as tea tree (TTEO) and is widely used in medicine as an antiseptic and anti-inflammatory, has a calming effect and stimulates the immune system, reducing contact dermatitis and improves wound healing [112,113]. The principal biologically active compounds of TTEO are terpenoids and terpene, including α -terpinene, terpinene-4-ol, 1,8-cineole and terpinolene [111]. In fact, TTEO can be classified into three major chemotypes: terpinen-4-ol, terpinolene, and 1,8-cineole [112]. The mechanism of action of this EO and its components is related to increasing the permeability and fluidity of the fungus cell membrane. These compounds incorporate into the lipid bilayer membrane, eventually disrupting its structure [113,114].

A recent review by Silva *et al.* [114] grouped several reports proving that TTEO has antifungal effects *in vitro* against various *Candida* species, including *C. albicans*, *C. krusei*, *C. orthopsilosis*, *C. lusitaniae*, *C. parapsilosis* and *C. tropicalis*. Mainly when used topically for the oral candidiasis treatment [114]. The TTEO at a concentration of 0.5 % (v/v) (MIC_{50}) has the capability to impede the initial adhesion of *C. albicans* as well as the subsequent stages of its biofilm formation on both abiotic and biotic surfaces. This inhibition includes impeding the formation of germ tubes or mycelial conversion and suppressing respiration [112–114]. In *in vivo* studies, the reduction of *C. albicans* colonization in the oral cavity occurred in different sensitivity profiles after applying TTEO [114,115]. In a mouse trial, 4 % (v/v) of TTEO for 2 days induced a protective action against oral candidiasis by FLC-resistant *C. albicans* isolates [114,115]. Evidence from nearly 80 years suggests that topical use of TTEO oil is relatively safe. However, published data indicate that TTEO is toxic if ingested at higher concentrations and may also cause skin irritation. Despite this, TTEO and its components are not genotoxic [116].

4.1.1.2 *Melaleuca quinquenervia* (Niaouli Essential Oil)

M. quinquenervia (Figure 6.B) usually known as the broad-leaved paperbark native to Australia, New Caledonia and the French Pacific Islands, is a medicinal plant known for its applications in aromatherapy, cosmetics, food industry and pharmaceutical preparations for rheumatism, coughs, colds and neuralgia [117,118]. Furthermore, it possesses antifungal activity [118]. The EO of *M. quinquenervia*, known as niaouli, although less widely recognized than TTEO, has been identified as effective against both gram-positive and gram-negative bacteria, as well as a variety of fungi. Its effectiveness was observed at concentrations similar to those of TTEO [119]. Despite niaouli EO presenting a wide variation in chemical composition, chemical analysis reports 1,8-cineole, p-cymen-8-ol, p-cymene, E-nerolidol and limonene as a major component, depending on chemotypes [113,117,118]. Valková *et al.* [120] could conclude that the presence of 1,8-cineole (40.8 %) in niaouli EO may contribute significantly to its

antifungal efficacy. Niaouli EO is generally considered a safe, non-toxic, non-irritant and non-sensitizing oil [121].

4.1.1.3 *Melaleuca leucadendra* (Cajeput Essential Oil)

M. leucadendra (Figure 6.C), commonly known as weeping paperbark, despite being native to tropical Australia has been widely cultivated in several parts of the world showing different biological properties [122]. The leaves and bark are used in traditional medicine as sedating, tranquilizing and pain-relieving agents [122]. Cajeput EO is extracted from the *M. leucadendra* and used for its calming properties and is also recognized for its respiratory tract cleansing properties [50]. Giang An *et al.* [123] observed that the EO of *M. leucadendra*, with α -eudesmol (24.1 %) and guaialol (11.3 %), showed good antibacterial activity against *E. faecalis*. Furthermore, it showed MIC's and IC₅₀ between 64- 256 $\mu\text{g mL}^{-1}$ and 34- 128 $\mu\text{g mL}^{-1}$, respectively, in *C. albicans*. However, Monzote *et al.* [122] observed that cajeput EO, with 1,8-cineole (61 %), did not exert activity on *E. faecalis*, *Staphylococcus aureus*, *Escherichia coli*, *Aspergillus niger*, *Pseudomonas aeruginosa* and *C. parapsilosis*. On the other hand, Zhang *et al.* [110] confirmed that *M. leucadendra* EO (4-Terpineol is the most abundant component) possesses fungicidal effects on *C. albicans*, forming mesosome-like structures around the cell membrane and spread across the whole cytoplasm. Furthermore, the synergistic effects of this EO with four drugs from azoles family were observed. This synergistic effect may result from EO-induced membrane damage and permeability alteration facilitating drug penetration [110].

The *Lamiaceae* family is one of the most widespread and diverse plant families in ethnomedicine (Figure 7). This species is an important source of EOs, with 10 subfamilies, more than 220 genus, and about 3500 species distributed worldwide. Many of these species, including *Thymus* species and *Origanum* species, have a wide range of pharmacological activities with the potential to be developed as an alternative to antibiotics [67]. In silico toxicity, research suggests that most of the main compounds of EOs from *Lamiaceae* species probably do not exhibit mutagenicity, carcinogenicity or cytotoxicity [124].



Figure 7. Images of plants of the *Lamiaceae* family: (A) *Thymus* species and (B) *Origanum* species.

4.1.1.4 *Thymus species (Thyme Essential Oil)*

The *Thymus* species (thyme EO) (Figure 7.A) are native to temperate regions in North Africa, Asia and Europe being a popular medicinal plant with several pharmaceutical and therapeutic applications, including bronchiolitic, antiseptic, antispasmodic and antimicrobial, including antifungal [125–127]. Its medicinal potential is associated with its constitution of thymol, carvacrol, flavonoids, aliphatic phenols, eugenol, saponins, flavones, and tetramethoxylated luteolin [125]. Thyme EO has several advantages such as pleasant odor and flavor, low cost and side effects, and insignificant toxicity [125,126]. *Thymus* species, rich in carvacrol and thymol, such as *Thymus pulegioides*, *Thymus vulgaris* (the most common) and *Thymus zygis*, exhibited a potent anti-*Candida* activity, classified as fungicidal [106,127]. Thymus is an oxygenated phenolic monoterpene, that interferes with the formation and viability of *Candida* mycelium hyphae and may cause dysfunction of enzymes on the cell membrane and wall, alterations in the cell membrane permeability of the cell membrane leading to wall disruption and, consequently, cell death [128,129]. Alshaikh *et al.* [125] reported that EO from *T. vulgaris* reduced the growth of *C. albicans* isolates and proved that this EO is fungistatic and fungicidal, at very low doses. Thyme EO also inhibited germ tube formation and the displacement of fungal pathogens [125].

4.1.1.5 *Origanum species (Oregano Essential Oil)*

The genus *Origanum* (Figure 7.B), which includes 43 species and 18 hybrids, is a perennial herb native to the rocky calcareous and dry soils in mountainous areas, most of which are found in the Western and Southwestern Eurasia and Mediterranean region [95,130]. Considering the naturally high volatility, biodegradability, and ephemeral characteristics of oregano EO (OEO), these compounds have been widely applied as agricultural products, food preservation agents and pharmaceuticals [131]. In contrast to the mode of action of antibiotics, OEOs can significantly reduce microbial resistance through complex mixtures of natural compounds [131]. Hao *et al.* [131] established a multidimensional analytical method to evaluate differences in chemical profiles and corresponding antibacterial ability from various OEOs. This study showed that OEOs exhibited wide chemodiversity, and three main groups, carvacrol, thymol, and sesquiterpene, were identified. Moreover, OEO groups rich in carvacrol and thymol showed potent inhibitory effects on the growth of common pathogenic bacteria [131]. Cid-Chevecich *et al.* [132] observed that OEO from *O. vulgare* L. inhibited morphogenesis, adhesion, and proliferation at least by 50 % for *C. albicans* ATCC-90029, *C. albicans* ATCC-10231, *C. dubliniensis* ATCC-CD36 and *C. krusei* ATCC-625. In addition, Stringaro *et al.* [133] demonstrated that the same EO, carvacrol, and thymol cause a reduction in fungal viability, alteration of hydrophobicity and induces damage to both cell wall (confirmed by SEM)

and cell biomembranes, being associated with the loss of intracellular materials. These authors suggested that these compounds can modify the yeast cell membrane structure by incorporating into the lipid monolayer, forming aggregates of molecules and lipids, and reducing their packing in the membrane monolayers [133]. Kaskatepe *et al.* [134] suggested that the EO from *O. majorana* L showed strong antifungal activities against planktonic and biofilm-forming *C. albicans* cells and had an influence on putative virulence factors, such as germ-tube formation, its length and on cell surface hydrophobicity. These two studies were confirmed on infected *Galleria mellonella* larvae, demonstrating these bioactive compounds' safety and their relevant antimicrobial activities [133,134].

Although the previously mentioned studies demonstrate that EOs has antimicrobial potential and are a possible substitute for antibiotics, the possible toxicity, strong taste, chemical instability, restricted administration routes and volatility of EO limit its application [131]. In this sense, nanotechnology is receiving particular attention to overcome these obstacles.

4.1.2 Encapsulated essential oil on *Candida* species

The incorporation of a controlled delivery system is a promising strategy to avoid the problems associated with EOs, making their application safer. So, nanoencapsulation of bioactive compounds, such as EOs, is a promising topic of nanotechnology [135]. In fact, this technology allows EOs protection from light and temperature alterations, increasing their solubility in aqueous environments, and improving their bioaccessibility and bioavailability [135]. The methods presented in the literature for the EO encapsulation consist of phase inversion precipitation, solvent evaporation, spray drying, hydrogels and in situ polymerization in the dispersed phase, for example nanoemulsion [136].

The studies related to the EO encapsulation against *Candida* species are summarized in Table 1, containing information about several factors that can influence the antifungal effect of the formulations, such as encapsulation structure and efficiency, particle size, polydispersity (PDI) and zeta potential.

Table 1. Essential oil encapsulation against *Candida* species, information about several factors that can influence the antifungal effect of the formulations. Encapsulation structure, particle size (nm), polydispersity (PDI), zeta potential (mV), encapsulation efficiency (% EE) and cytotoxicity

Encapsulating structure	Essential Oil/ bioactive compound	Size (nm)	PDI	zeta potential (mV)	% EE	Cytotoxicity	Anti- <i>Candida</i> effect	References
Physically cross-linked Chitosan/dextrin cryogels	<i>Thymus vulgaris</i>	W/I	W/I	W/I	W/I	W/I	ZI: 40 mm <i>C. parapsilosis</i>	[137]
Methylcellulose hydrogel	<i>Melissa officinalis</i>	W/I	W/I	W/I	W/I	W/I	ZI: 17.5 mm <i>C. albicans</i>	[138]
Polyvinyl alcohol/chitosan/gelatin thermo-responsive hydrogel	<i>Zataria multiflora</i>	1100 ± 600	W/I	W/I	98.66	High cell viability (over 96 %)	MIC ₉₀ : 128 µL mL ⁻¹ <i>C. albicans</i> and <i>C. dubliniensis</i> ; 64 µL mL ⁻¹ <i>C. glabrata</i> ; 4µL mL ⁻¹ <i>C. krusei</i> ; <i>C. tropicalis</i> ; 14 µL mL ⁻¹ <i>C. parapsilosis</i>	[139]
Nanoemulsions: tween 20 (emulsifier), and Butanaol (co-emulsifier)	Citronella	130 ± 5	0.127	-12.6	95.5 ± 4.8	W/I	MIC: 125 µg mL ⁻¹ <i>C. albicans</i>	[140]
Chitosan-aloe vera films	Thymol	197–389	0.2 to 0.6	22.8- 36.4	95.3	W/I	ZI: 0.5- 1.33 cm ² <i>C. albicans</i>	[141]
Oil-in-water emulsions: mannosylerythritol lipid B (emulsifier)	<i>T. vulgaris</i>	5000	W/I	W/I	W/I	W/I	MIC: 125 µg mL ⁻¹ <i>C. albicans</i>	[142]
	<i>Lippia sidoides</i>						MIC: 250 µg mL ⁻¹ <i>C. albicans</i>	
	<i>Cymbopogon citratos</i>						MIC: 500 µg mL ⁻¹ <i>C. albicans</i>	
Polyamide nano capsules	<i>Campsis radicans</i> (Guaiacol)	138.7	0.23	W/I	86	W/I	MIC: 0.133 µg mL ⁻¹ MFC: 0.071 µg mL ⁻¹ <i>C. albicans</i>	[143]
Oil-in-water emulsion: Chitosan-based films	Lemon	1.44 ± 0.03	W/I	W/I	73.8	W/I	ZI: 11 mm <i>C. albicans</i>	[144]
Nanostructured Lipid	<i>L. sidoides</i>	213.1- 445.5	0.3	-93.1 - -63.8	W/I	Not evidence toxicity in the <i>in vivo</i> model	MIC: 281- 563 µg mL ⁻¹ <i>C. auris</i>	[145]

Liposome: L- α -phosphatidylcholine and cholesterol	<i>Lavandula angustifolia</i>	177.2 $\pm$ 11.49	0.30 $\pm$ 0.05	-3.73 $\pm$ 0.10	100	W/I	Eradicate <i>C. auris</i> biofilms	[146]
Nanofibers containing collagen and chitosan	Lemon balm (<i>M. officinalis</i> L.)	926.7	0.51	5.53	21.7 - 56.5	Good biocompatibility in mice model	ZI: 15.69- 19.61 mm <i>C. albicans</i> ZI: 22.50- 51.12 mm <i>C. glabrata</i>	[147]
	Dill (<i>Anethum graveolens</i> L.)							
Gellan/polyvinyl alcohol (PVA) nanofibers	Cinnamaldehyde	278.5 $\pm$ 57.8	W/I	W/I	17.3 $\pm$ 4.1	W/I	Biofilm reduction: 89.29 % <i>C. glabrata</i> 50.45 % <i>C. albicans</i>	[148]
	Eucalyptol	132.05 $\pm$ 82.01	W/I	W/I	40 $\pm$ 6.2	W/I	Biofilm reduction: 70 % <i>C. albicans</i> and <i>C. glabrata</i>	[149]
Poly(ϵ -caprolactone) nanocapsules	<i>Thymus capitatus</i>	198	0.09	-11	84	Not cytotoxic on HaCaT human keratinocyte cell line	MIC: 0.125 mg ml mL ⁻¹ MFC: 0.250 mg mL ⁻¹ <i>C. albicans</i>	[150]
	<i>Origanum vulgare</i>	200	0.05	-10	51			
Chitosan microparticles	Lemongrass (<i>Cymbopogon flexuosus</i>)	4959	W/I	+30.75	11.45- 19.37	W/I	MIC: 8- 128 μ g mL ⁻¹ <i>C. albicans</i>	[151]
	Geranium (<i>Pelargonium graveolens</i>)	5009	W/I	+46.54	34.13- 39.58	W/I		
Terpolymer capsules: methyl methacrylate/ methacrylic acid	<i>Rosmarinus officinalis</i>	285	W/I	W/I	>84	Non-toxic	MIC: <0.03 -0.06 %; MFC: 0.03- 0.13 % <i>C. albicans</i> , <i>C. glabrata</i> and <i>C. parapsilosis</i>	[136]
Terpolymer capsules: methyl methacrylate/ methacrylic acid/ styrene		150			>90	Considerable toxicity		
Solid lipid nanoparticles: stearic acid	<i>Eugenia caryophyllata</i>	397.2 $\pm$ 10.1	0.22 $\pm$ 0.01	-20.9 $\pm$ 0.3	71.0 $\pm$ 03	W/I	MIC/MFC: 0.1- 0.25 μ g mL ⁻¹ <i>C. albicans</i>	[152]
Solid lipid nanoparticles: precinol/ glyceryl monostearate		506.4 $\pm$ 22	0.68 $\pm$ 0.02	-21.7 $\pm$ 0.2	W/I			

Solid lipid nanoparticles: glyceryl monostearate		786.9 ± 11	0.56 ± 0.08	-19.2 ± 0.5	69.17 ± 0.2			
Lipid-based nanocarriers	Citral	74.8	0.266 ± 0.08	W/I	99.84	W/I	No effect on <i>Candida</i> species	[153]
Mesoporous silica nanostructures	Eucalyptus	200 to 500	W/I	W/I	W/I	MIC value were proved to be safe for eukaryotic cells	MIC: 0.18 µg mL ⁻¹ <i>C. albicans</i>	[154]
	Orange						MIC: 5 µg mL ⁻¹ <i>C. albicans</i>	
	Cinnamon						MIC: 1.25 µg mL ⁻¹ <i>C. albicans</i>	
Chitosan Polymer Matrix	<i>Mentha spicata</i> L. var. <i>viridis</i>	835820	W/I	W/I	81.20	W/I	ZI: 10- 18.67 mm <i>C. albicans</i> , <i>C. kefyr</i> , <i>C. tropicalis</i>	[155]
Sodium alginate matrix	Elicriso italic	5 x 10 ⁻³ - 1 x 10 ⁻⁵	W/I	W/I	66	W/I	ZI: 0,5 mm <i>C. albicans</i>	[156]
	Chamomile blue						No effect on <i>C. albicans</i>	
	Cannella corteccia (cinnamon)						ZI: 18 mm <i>C. albicans</i>	
	Levanda vera (lavender)						ZI: 2 mm <i>C. albicans</i>	
	Tea tree						ZI: 2 mm <i>C. albicans</i>	
	Peppermint						ZI: 6 mm <i>C. albicans</i>	
	Eucalyptus globulus juvenile						ZI: 1 mm <i>C. albicans</i>	
	Lemongrass						ZI: All zone <i>C. albicans</i>	
	Lemon						ZI: 1 mm <i>C. albicans</i>	
Self-nanoemulsifying	Oregano	150- 500	0.1- 0.4	W/I	W/I	W/I	ZI: 17 mm <i>C. albicans</i>	[66]

IZ Inhibition zone.

W/I without information.

Biomaterial-based hydrogels, particularly intelligent hydrogels with the ability to react to environmental stimuli such as temperature, are promising platforms for the EO encapsulation and have been widely used in biomedical applications [139]. In this sense, Torabiardekani *et al.* [139] demonstrated that encapsulation of *Zataria multiflora* EO in a polyvinyl alcohol/chitosan/gelatin thermo-responsive hydrogel (gel-sol viscoelastic transition at a temperature of 24.5 °C) inhibits the metabolic activity of *C. albicans* biofilms by approximately 60- 80 %, which may be related to the antifungal activity of both EO constituents and chitosan. This formulation is biocompatible with high cell viability and a possible application of this system is to control cutaneous candidiasis [139]. Also, Serra *et al.* [138] synthesized a hydrogel with 10 % (v/ v) methylcellulose and 2 % (v/ v) *Melissa officinalis*, which significantly reduced the retention of *C. albicans*. These hydrogels have the ability to gel at body temperature in less than 3 minutes and may provide a novel antimicrobial therapy for the treatment of oral candidiasis [138]. Another approach in this sense is the cross-linked chitosan/dextrin cryogels entrapping *T. vulgaris*, Dinu *et al.* [137] showed that this 3D cryogenically-structured network, constructed by hydrogen bridges formed by mixing two naturally polysaccharides, chitosan and dextrin, induces a 40 mm inhibition zone for *C. parapsilosis*. Furthermore, Balaure *et al.* [154] observed a remarkable inhibitory effect against the biofilm formation of *C. albicans* ATCC 10231 of mesoporous silica nanostructures incorporating eucalyptus, orange and cinnamon EOs. The incorporation of orange showed the most intense inhibitory effect on biofilm production, with inhibition of biofilm formation starting at a concentration of 0.31 % [154].

Also, nanoemulsions, consisting of very small oil droplets held in aqueous environments by surfactants, have been extensively developed as a delivery mechanism for plant-derived bioactive compounds [140]. Incorporating citronella EO into nanoemulsion decreased its MIC against *C. albicans* by half, i.e. 250 µg mL⁻¹ for citronella EO and 125 µg mL⁻¹ for nanoemulsion with citronella EO [140]. The same MIC values to *C. albicans* were observed for the nanoencapsulation of *Lippia sidoides* and *T. vulgaris* in mannosylerythritol [142]. Hosny *et al.* [66] demonstrated that oregano EO- nanoemulsions can protect against oral infections caused by *C. albicans*. being able to inhibit ulcer formation by *C. albicans* in the buccal mucosa.

The development of easily removable, plant-based antimicrobial films formed using a component of thymol EO, chitosan biopolymer and the medicinal plant aloe vera increases the spaces between the biopolymer chains, facilitating greater water intrusion, conducive to preventing the possibility of bacterial infection. However, this formulation had a tiny effect on *C. albicans* [141]. Also, chitosan films with lemon EO microspheres and cranberry juice were developed, obtaining zones of inhibition in the disk diffusion

method for *C. albicans* of 11 mm [143]. The antimicrobial effect of guaiacol, the active ingredient of *Campsis radicans* EO, both free and in guaiacol-polyamide nanocapsules, was evaluated on FLC-resistant *C. albicans*, obtaining a lower MIC when encapsulated of 0.133 $\mu\text{g mL}^{-1}$ and MFC of 0.071 $\mu\text{g mL}^{-1}$ [143]. Also, the inclusion of eucalyptol/ β -cyclodextrin in gellan/polyvinyl alcohol nanofibers inhibited approximately 70 % of the biofilm of *C. albicans* and *C. glabrata*. Electron microscopy studies revealed that this formulation disrupted the *Candida* cell surface [149]. The same encapsulation system with cinnamaldehyde, can effectively eradicate 50.45 % and 89.29 % of *C. albicans* and *C. glabrata* biofilm, respectively [149].

On a study performed on *C. auris*, Baldim *et al.* [145] demonstrated the potent antifungal activity of nanostructured lipid carriers loaded with *L. sidoides* EO, with MIC between 281 and 563 $\mu\text{g mL}^{-1}$, without evidencing toxicity in the *G. mellonella* model. In a study by Alteriis *et al.* [146], low concentrations of free and liposome-encapsulated *L. angustifolia* EOs inhibited the growth of *C. auris*. In fact, using liposomes or nanoliposomes for EO encapsulation can protect biomolecules from the environment with the advantages of being non-toxic and biodegradable [157]. Furthermore, the same compounds, liposome-encapsulated *L. angustifolia* EOs, were effective in eradicating *C. auris* biofilms formed by, both biofilms developed from planktonic cells and that developed from persistent cells [146]. The authors observed that the *ERG11* gene was down-regulated during eradication with the encapsulated oil, which may be attributed to its cell membrane targeting activity. They also observed that the *HOG1* gene, involved in the oxidative stress response, was positively regulated when the encapsulated oil was used [146]. In addition to these genes, the expression of *ALS5* gene, responsible for hyphae growth, in persistent biofilm cells treated with encapsulated EO was negatively regulated [146].

However, further research is needed to delve deeper into the efficacy, toxicity and dosimetry of encapsulating EOs. Thorough exploration of these aspects is crucial to scientifically establishing the scope and value of encapsulating these compounds for new applications in the fight against *Candida* species. The integration of *in vivo* testing is also essential to association laboratory findings and real-world applicability, providing a deeper understanding of the potential benefits and limitations of EOs encapsulation in the treatment of *Candida* infections.

5. Conclusion

The virulence factors of *Candida* species are relevant therapeutic targets, as they are responsible for increasing resistance and pathogenicity. Mainstream commercial antifungals often fail to inhibit these factors, and their effectiveness diminishes over time due to rising resistance. The data collected here

underscore the antifungal potential of EOs as complementary strategies or alternative treatments to commonly used commercial antifungals. EOs have been widely studied through direct use as biological agents or as a basis for developing new drugs with promising therapeutic applications. Furthermore, the encapsulation of EOs offers additional advantages, such as protection from light and temperature alterations, increasing their solubility in aqueous environments, and improving their bioaccessibility and bioavailability.

References

- [1] Global report on infection prevention and control. <https://www.who.int/publications/i/item/9789240051164> (accessed February 16, 2023).
- [2] Ciurea CN, Kosovski IB, Mare AD, *et al.* *Candida* and Candidiasis-Opportunism Versus Pathogenicity: A Review of the Virulence Traits. *Microorganisms* 2020;8:1–17. <https://doi.org/10.3390/MICROORGANISMS8060857>.
- [3] Mora C, Tittensor DP, Adl S, *et al.* How many species are there on Earth and in the ocean? *PLoS Biol* 2011;9. <https://doi.org/10.1371/JOURNAL.PBIO.1001127>.
- [4] Naveen KV, Saravanakumar K, Sathiyaseelan A, *et al.* Human Fungal Infection, Immune Response, and Clinical Challenge—a Perspective During COVID-19 Pandemic. *Appl Biochem Biotechnol* 2022;194:4244. <https://doi.org/10.1007/S12010-022-03979-5>.
- [5] Benedict K, Whitham HK, Jackson BR. Economic Burden of Fungal Diseases in the United States. *Open Forum Infect Dis* 2022;9. <https://doi.org/10.1093/OFID/OFAC097>.
- [6] Sharma M, Chakrabarti A. Candidiasis and Other Emerging Yeasts. *Curr Fungal Infect Rep* 2023;17:15–24. <https://doi.org/10.1007/S12281-023-00455-3>.
- [7] Suleyman G, Alangaden GJ. Nosocomial Fungal Infections: Epidemiology, Infection Control, and Prevention. *Infect Dis Clin North Am* 2021;35:1027–53. <https://doi.org/10.1016/J.IDC.2021.08.002>.
- [8] Vallabhaneni S, Chiller TM. Fungal Infections and New Biologic Therapies. *Curr Rheumatol Rep* 2016;18:1–10. <https://doi.org/10.1007/S11926-016-0572-1/METRICS>.
- [9] Global Invasive Fungal Infections Market Report 2023: <https://www.globenewswire.com/en/news-release/2023/05/15/2668440/28124/en/Global-Invasive-Fungal-Infections-Market-Report-2023-Increasing-Invasive-Fungal-Infections-with-Life-threatening-Conditions-Bolsters-Growth.html> (accessed July 7, 2023).
- [10] Brown GD, Denning DW, Gow NAR, *et al.* Hidden killers: human fungal infections. *Sci Transl Med* 2012;4. <https://doi.org/10.1126/SCITRANSLMED.3004404>.
- [11] Kubin CJ, McConville TH, Dietz D, *et al.* Characterization of Bacterial and Fungal Infections in Hospitalized Patients With Coronavirus Disease 2019 and Factors Associated With Health Care-Associated Infections. *Open Forum Infect Dis* 2021;8. <https://doi.org/10.1093/OFID/OFAB201>.
- [12] Robertson KD, Nagra N, Mehta D. Esophageal Candidiasis. *Video Journal and Encyclopedia of GI Endoscopy* 2022;1:64–5. [https://doi.org/10.1016/S2212-0971\(13\)70029-3](https://doi.org/10.1016/S2212-0971(13)70029-3).
- [13] About Fungal Diseases | Fungal Diseases | CDC n.d. <https://www.cdc.gov/fungal/about-fungal-diseases.html> (accessed May 29, 2023).
- [14] Donors making a difference: Climate change and its impact on health n.d. <https://www.who.int/news-room/feature-stories/detail/donors-making-a-difference-climate-change-and-its-impact-on-health> (accessed May 17, 2023).
- [15] Zhangid Z, Bills GF, Anid Z. Advances in the treatment of invasive fungal disease. *PLoS Pathog* 2023;19:e1011322. <https://doi.org/10.1371/JOURNAL.PPAT.1011322>.
- [16] Bitar I, Khalaf RA, Harastani H, *et al.* Identification, Typing, Antifungal Resistance Profile, and Biofilm Formation of *Candida albicans* Isolates from Lebanese Hospital Patients. *Biomed Res Int* 2014;2014. <https://doi.org/10.1155/2014/931372>.
- [17] Statistics | Invasive Candidiasis | Candidiasis | Types of Diseases | Fungal Diseases | CDC. <https://www.cdc.gov/fungal/diseases/candidiasis/invasive/statistics.html> (accessed July 7, 2023).
- [18] Buchacz K, Lau B, Jing Y, *et al.* Incidence of AIDS-Defining Opportunistic Infections in a Multicohort Analysis of HIV-infected Persons in the United States and Canada, 2000-2010. *J Infect Dis* 2016;214:862–72. <https://doi.org/10.1093/INFDIS/JIW085>.
- [19] Deng Z, Kiyuna A, Hasegawa M, *et al.* Oral candidiasis in patients receiving radiation therapy for head and neck cancer. *Otolaryngol Head Neck Surg* 2010;143:242–7. <https://doi.org/10.1016/J.OTOHNS.2010.02.003>.
- [20] Kullberg BJ, Arendrup MC. Invasive Candidiasis. *N Engl J Med* 2015;373:1445–56. <https://doi.org/10.1056/NEJMRA1315399>.

- [21] Fernandes Â, Azevedo N, Valente A, *et al.* Vulvovaginal candidiasis and asymptomatic vaginal colonization in Portugal: epidemiology, risk factors and antifungal pattern. *Med Mycol* 2022;60. <https://doi.org/10.1093/MMY/MYAC029>.
- [22] Disha T, Haque F. Prevalence and Risk Factors of Vulvovaginal Candidosis during Pregnancy: A Review. *Infect Dis Obstet Gynecol* 2022;2022. <https://doi.org/10.1155/2022/6195712>.
- [23] Denning DW, Kneale M, Sobel JD, *et al.* Global burden of recurrent vulvovaginal candidiasis: a systematic review. *Lancet Infect Dis* 2018;18:e339–47. [https://doi.org/10.1016/S1473-3099\(18\)30103-8](https://doi.org/10.1016/S1473-3099(18)30103-8).
- [24] Castanheira M, Deshpande LM, Messer SA, *et al.* Analysis of global antifungal surveillance results reveals predominance of Erg11 Y132F alteration among azole-resistant *Candida parapsilosis* and *Candida tropicalis* and country-specific isolate dissemination. *Int J Antimicrob Agents* 2020;55:105799. <https://doi.org/10.1016/J.IJANTIMICAG.2019.09.003>.
- [25] Hof H. Developments in the epidemiology of invasive fungal infections – implications for the empiric and targeted antifungal therapy. *Mycoses* 2008;51:1–6. <https://doi.org/10.1111/J.1439-0507.2008.01522.X>.
- [26] *Candida* infections of the mouth, throat, and esophagus | Fungal Diseases | CDC. <https://www.cdc.gov/fungal/diseases/candidiasis/thrush/index.html> (accessed May 17, 2023).
- [27] Gharehbolagh SA, Fallah B, Izadi A, *et al.* Distribution, antifungal susceptibility pattern and intra-*Candida albicans* species complex prevalence of *Candida africana*: A systematic review and meta-analysis. *PLoS One* 2020;15:e0237046. <https://doi.org/10.1371/JOURNAL.PONE.0237046>.
- [28] Kołaczowska A, Kołaczowski M. Drug resistance mechanisms and their regulation in non-*albicans* *Candida* species. *Journal of Antimicrobial Chemotherapy* 2016;71:1438–50. <https://doi.org/10.1093/JAC/DKV445>.
- [29] Mitchell AP. Microbiology: Fungus produces a toxic surprise. *Nature* 2016;532:41–2. <https://doi.org/10.1038/nature17319>.
- [30] Williams DW, Kuriyama T, Silva S, *et al.* *Candida* biofilms and oral candidosis: Treatment and prevention. *Periodontol* 2000 2011;55:250–65. <https://doi.org/10.1111/j.1600-0757.2009.00338.x>.
- [31] Jayatilake JAMS, Samaranyake YH, Cheung LK, *et al.* Quantitative evaluation of tissue invasion by wild type, hyphal and SAP mutants of *Candida albicans*, and non-*albicans* *Candida* species in reconstituted human oral epithelium. *Journal of Oral Pathology and Medicine* 2006;35:484–491. <https://doi.org/10.1111/j.1600-0714.2006.00435.x>.
- [32] Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. *Science* 1999;284:1318–22. <https://doi.org/10.1126/SCIENCE.284.5418.1318>.
- [33] Hajiagha MN, Kafil HS. Efflux pumps and microbial biofilm formation. *Infect Genet Evol* 2023;112. <https://doi.org/10.1016/J.MEEGID.2023.105459>.
- [34] Cuéllar-Cruz M, López-Romero E, Villagómez-Castro JC, *et al.* *Candida* species: new insights into biofilm formation. *Future Microbiol* 2012;7:755–71. <https://doi.org/10.2217/fmb.12.48>.
- [35] Araújo D, Henriques M, Silva S. Portrait of *Candida* Species Biofilm Regulatory Network Genes. *Trends Microbiol* 2017;25:62–75. <https://doi.org/10.1016/J.TIM.2016.09.004>.
- [36] Alonso VPP, Lemos JG, Nascimento M da S do. Yeast biofilms on abiotic surfaces: Adhesion factors and control methods. *Int J Food Microbiol* 2023;400. <https://doi.org/10.1016/J.IJFOODMICRO.2023.110265>.
- [37] Li Y, Huang S, Du J, *et al.* Current and prospective therapeutic strategies: tackling *Candida albicans* and *Streptococcus* mutans cross-kingdom biofilm. *Front Cell Infect Microbiol* 2023;13:1106231. <https://doi.org/10.3389/FCIMB.2023.1106231/BIBTEX>.
- [38] Gaziano R, Sabbatini S, Monari C. The Interplay between *Candida albicans*, Vaginal Mucosa, Host Immunity and Resident Microbiota in Health and Disease: An Overview and Future Perspectives. *Microorganisms* 2023;11. <https://doi.org/10.3390/MICROORGANISMS11051211>.
- [39] Fernandes L, Costa R, Henriques M, *et al.* Simulated Vaginal Fluid: *Candida* resistant strains' biofilm characterization and vapor phase of essential oil effect. *J Mycol Med* 2022;101329. <https://doi.org/10.1016/J.MYCMED.2022.101329>.
- [40] Kean R, Delaney C, Rajendran R, *et al.* Gaining Insights from *Candida* Biofilm Heterogeneity: One Size Does Not Fit All. *J Fungi (Basel)* 2018;4. <https://doi.org/10.3390/JOF4010012>.

- [41] Takashima M, Sugita T. Taxonomy of Pathogenic Yeasts *Candida*, *Cryptococcus*, *Malassezia*, and *Trichosporon*. *Med Mycol J* 2022;63:119–32. <https://doi.org/10.3314/MMJ.22.004>.
- [42] Gómez-Gaviria M, Ramírez-Sotelo U, Mora-Montes HM. Non-*albicans* *Candida* Species: Immune Response, Evasion Mechanisms, and New Plant-Derived Alternative Therapies. *J Fungi* 2023, Vol 9, Page 11 2022;9:11. <https://doi.org/10.3390/JOF9010011>.
- [43] de Souza CM, Moreira dos Santos M, Furlaneto-Maia L, *et al.* Adhesion and biofilm formation by the opportunistic pathogen *Candida tropicalis*: what do we know? <https://doi.org/10.1139/CJM-2022-0195> 2023. <https://doi.org/10.1139/CJM-2022-0195>.
- [44] Cooper CR. Yeasts Pathogenic to Humans. *The Yeasts* 2011;1:9–19. <https://doi.org/10.1016/B978-0-444-52149-1.00002-1>.
- [45] Faria-Gonçalves P, Rolo J, Gaspar C, *et al.* Recurrent vulvovaginal *Candida* spp isolates phenotypically express less virulence traits. *Microb Pathog* 2020;148:104471. <https://doi.org/10.1016/J.MICPATH.2020.104471>.
- [46] Ghasemi R, Lotfali E, Rezaei K, *et al.* *Meyerozyma guilliermondii* species complex: review of current epidemiology, antifungal resistance, and mechanisms. *Braz J Microbiol* 2022;53:1761–79. <https://doi.org/10.1007/S42770-022-00813-2>.
- [47] Chu Y, Li M, Jin J, *et al.* Advances in the Application of the Non-Conventional Yeast *Pichia kudriavzevii* in Food and Biotechnology Industries. *J. Fungi* 2023, Vol 9, Page 170 2023;9:170. <https://doi.org/10.3390/JOF9020170>.
- [48] Daneshnia F, de Almeida Júnior JN, Ilkit M, *et al.* Worldwide emergence of fluconazole-resistant *Candida parapsilosis*: current framework and future research roadmap. *Lancet Microbe* 2023;4. [https://doi.org/10.1016/S2666-5247\(23\)00067-8](https://doi.org/10.1016/S2666-5247(23)00067-8).
- [49] Branco J, Miranda IM, Rodrigues AG. *Candida parapsilosis* Virulence and Antifungal Resistance Mechanisms: A Comprehensive Review of Key Determinants. *J Fungi (Basel)* 2023;9. <https://doi.org/10.3390/JOF9010080>.
- [50] Fernandes L, Ribeiro R, Henriques M, *et al.* *Candida auris*, a singular emergent pathogenic yeast: its resistance and new therapeutic alternatives. *Eur J Clin Microbiol Infect Dis* 2022;41:1371–85. <https://doi.org/10.1007/S10096-022-04497-2>.
- [51] Jeffery-Smith A, Taori SK, Schelenz S, *et al.* *Candida auris*: A review of the literature. *Clin Microbiol Rev* 2018;31. <https://doi.org/10.1128/CMR.00029-17/ASSET/A4EF16DA-232A-403D-AE90-E90B9247DB73/ASSETS/GRAPHIC/ZCM0011826100009.JPEG>.
- [52] Vandeputte P, Pineau L, Larcher G, *et al.* Molecular mechanisms of resistance to 5-fluorocytosine in laboratory mutants of *Candida glabrata*. *Mycopathologia* 2011;171:11–21. <https://doi.org/10.1007/S11046-010-9342-1>.
- [53] Phillips NA, Rocktaschel M, Merjanian L. Ibrexafungerp for the Treatment of Vulvovaginal Candidiasis: Design, Development and Place in Therapy. *Drug Des Devel Ther* 2023;17:363. <https://doi.org/10.2147/DDDT.S339349>.
- [54] Bhattacharya S, Sae-Tia S, Fries BC. Candidiasis and Mechanisms of Antifungal Resistance. *Antibiotics* 2020;9:1–19. <https://doi.org/10.3390/ANTIBIOTICS9060312>.
- [55] Arendrup MC, Patterson TF. Multidrug-Resistant *Candida*: Epidemiology, Molecular Mechanisms, and Treatment. *J Infect Dis* 2017;216:S445–51. <https://doi.org/10.1093/INFDIS/JIX131>.
- [56] Rodrigues ME, Silva S, Azeredo J, *et al.* Novel strategies to fight *Candida* species infection. *Crit Rev Microbiol* 2014;7828:1–13. <https://doi.org/10.3109/1040841X.2014.974500>.
- [57] Cleveland AA, Harrison LH, Farley MM, *et al.* Declining incidence of candidemia and the shifting epidemiology of *Candida* resistance in two US metropolitan areas, 2008-2013: Results from population-based surveillance. *PLoS One* 2015;10:1–12. <https://doi.org/10.1371/journal.pone.0120452>.
- [58] Rapp RP. Changing Strategies for the Management of Invasive Fungal Infections. *Pharmacotherapy* 2004;24:4S–28S. <https://doi.org/10.1592/phco.24.3.4S.33151>.
- [59] Raut JS, Karuppayil SM. A status review on the medicinal properties of essential oils. *Ind Crops Prod* 2014;62:250–264. <https://doi.org/10.1016/j.indcrop.2014.05.055>.
- [60] Al-Mijalli SH, Mrabti NN, Ouassou H, *et al.* Chemical Composition and Antioxidant, Antimicrobial, and Anti-Inflammatory Properties of *Origanum compactum* Benth Essential Oils from Two Regions: *In Vitro* and *In*

- Vivo* Evidence and *In Silico* Molecular Investigations. *Molecules* 2022, Vol 27, Page 7329 2022;27:7329. <https://doi.org/10.3390/MOLECULES27217329>.
- [61] Pappas PG, Rex JH, Sobel JD, *et al.* Guidelines for treatment of candidiasis. *Clin Infect Dis* 2004;38:161–89. <https://doi.org/10.1097/01.idc.0000130888.27859.52>.
- [62] Vazquez J. Combination antifungal therapy against *Candida* species: the new frontier—are we there yet? *Medical Mycology: Official Publication of the International Society for Human and Animal Mycology* 2003;41:355–68. <https://doi.org/10.1080/13693780310001616528>.
- [63] Hodgetts S, Nooney L, Al-Akeel R, *et al.* Efungumab and caspofungin: pre-clinical data supporting synergy. *Journal of Antimicrobial Chemotherapy* 2008;61:1132–9. <https://doi.org/10.1093/JAC/DKN075>.
- [64] Donnelly RF, McCarron PA, Tunney MM. Antifungal photodynamic therapy. *Microbiol Res* 2008;163:1–12. <https://doi.org/10.1016/j.micres.2007.08.001>.
- [65] Černáková L, Light C, Salehi B, *et al.* Novel Therapies for Biofilm-Based *Candida* spp. Infections. *Adv Exp Med Biol* 2019;1214:93–123. https://doi.org/10.1007/5584_2019_400.
- [66] Hosny K, Asfour H, Rizg W, *et al.* Formulation, Optimization, and Evaluation of Oregano Oil Nanoemulsions for the Treatment of Infections Due to Oral Microbiota. *Int J Nanomedicine* 2021;16:5465–78. <https://doi.org/10.2147/IJN.S325625>.
- [67] Liu M, Luo F, Qing Z, Yang H, *et al.* Chemical Composition and Bioactivity of Essential Oil of Ten *Labiatae* Species. *Molecules* 2020, Vol 25, Page 4862 2020;25:4862. <https://doi.org/10.3390/MOLECULES25204862>.
- [68] Imran M, Iqbal A, Badshah SL, *et al.* Chemical and Nutritional Profiling of the Seaweed *Dictyota dichotoma* and Evaluation of Its Antioxidant, Antimicrobial and Hypoglycemic Potentials. *Mar Drugs* 2023;21:273. <https://doi.org/10.3390/MD21050273/S1>.
- [69] Gheda S, El-Zaher EHFA, Abou-Zeid AM, *et al.* Potential Activity of *Arthrospira platensis* as Antioxidant, Cytotoxic and Antifungal against Some Skin Diseases: Topical Cream Application. *Marine Drugs* 2023, Vol 21, Page 160 2023;21:160. <https://doi.org/10.3390/MD21030160>.
- [70] Ramli I, Zerizer S, Gali L, *et al.* *In vitro* and *in vivo* bioactivities of *Ambrosia maritima* and *Bituminaria bituminosa* organic extracts from Algeria. *The Journal of Infection in Developing Countries* 2022;16:1064–74. <https://doi.org/10.3855/jidc.16788>.
- [71] Saddiq AA, Tag HM, Doleib NM, *et al.* Antimicrobial, Antigenotoxicity, and Characterization of *Calotropis procera* and Its Rhizosphere-Inhabiting Actinobacteria: *In Vitro* and *In Vivo* Studies. *Molecules* 2022;27. <https://doi.org/10.3390/MOLECULES27103123>.
- [72] Liu Y, Ren H, Wang D, *et al.* The synergistic antifungal effects of gypenosides combined with fluconazole against resistant *Candida albicans* via inhibiting the drug efflux and biofilm formation. *Biomed Pharmacother* 2020;130. <https://doi.org/10.1016/J.BIOPHA.2020.110580>.
- [73] Wang ZJ, Zhu YY, Yi X, *et al.* Bioguided isolation, identification and activity evaluation of antifungal compounds from *Acorus tatarinowii* Schott. *J Ethnopharmacol* 2020;261. <https://doi.org/10.1016/J.JEP.2020.113119>.
- [74] Rajamohamed BS, Siddharthan S. Modulatory effects of *Amukkara Choornam* on *Candida albicans* biofilm: *in vitro* and *in vivo* study. *Mol Biol Rep* 2019 46:3 2019;46:2961–9. <https://doi.org/10.1007/S11033-019-04757-Z>.
- [75] Abirami G, Alexpandi R, Durgadevi R, *et al.* Inhibitory Effect of Morin Against *Candida albicans* Pathogenicity and Virulence Factor Production: An *in vitro* and *in vivo* Approaches. *Front Microbiol* 2020;11. <https://doi.org/10.3389/FMICB.2020.561298/FULL>.
- [76] Alrumaihi F, Allemailem KS, Almatroudi A, *et al.* *Tinospora cordifolia* Aqueous Extract *Alleviates Cyclophosphamide*- Induced Immune Suppression, Toxicity and Systemic Candidiasis in Immunosuppressed Mice: *In vivo* Study in Comparison to Antifungal Drug Fluconazole. *Curr Pharm Biotechnol* 2019;20:1055–63. <https://doi.org/10.2174/1389201019666190722151126>.
- [77] Abdallah BM, Ali EM. Therapeutic Effect of Green Synthesized Silver Nanoparticles Using *Erodium glaucophyllum* Extract against Oral Candidiasis: *In Vitro* and *In Vivo* Study. *Molecules* 2022;27:4221. <https://doi.org/10.3390/MOLECULES27134221/S1>.

- [78] de Oliveira Zoccolotti J, Cavaleiro AJ, Tasso CO, *et al.* *In vivo* antifungal activity and biocompatibility of *Cryptocarya moschata*. Arch Microbiol 2022;204:1–9. <https://doi.org/10.1007/S00203-022-03177-1/METRICS>.
- [79] Silva DR, Rosalen PL, Freires IA, *et al.* *Anadenanthera Colubrina* vell Brenan: anti-*Candida* and antibiofilm activities, toxicity and therapeutical action. Braz Oral Res 2019;33:e023. <https://doi.org/10.1590/1807-3107BOR-2019.VOL33.0023>.
- [80] Campos LM, Silva TP, de Oliveira Lemos AS, *et al.* Antibiofilm potential of *Annona muricata* L. ethanolic extract against multi-drug resistant *Candida albicans*. J Ethnopharmacol 2023;315. <https://doi.org/10.1016/J.JEP.2023.116682>.
- [81] Fabri RL, Freitas JCO, Lemos ASO, *et al.* Spilanthol as a promising antifungal alkylamide for the treatment of vulvovaginal candidiasis. Med Mycol 2021;59:1210–24. <https://doi.org/10.1093/MMY/MYAB054>.
- [82] Li L, Qiu J, Wei M, *et al.* Inhibition of *Candida albicans* and induced vaginitis by sapindus water extract. Nat Prod Res 2021;35:2987–91. <https://doi.org/10.1080/14786419.2019.1679136>.
- [83] Campos LM, de Oliveira Lemos AS, da Cruz LF, *et al.* Development and *in vivo* evaluation of chitosan-gel containing *Mitracarpus frigidus* methanolic extract for vulvovaginal candidiasis treatment. Biomedicine & Pharmacotherapy 2020;130:110609. <https://doi.org/10.1016/J.BIOPHA.2020.110609>.
- [84] Saghafi N, Karjalian M, Ghazanfarpour M, *et al.* The effect of a vaginal suppository formulation of dill (*Anethum graveolens*) in comparison to clotrimazole vaginal tablet on the treatment of vulvovaginal candidiasis. J Obstet Gynaeco 2018;38:985–8. <https://doi.org/10.1080/01443615.2018.1432578>.
- [85] Akroum S. Anti-microbial activity of *Rosmarinus officinalis* and *Zingiber officinale* extracts on the species of the genus *Candida* and on *Streptococcus pneumonia*. Ann Pharm Fr 2020;79:62–9. <https://doi.org/10.1016/J.PHARMA.2020.06.003>.
- [86] Raj S, Vinod V, Jayakumar J, *et al.* Antifungal activity of *Syzygium samarangense* leaf extracts against *Candida*. Lett Appl Microbiol 2021;73:31–8. <https://doi.org/10.1111/LAM.13471>.
- [87] Güzel S, Özay Y, Kumaş M, *et al.* Wound healing properties, antimicrobial and antioxidant activities of *Salvia kronenburgii* Rech. f. and *Salvia euphratica* Montbret, Aucher & Rech. f. var. *euphratica* on excision and incision wound models in diabetic rats. Biomedicine & Pharmacotherapy 2019;111:1260–76. <https://doi.org/10.1016/J.BIOPHA.2019.01.038>.
- [88] Akroum S. Antifungal activity of *Camellia sinensis* crude extracts against four species of *Candida* and *Microsporum persicolor*. J Mycol Med 2018;28:424–7. <https://doi.org/10.1016/J.MYCMED.2018.06.003>.
- [89] Alves CT, Ferreira ICFR, Barros L, *et al.* Antifungal activity of phenolic compounds identified in flowers from North Eastern Portugal against *Candida* species. Future Microbiol 2014;9:139–46. <https://doi.org/10.2217/FMB.13.147>.
- [90] Cowan MM. Plant products as antimicrobial agents. Clin Microbiol Rev 1999;12:564–582. <https://doi.org/10.1128/cmr.12.4.564>.
- [91] Lang G, Buchbauer G. A review on recent research results (2008-2010) on essential oils as antimicrobials and antifungals. A review. Flavour Fragr J 2012;27:13–39. <https://doi.org/10.1002/ffj.2082>.
- [92] Marino A, Nostro A, Mandras N, *et al.* Evaluation of antimicrobial activity of the hydrolate of *Coridothymus capitatus* (L.) Reichenb. fil. (*Lamiaceae*) alone and in combination with antimicrobial agents. BMC Complement Med Ther 2020;20:89. <https://doi.org/10.1186/s12906-020-2877-x>.
- [93] Bakkali F, Averbeck S, Averbeck D, *et al.* Biological effects of essential oils - A review. Food and Chemical Toxicology 2008;46:446–475. <https://doi.org/10.1016/j.fct.2007.09.106>.
- [94] Nostro A, Roccaro AS, Bisignano G, *et al.* Effects of oregano, carvacrol and thymol on *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms. J Med Microbiol 2007;56:519–23. <https://doi.org/10.1099/jmm.0.46804-0>.
- [95] Hao Y, Li J, Zhang W, *et al.* Analysis of the Chemical Profiles and Anti-*S. aureus* Activities of Essential Oils Extracted from Different Parts of Three Oregano Cultivars. Foods 2021, Vol 10, Page 2328 2021;10:2328. <https://doi.org/10.3390/FOODS10102328>.
- [96] Baser K, Demitri FC. Chemistry of essential oils. In Flavours and Fragrances - chemistry, bioprocessing and sustainability. Berlin, Germany: Springer; 2007.

- [97] Koroch AR, Rodolfo Juliani H, Zygodlo JA. Bioactivity of essential oils and their components. *Flavours and Fragrances: Chemistry, Bioprocessing and Sustainability*, 2007, p. 87–115. https://doi.org/10.1007/978-3-540-49339-6_5.
- [98] Burt S. Essential oils: Their antibacterial properties and potential applications in foods - A review. *Int J Food Microbiol* 2004;94:223–53. <https://doi.org/10.1016/j.ijfoodmicro.2004.03.022>.
- [99] Masotti V, Juteau F, Bessière JM, *et al.* Seasonal and Phenological Variations of the Essential Oil from the Narrow Endemic Species *Artemisia molinieri* and Its Biological Activities. *J Agric Food Chem* 2003;51:7115–7121. <https://doi.org/10.1021/jf034621y>.
- [100] Copetta A, Lingua G, Berta G. Effects of three AM fungi on growth, distribution of glandular hairs, and essential oil production in *Ocimum basilicum* L. var. Genovese. *Mycorrhiza* 2006;16:485–494. <https://doi.org/10.1007/s00572-006-0065-6>.
- [101] Milenković L, Ilić ZS, Šunić L, *et al.* Modification of light intensity influence essential oils content, composition and antioxidant activity of thyme, marjoram and oregano. *Saudi J Biol Sci* 2021;28:6532–43. <https://doi.org/10.1016/J.SJBS.2021.07.018>.
- [102] Caputo L, Amato G, de Bartolomeis P, *et al.* Impact of drying methods on the yield and chemistry of *Origanum vulgare* L. essential oil. *Sci Rep* 2022;12. <https://doi.org/10.1038/S41598-022-07841-W>.
- [103] Russo M, Serra D, Suraci F, *et al.* Effectiveness of electronic nose systems to detect bergamot (*Citrus bergamia* Risso et Poiteau) essential oil quality and genuineness. *J Essent Oil Res* 2012;24:137–51. <https://doi.org/10.1080/10412905.2012.659530>.
- [104] Feyaerts AF, Mathé L, Luyten W, *et al.* Comparison between the vapor-phase-mediated anti-*Candida* activity of conventional and organic essential oils. *Volatiles & Essent Oils* 2018;5:1–6.
- [105] Salvo A, La Torre GL, Rotondo A, *et al.* Multiple analytical approaches for the organic and inorganic characterization of *Origanum vulgare* L. samples. *Nat Prod Res* 2019;33:2815–22. <https://doi.org/10.1080/14786419.2018.1503269>.
- [106] Pina-Vaz C, Rodrigues AG, Pinto E, *et al.* Antifungal activity of Thymus oils and their major compounds. *J. Eur. Acad. Dermatol. Venereol.* 2004;18:73–8. <https://doi.org/10.1111/j.1468-3083.2004.00886.x>.
- [107] Pinto E, Pina-Vaz C, Salgueiro L, *et al.* Antifungal activity of the essential oil of *Thymus pulegioides* on *Candida*, *Aspergillus* and dermatophyte species. *J Med Microbiol* 2006;55:1367–73. <https://doi.org/10.1099/JMM.0.46443-0>.
- [108] Villar Rodríguez J, Pérez-Pico AM, Mingorance-Álvarez E, *et al.* Meta-analysis of the antifungal activities of three essential oils as alternative therapies in dermatophytosis infections. *J Appl Microbiol* 2022;133:241–53. <https://doi.org/10.1111/JAM.15539>.
- [109] Rajkowska K, Nowak A, Kunicka-Styczyńska A, *et al.* Biological effects of various chemically characterized essential oils: Investigation of the mode of action against: *Candida albicans* and HeLa cells. *RSC Adv* 2016;6:97199–207. <https://doi.org/10.1039/c6ra21108a>.
- [110] Zhang J, Wu H, Jiang D, *et al.* The antifungal activity of essential oil from *Melaleuca leucadendra* (L.) L. grown in China and its synergistic effects with conventional antibiotics against *Candida*. *Nat Prod Res* 2019;33:2545–8. https://doi.org/10.1080/14786419.2018.1448979/SUPPL_FILE/GNPL_A_1448979_SM3660.PDF.
- [111] Garzoli S, Filatov VA, Kulyak OY, *et al.* Chemical Composition and Antimicrobial Potential of a Plant-Based Substance for the Treatment of Seborrheic Dermatitis. *Pharmaceutics* 2023, Vol 16, Page 328 2023;16:328. <https://doi.org/10.3390/PH16030328>.
- [112] Borotová P, Galovičová L, Vukovic NL, *et al.* Chemical and Biological Characterization of *Melaleuca alternifolia* Essential Oil. *Plants* 2022, Vol 11, Page 558 2022;11:558. <https://doi.org/10.3390/PLANTS11040558>.
- [113] Carson CF, Hammer KA, Riley T V. *Melaleuca alternifolia* (tea tree) oil: A review of antimicrobial and other medicinal properties. *Clin Microbiol Rev* 2006;19:50–62. <https://doi.org/10.1128/CMR.19.1.50-62.2006>.
- [114] da Silva RA, Antonietti FMPM, Röder DVD de B, *et al.* Essential Oils of *Melaleuca*, *Citrus*, *Cupressus*, and *Litsea* for the Management of Infections Caused by *Candida* Species: A Systematic Review. *Pharmaceutics* 2021;13. <https://doi.org/10.3390/PHARMACEUTICS13101700>.

- [115] de Campos Rasteiro MM, da Costa PCBP, Araújo FF, *et al.* Essential oil of *Melaleuca alternifolia* for the treatment of oral candidiasis induced in an immunosuppressed mouse model. BMC Complement Altern Med 2014;14. <https://doi.org/10.1186/1472-6882-14-489>.
- [116] Hammer KA, Carson CF, Riley TV, *et al.* A review of the toxicity of *Melaleuca alternifolia* (tea tree) oil. Food Chem Toxicol 2006;44:616–25. <https://doi.org/10.1016/J.FCT.2005.09.001>.
- [117] Thoma JL, Cantrell CL, Zheljazkov VD. Evaluation of Essential Oils as Sprout Suppressants for Potato (*Solanum tuberosum*) at Room Temperature Storage. Plants 2022;11. <https://doi.org/10.3390/PLANTS11223055>.
- [118] Siddique S, Mazhar S, Parveen Z. Chemical characterization, antioxidant and antimicrobial activities of essential oil from *Melaleuca quinquenervia* leaves. Indian J Exp Biol 2018;56:686–93.
- [119] Wilkinson JM, Cavanagh HMA. Antibacterial activity of essential oils from Australian native plants. Phytother Res 2005;19:643–6. <https://doi.org/10.1002/PTR.1716>.
- [120] Valková V, Ďúranová H, Vukovic NL, *et al.* Assessment of Chemical Composition and Anti-Penicillium Activity of Vapours of Essential Oils from *Abies Alba* and Two *Melaleuca* Species in Food Model Systems. Molecules 2022;27. <https://doi.org/10.3390/MOLECULES27103101>.
- [121] Tisserand R, Young R. Essential oil safety : a guide for health care professionals. 2014 Second ed. Edinburgh: Elsevier Limited.
- [122] Monzote L, Scherbakov AM, Scull R, *et al.* Essential Oil from *Melaleuca leucadendra*: Antimicrobial, Antikinetoplastid, Antiproliferative and Cytotoxic Assessment. Molecules 2020, Vol 25, Page 5514 2020;25:5514. <https://doi.org/10.3390/MOLECULES25235514>.
- [123] Giang An NT, Huong LT, Satyal P, *et al.* Mosquito Larvicidal Activity, Antimicrobial Activity, and Chemical Compositions of Essential Oils from Four Species of *Myrtaceae* from Central Vietnam. Plants 2020, Vol 9, Page 544 2020;9:544. <https://doi.org/10.3390/PLANTS9040544>.
- [124] Karpiński TM, Ożarowski M, Seremak-Mrozikiewicz A, *et al.* Anti-*Candida* and Antibiofilm Activity of Selected *Lamiaceae* Essential Oils. Front Biosci (Landmark Ed) 2023;28:28. <https://doi.org/10.31083/J.FBL2802028>.
- [125] Alshaikh NA, Perveen K. Susceptibility of Fluconazole-Resistant *Candida albicans* to Thyme Essential Oil. Microorganisms 2021;9. <https://doi.org/10.3390/MICROORGANISMS9122454>.
- [126] Naseri N, Kalantari Khandani A, Baherimoghadam T, *et al.* The Effect of *Thymus Vulgaris* Essential Oil and Chlorhexidine on *Candida Albicans* Accumulated on Removable Orthodontic Appliance: A Clinical Trial. J Dent 2022;23:190–7. <https://doi.org/10.30476/DENTJODS.2021.89317.1404>.
- [127] Shanaida M, Hudz N, Białoń M, *et al.* Chromatographic profiles and antimicrobial activity of the essential oils obtained from some species and cultivars of the *Menthae* tribe (*Lamiaceae*). Saudi J Biol Sci 2021;28:6145. <https://doi.org/10.1016/J.SJBS.2021.06.068>.
- [128] Beicu R, Alexa E, Obişcioiu D, *et al.* Antimicrobial potential and phytochemical profile of wild and cultivated populations of thyme (*Thymus* spp.) growing in western romania. Plants 2021;10:1833. <https://doi.org/10.3390/PLANTS10091833/S1>.
- [129] Córdoba S, Vivot W, Szusz W, *et al.* Antifungal Activity of Essential Oils Against *Candida* Species Isolated from Clinical Samples. Mycopathologia 2019;184:615–23. <https://doi.org/10.1007/S11046-019-00364-5/TABLES/2>.
- [130] Demirci B, Kırıcı D, Öztürk G, *et al.* Effect of Extraction Time on *Origanum onites* L. Infusions and Essential Oils - Biological Evaluation, Statistical Principal Component and Hierarchical Cluster Analyses. Chem Biodivers 2022;19. <https://doi.org/10.1002/CBDV.202200482>.
- [131] Hao Y, Kang J, Yang R, *et al.* Multidimensional exploration of essential oils generated via eight oregano cultivars: Compositions, chemodiversities, and antibacterial capacities. Food Chem 2022;374:131629. <https://doi.org/10.1016/J.FOODCHEM.2021.131629>.
- [132] Cid-Chevecich C, Müller-Sepúlveda A, Jara JA, *et al.* *Origanum vulgare* L. essential oil inhibits virulence patterns of *Candida* spp. and potentiates the effects of fluconazole and nystatin *in vitro*. BMC Complement Med Ther 2022;22. <https://doi.org/10.1186/S12906-022-03518-Z>.
- [133] Stringaro A, Colone M, Cecchetti S, *et al.* “*In vivo*” and “*in vitro*” antimicrobial activity of *Origanum vulgare* essential oil and its two phenolic compounds on clinical isolates of *Candida* spp. Arch Microbiol 2023;205:1–12. <https://doi.org/10.1007/S00203-022-03355-1/FIGURES/5>.

- [134] Kaskatepe B, Erdem SA, Ozturk S, *et al.* Antifungal and Anti-Virulent Activity of *Origanum majorana* L. Essential Oil on *Candida albicans* and *In Vivo* Toxicity in the *Galleria mellonella* Larval Model. *Molecules* 2022;27. <https://doi.org/10.3390/MOLECULES27030663>.
- [135] Kapustová M, Granata G, Napoli E, *et al.* Nanoencapsulated Essential Oils with Enhanced Antifungal Activity for Potential Application on Agri-Food, Material and Environmental Fields. *Antibiotics (Basel)* 2021;10:1–12. <https://doi.org/10.3390/ANTIBIOTICS10010031>.
- [136] Neves JS, Lopes-Da-Silva Z, De Sousa Brito Neta M, *et al.* Preparation of terpolymer capsules containing *Rosmarinus officinalis* essential oil and evaluation of its antifungal activity. *RSC Adv* 2019;9:22586. <https://doi.org/10.1039/C9RA02336D>.
- [137] Dinu MV, Gradinaru AC, Lazar MM, *et al.* Physically cross-linked chitosan/dextrin cryogels entrapping *Thymus vulgaris* essential oil with enhanced mechanical, antioxidant and antifungal properties. *Int J Biol Macromol* 2021;184:898–908. <https://doi.org/10.1016/J.IJBIOMAC.2021.06.068>.
- [138] Serra E, Saubade F, Ligorio C, *et al.* Methylcellulose Hydrogel with *Melissa officinalis* Essential Oil as a Potential Treatment for Oral Candidiasis. *Microorganisms* 2020, Vol 8, Page 215 2020;8:215. <https://doi.org/10.3390/MICROORGANISMS8020215>.
- [139] Torabiardekani N, Karami F, Khorram M, *et al.* Encapsulation of *Zataria multiflora* essential oil in polyvinyl alcohol/chitosan/gelatin thermo-responsive hydrogel: Synthesis, physico-chemical properties, and biological investigations. *Int J Biol Macromol* 2023;243. <https://doi.org/10.1016/J.IJBIOMAC.2023.125073>.
- [140] Jawaid T, Alaseem AM, Khan MM, *et al.* Preparation and Evaluation of Nanoemulsion of Citronella Essential Oil with Improved Antimicrobial and Anti-Cancer Properties. *Antibiotics (Basel)* 2023;12:478. <https://doi.org/10.3390/ANTIBIOTICS12030478>.
- [141] Sharma K, Munjal M, Sharma RK, *et al.* Thymol encapsulated chitosan-Aloe vera films for antimicrobial infection. *Int J Biol Macromol* 2023;235:123897. <https://doi.org/10.1016/J.IJBIOMAC.2023.123897>.
- [142] Zanutto AW, Kanemaru MYS, de Souza FG, *et al.* Enhanced antimicrobial and antioxidant capacity of *Thymus vulgaris*, *Lippia sidoides*, and *Cymbopogon citratus* emulsions when combined with mannosylerythritol a lipid biosurfactant. *Food Res Int* 2023;163. <https://doi.org/10.1016/J.FOODRES.2022.112213>.
- [143] Ramtin M, Sharifniya F, Larypoor M, *et al.* Evaluation of the Active Ingredient of Campsis radicans Essential Oils and its Antimicrobial Evaluation Against Pathogenic Bacteria. *Curr Microbiol* 2022;79:1–12. <https://doi.org/10.1007/S00284-022-03042-W/TABLES/4>.
- [144] Odjo K, Al-Maqtari QA, Yu H, *et al.* Preparation and characterization of chitosan-based antimicrobial films containing encapsulated lemon essential oil by ionic gelation and cranberry juice. *Food Chem* 2022;397. <https://doi.org/10.1016/J.FOODCHEM.2022.133781>.
- [145] Baldim I, Paziani MH, Grizante Barião PH, *et al.* Nanostructured Lipid Carriers Loaded with *Lippia sidoides* Essential Oil as a Strategy to Combat the Multidrug-Resistant *Candida auris*. *Pharmaceutics* 2022;14. <https://doi.org/10.3390/PHARMACEUTICS14010180>.
- [146] de Alteriis E, Maione A, Falanga A, *et al.* Activity of Free and Liposome-Encapsulated Essential Oil from *Lavandula angustifolia* against Persister-Derived Biofilm of *Candida auris*. *Antibiotics (Basel)* 2021;11. <https://doi.org/10.3390/ANTIBIOTICS11010026>.
- [147] Râpă M, Gaidau C, Mititelu-Tartau L, *et al.* Bioactive collagen hydrolysate-chitosan/essential oil electrospun nanofibers designed for medical wound dressings. *Pharmaceutics* 2021;13. <https://doi.org/10.3390/PHARMACEUTICS13111939/S1>.
- [148] Mishra P, Gupta P, Pruthi V. Cinnamaldehyde incorporated gellan/PVA electrospun nanofibers for eradicating *Candida* biofilm. *Materials Science and Engineering: C* 2021;119:111450. <https://doi.org/10.1016/J.MSEC.2020.111450>.
- [149] Mishra P, Gupta P, Srivastava AK, *et al.* Eucalyptol/ β -cyclodextrin inclusion complex loaded gellan/PVA nanofibers as antifungal drug delivery system. *Int J Pharm* 2021;609. <https://doi.org/10.1016/J.IJPHARM.2021.121163>.
- [150] Kapustová M, Puškárová A, Bučková M, *et al.* Biofilm inhibition by biocompatible poly(ϵ -caprolactone) nanocapsules loaded with essential oils and their cyto/genotoxicity to human keratinocyte cell line. *Int J Pharm* 2021;606:120846. <https://doi.org/10.1016/J.IJPHARM.2021.120846>.

-
- [151] Garcia LGS, da Rocha MG, Lima LR, *et al.* Essential oils encapsulated in chitosan microparticles against *Candida albicans* biofilms. *Int J Biol Macromol* 2021;166:621–32. <https://doi.org/10.1016/J.IJBIOMAC.2020.10.220>.
 - [152] Fazly Bazzaz BS, Khameneh B, Namazi N, *et al.* Solid lipid nanoparticles carrying *Eugenia caryophyllata* essential oil: the novel nanoparticulate systems with broad-spectrum antimicrobial activity. *Lett Appl Microbiol* 2018;66:506–13. <https://doi.org/10.1111/LAM.12886>.
 - [153] Mokarizadeh M, Kafil H, Ghanbarzadeh S, *et al.* Improvement of citral antimicrobial activity by incorporation into nanostructured lipid carriers: a potential application in food stuffs as a natural preservative. *Res Pharm Sci* 2017;12:409. <https://doi.org/10.4103/1735-5362.213986>.
 - [154] Balaure PC, Boarca B, Popescu RC, *et al.* Bioactive mesoporous silica nanostructures with anti-microbial and anti-biofilm properties. *Int J Pharm* 2017;531:35–46. <https://doi.org/10.1016/J.IJPHARM.2017.08.062>.
 - [155] Mishra N, Rai VK, Yadav KS, *et al.* Encapsulation of Mentha Oil in Chitosan Polymer Matrix Alleviates Skin Irritation. *AAPS PharmSciTech* 2016;17:482–92. <https://doi.org/10.1208/S12249-015-0378-X>.
 - [156] Liakos I, Rizzello L, Scurr DJ, *et al.* All-natural composite wound dressing films of essential oils encapsulated in sodium alginate with antimicrobial properties. *Int J Pharm* 2014;463:137–45. <https://doi.org/10.1016/J.IJPHARM.2013.10.046>.
 - [157] Kryeziu TL, Haloci E, Loshaj-Shala A, *et al.* Nanoencapsulation of *Origanum vulgare* essential oil into liposomes with anticancer potential. *Pharmazie* 2022;77:172–8. <https://doi.org/10.1691/PH.2022.1230>.

CHAPTER II

EFFECT OF ESSENTIAL OILS ON DRUG-RESISTANT *CANDIDA* STRAINS

Over the time there has been an increase in *Candida* strains resistant to conventional antifungals. Highlighting *Candida auris*, a recently found *Candida* species, mainly associated with nosocomial outbreaks in intensive care hospital settings, and unlike other *Candida* species, it can be transmitted through person-to-person or by contact with surfaces. This yeast pathogen is responsible for many invasive fungal infections due to its multidrug-resistant character and its huge transmission ability. Indeed, *C. auris* is described as resistant to first-line antifungals and, consequently, associated with high mortality.

Despite the impact and importance that *C. auris* has recently demonstrated, *Candida albicans* remains the *Candida* species most frequently found and associated with vulvovaginal candidiasis (VVC). Moreover, there is also growing concern about the incidence of non-*Candida albicans Candida* (NCAC) species, such as *Candida glabrata*, *Candida parapsilosis*, *Candida krusei* or recently named *Pichia kudriavzevii*. VVC is a disease with high incidence, a huge impact on the quality of life and health of women, and which represents a great challenge to treat. The growing need to apply antifungal intensive therapies have contributed to an emergence of drug-resistant *Candida* strains.

Effective therapeutic options, to meet the antifungal-resistance challenge and to control high resilient biofilms, are urgently needed. Nowadays, essential oils (EOs), obtained from several aromatic plants, are known to be effective against fungal infections. For example, *Thymus* species are known by their antifungal effect due to the presence of some volatile compounds in their EOs, such as carvacrol, thymol, linalool and γ -terpinene. However, the efficiency of the EOs can vary significantly even within the same genus. Therefore, the selection of natural therapies should always have in account EOs composition.

CHAPTER II.1

ESSENTIAL OILS AS A GOOD WEAPON AGAINST DRUG-RESISTANT *CANDIDA AURIS*

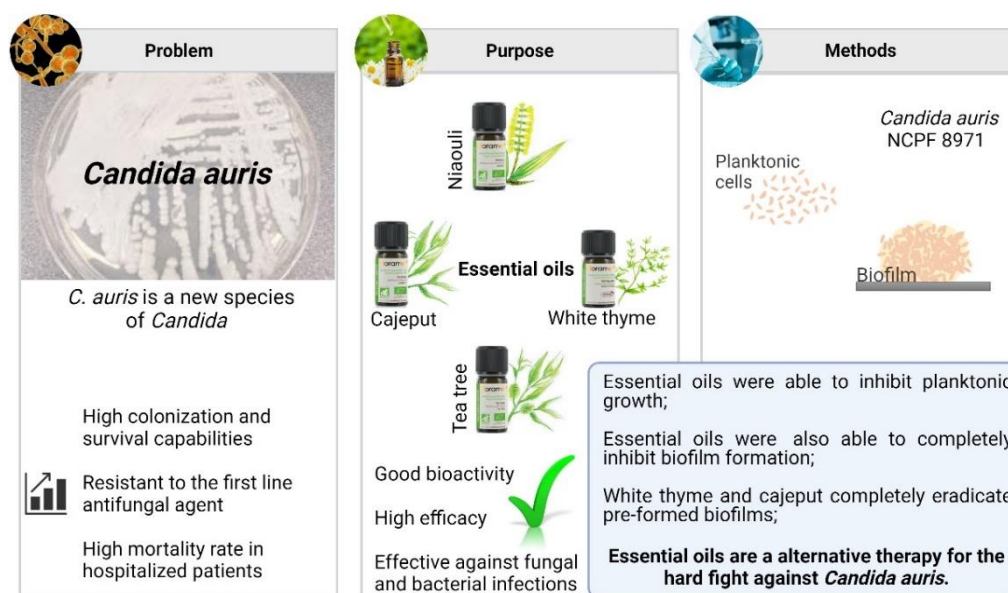


Figure 8. Subchapter II.1 workflow.

Candida auris, a recently discovered *Candida* species, is primarily linked to nosocomial outbreaks within intensive care hospital settings. Thus, this subchapter evaluated four specific essential oils based on clinical evidence and their individual characteristics: tea tree, niaouli, white thyme and cajepout oils. The main objective of this research was to evaluate the impact of these essential oils against planktonic growth and biofilms of *Candida auris*.

This subchapter is based on the following publication: Fernandes L, Ribeiro R, Costa R, Henriques M, Rodrigues ME. Essential Oils as a Good Weapon against Drug-Resistant *Candida auris*. Antibiotics 2022, Vol 11, Page 977 2022;11:977. <https://doi.org/10.3390/ANTIBIOTICS11070977>.

1. Introduction

In recent years, the incidence and prevalence of fungal infections became a worrying problem in hospital environments. These invasive fungal infections are reported to be one of the most common diseases in humans, affecting nearly 25 % of the population [1]. *Candida auris* is a new *Candida* species recently found, after being isolated from the external auditory canal of a Japanese patient, and since then it has been found and reported in more than 6 continents and 30 countries [2–4]. *C. auris* is mainly associated with nosocomial outbreaks in intensive care settings, where it has been recovered from the skin of healthcare workers and patients, areas that have been in contact with infected patients and a wide variety of surfaces [5]. *C. auris* has shown high colonization and survival capabilities and, unlike the majority of the other *Candida* species, can be transmitted through person-to-person or through contact with surfaces or contaminated equipment [5]. This yeast causes highly invasive infections with a high mortality rate in hospitalized patients, with immunosuppression and underlying chronic disease being the main risk factors [6]. In fact, several virulence factors commonly associated with *Candida albicans* (considered as the most virulent member of the genus) have been identified in *C. auris*, such as phospholipase and proteinase activities and biofilm formation, although *C. auris* isolates produce only rudimentary pseudohyphae [7]. In addition, *C. auris* has already been described as resistant to the first line antifungal agent, fluconazole (FLC), exhibiting variable susceptibility to other azoles and echinocandins [2]. Thus, the search for alternative therapies from natural sources featuring new mechanisms of action combined with new strategies has become crucial. In this sense, essential oils (EOs) have emerged due to their many useful properties for human complications, including antiviral, anti-inflammatory and antifungal activity. The strong antimicrobial activities of EOs and their components are described in several studies [8–11]. EOs have the ability to hinder the growth of pathogens, increasing membrane permeability, disrupting the cell membrane, inducing leakage of vital intracellular constituents, and interrupting cell metabolism and enzymatic kinetics [10]. Tran *et al.* [11] demonstrated for the first time the potential of EO from *Cinnamomum zeylanicum* to exert antifungal activity against planktonic *C. auris* cells, highlighting their ability to damage the membranous structures of fungal cells and haemolytic activities. In addition, it was also demonstrated that EO arborvitae led to a significant decrease in the intrinsic planktonic growth rate of *C. auris*. However, in general there are very few studies on the effect of EOs on *C. auris* especially on *C. auris* biofilm. In this study, four EOs were evaluated, the choice of these four specific EOs was based on clinical evidence provided by a clinic (Costa Raquel, Aromas Aqua Spa – Health Clinic) and due to their characteristics: tea tree EO that is recognized for its anti-fungal, antiseptic, anti-inflammatory and anti-infective properties, it also stimulates the immune system and has

a calming effect; cajeput and white thyme EOs are recognized for its respiratory tract cleansing properties and is also used for its calming properties and niaouli EO that is an expectorant, decongestant and strengthens the immune system and can be used for skin problems such as acne, itching, dermatitis or sunburn [12]. So, the aim of this work was to evaluate the effect of tea tree, cajeput, niaouli and white thyme EOs against *C. auris* species.

2. Materials and Methods

2.1 Essential oils

This study evaluated the antifungal activity of four EOs, namely cajeput (*Melaleuca cajaputi* (leaf); florame® (Lot 801736), Portugal); niaouli (*Melaleuca quinquenervia* (leaf); florame® (Lot 100378), Portugal); tea tree (*Melaleuca alternifolia* (leaf); florame® (Lot 903025), Portugal) and white thyme (*Thymus satureioides* (flowering tops); florame® (Lot 800180), Portugal) (all with 100 % purity). All EO samples were stored wrapped in aluminum foil, in order to protect from light, at room temperature.

Analysis of the EOs was carried out by florame® (Saint-Rémy-de-Provence, France) (Annex I.1-4), using a Gas Chromatography (GC) with Flame-Ionization Detection (FID) at the following conditions: the hydrogen carrier gas; the polar Elite-WAX column (100 % polyethylene glycol) (60 m / 0.25 mm / 0.25 µm) and the non-polar Elite-5 columns (5 % diphenyl, 95 % dimethylpolysiloxane) (60 m / 0.25 mm / 0.25 µm) for tea tree, white thyme and cajeput EOs. Analysis of the niaouli EO was carried out using a GC 6890 MS 5975 instrument at the following conditions: the injected sample volume was 1 µL; the helium carrier gas flow rate was 1 mL min⁻¹; a HP5 ms capillary column (30 m / 0.25 mm) was used, with a film thickness of 0.25 µm; the column temperature was 60 °C increasing at 2 °C min⁻¹ to 250 °C; and the mass range was 40- 450 m z⁻¹.

2.2 Microorganisms and culture conditions in this study

Candida auris NCPF 8971 was stored at $- 80 \pm 2$ °C in Sabouraud Dextrose Broth (SDB; Liofilchem) with 20 % (v/ v) glycerol. Before each assay, *C. auris* was subcultured onto Sabouraud Dextrose Agar (SDA; Liofilchem) and incubated aerobically at 37 °C for 18- 24 h. SDA plates were prepared from SDB supplemented with 2 % (w/ v) agar (Liofilchem).

2.3 Antifungal susceptibility testing

2.3.1 Fluconazole

Minimum Inhibitory Concentrations (MICs) and Minimum fungicidal concentrations (MFCs) of FLC were determined using broth micro dilution method according to Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI M27-A4), with some modifications [13]. Briefly, the initial cell concentration for *C. auris* was adjusted for 2×10^5 Colony-forming units (CFU) mL⁻¹ in RPMI 1640 and dispensed into 96-well round bottom microtiter plates with the FLC solution, both in a proportion of 1: 2. Positive (*C. auris* suspension) and negative (broth medium RPMI 1640) controls were included. All microtiter plates were incubated for 24- 48 h at 37 °C. MIC was obtained by visual observation of the turbidity gradient and by the determination of the optical density at 570 nm. Minimum Inhibitory Concentrations (MIC₅₀) endpoints were established as the lowest concentration of FLC that resulted in decrease of growth by ≥ 50 % relative to the positive control (*C. auris* suspension). To determine MFC, each well was sub-cultured onto SDA plates and incubated for 24 h at 37 °C. MFC endpoints were defined as the concentration in the first well with no growth on SDA plate. The experiment was performed in three independent assays in triplicate.

2.3.2 Essential oils

2.3.2.1 Planktonic Antimicrobial Susceptibilities

MICs and MFCs of cajeput, niaouli, tea tree and white thyme EOs were determined as described above, with some modifications due to the use of volatile compounds. After the concentration was adjusted, the 1 mL of cell suspension was dispensed into glass wells (Figure 9.A) into glass petri dishes (Figure 9.B) with the EOs diluted (12.5 % (v/ v) - 0.02 % (v/ v)) in a vegetable oil, almond oil (*Prunus amygdalus*, AB- Agriculture Biologique, France), with both at a ratio of 1: 2. EOs are extremely strong organic solvents that can cause damage and deterioration, and even melting, to most plastic materials, such as polystyrene, normally used in this type of assays. So, the use of a system with a glass well for cell culture inside glass Petri dishes (Figure 9.C) allows the direct application and/or the use of vapor-phase of EOs. Positive (*C. auris* suspension with almond oil) and negative (RPMI broth medium) controls were included. The set glass wells into glass petri dishes were incubated for 48 h at 37 °C. MIC₅₀ and MFC was determined as described above.

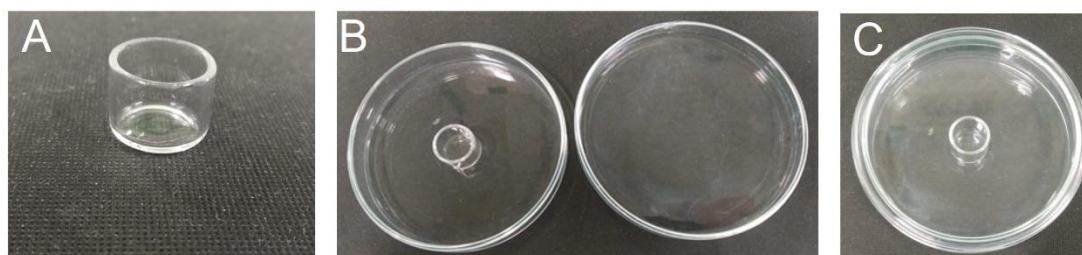


Figure 9. Glass system: **(A)** well, **(B)** well and petri dishes and **(C)** glass well inside glass petri dishes.

The agar disc diffusion method described by Tran *et al.* and Touati *et al.* [11,14], with some modifications, was used for determination of the antifungal activities of cajeput, niaouli, tea tree and white thyme oils. Briefly, a swab dipped in cell suspensions (pre-inocula) adjusted to 1×10^8 cells mL^{-1} was spread onto SDA plates. Then, sterile 6 mm blank disks (Liofilchem) impregnated with 25 μL of each oil (100 % (v/ v)) were placed over the agar surface, and plates were incubated at 37 °C for 24 h. Plates with disks loaded with ethanol 70 % (v/ v) and almond oil were also included as positive and negative controls, respectively. The inhibition zones induced by different EOs were measured (mm). Both experiments were performed in triplicate and in three independent assays.

2.3.2.2 Biofilms antimicrobial susceptibilities

C. auris biofilms were developed as described by Stepanović *et al.* [15], with some modifications due to the use of volatile compounds. Briefly, pre-inocula (pure liquid cultures) of *C. auris* were maintained in SDB for 18 h at 37 °C under agitation (120 rev/ min). Then, the initial cell concentration was adjusted for 1×10^5 cells mL^{-1} . The cellular suspension was transferred, under aseptic conditions, to glass wells inside glass petri plates (1 mL / well) (Figure 9.C). The *C. auris* biofilm culture was incubated aerobically for 24 h on a horizontal shaker at 120 rpm and 37 °C. The effect of four EOs (100 %) (tea tree, niaouli, white thyme and cajeput) was evaluated on biofilm formation and on 24 h-old biofilms. For this, two drops (25 μL with a final concentration of 2.4 % (v/ v)) of each oil were added to the wells containing a cell suspension (biofilm formation) or a pre-formed biofilms (24 h old biofilms). Positive (*C. auris* suspension) and negative (SDB broth medium) controls were included. All plates were then incubated in the same conditions. After 24 h the wells were washed with Phosphate-buffered saline (PBS) to discard the planktonic fraction and the microdrop technique was used. Biofilm cell suspensions were serially diluted in PBS and plated on SDA plates (incubated aerobically for 24 h at 37°C) and the cultivable cells were enumerated. Values of cultivable sessile cells were expressed as Log CFU per mL (Log_{10} (CFU mL^{-1})). The biofilm experiment was performed in triplicate with three independent assays.

2.4 Statistical analysis

Biofilm antimicrobial susceptibility results were analyzed using the Prism software package (GraphPad Software version 6.01 for Macintosh). Two-way ANOVA (analysis of variance) tests were performed, and means were compared by applying Tukey's multiple comparison test. Statistically significant differences were considered significant when $P < 0.05$.

3. Results and Discussion

The general resistance of *Candida* species to most available antifungal drugs and the emergence of multidrug-resistant species, such as *C. auris*, make it urgent to develop new drugs or more effective therapies. So, this study aimed to evaluate the antifungal activity of four EOs against *C. auris* in both planktonic cells and biofilms (on its formation and on pre-formed biofilms).

The identified compounds in the four EOs and their relative contents are listed in Table 2 according to their percentage ascending on a nonpolar column. The major compounds of the EOs are terpinen-4-ol (42 %), γ -terpinene (21 %) and α -terpinene (10 %) in tea tree oil, cineole (65 %), α -terpineol (11 %) and linalool (3 %) in cajeput oil, p-cymene, limonene and 1,8-cineole (57 %) in niaouli oil, and borneol (32 %), α -terpineol (16 %) and carvacrol (9 %) in white thyme oil.

To verify *C. auris* resistance to FLC, both MIC₅₀ and MFC were firstly evaluated. The results showed a MIC₅₀ of 125 $\mu\text{g mL}^{-1}$ and a MFC of $\geq 125 \mu\text{g mL}^{-1}$. These results confirm, that, as expected, *C. auris* is highly resistant to FLC (sensitive $< 32 \mu\text{g mL}^{-1}$; resistant $\geq 32 \mu\text{g mL}^{-1}$ [16]). Recently, genetic analyses of the *C. auris* species have reported mutations in genes related to the development of triazole resistance. In addition, the presence of genes encoding proteins involved in the acquisition of resistance, such as protein kinases, efflux pumps and major facilitator superfamilies have also been reported [17]. Thus, considering the still increasing problem of *C. auris* drug resistance, the antifungal properties of the EOs can be considered a promising alternative.

Regarding the antifungal activity of EOs against planktonic *C. auris* cells, the MIC₅₀ and MFC of white thyme, tea tree, cajeput and niaouli EOs were determined. A lower MIC₅₀ was observed for tea tree EO (0.78 % (v/v)), in comparison to the other EOs tested, with a MIC₅₀ value of 1.56 % (v/v). Similarly, the MFC recorded for the tea tree EO was lower (1.56 % (w/v)) than for white thyme, cajeput and niaouli EOs (3.12 % (v/v)). The four EOs tested showed inhibitory and fungicidal properties at low concentrations. Tran *et al.* [11], one of the few studies that presents the MIC and MFC values of EOs in *C. auris*, also

verified very low MIC and MFC values for cinnamon EOs of <0.03-0.13 % (v/ v) and 0.25 % (v/ v), respectively.

Table 2. Identification of the component of tea tree, cajeput, niaouli and white thyme essential oils expressed as a percentage (%) of individual compound in the total essential oil sample

Tea tree oil		Cajeput oil		Niaouli oil		White thyme oil	
Compounds	(%)	Compounds	(%)	Compounds	(%)	Compounds	(%)
Terpinen-4-ol	42.20	Cineole	64.83	p-Cymene	57.14	Borneol	31.86
γ -terpinene	21.38	α -terpineol	11.19	+Limonene+1,8-cineole	12.00	α -terpineol	15.95
α -terpinene	10.19	Linalool	3.21	α -terpineol	8.55	Carvacrol	8.63
Terpinolene	3.46	Myrcene	1.81	Viridiflorol	7.29	Camphene	7.26
α -terpineol	3.25	α -pinene	1.71	β -pinene	3.46	trans- β -Caryophyllene	6.62
1,8-cineole	3.03	γ -terpinene	1.71	β -Caryophyllene	1.22	Bornyl acetate + Thymol	4.54
α -pinene	2.52	Terpinolene	1.31	Ledene	1.19	α -pinene	4.12
p-Cymene	1.49	β -selinene	1.28	γ -terpinene	1.04	Linalool	2.92
Limonene	1.08	Sabinene	1.23	Terpinen-4-ol	1.01	p-Cymene	2.14
Aromadendrene	1.00	α -caryophyllene	0.92	Nerolidol	0.73	Terpinen-4-ol	1.85
Bicyclogermacrene	0.85	Terpinen-4-ol	0.91	Ledol	0.73	γ -terpinene	1.83
Ledene	0.82	α -selinene	0.89	β -myrcene	0.56	Camphor	1.27
δ -cadinene	0.78	Caryophyllene oxide	0.89	Alloaromadendrene	0.38	Limonene	1.07
Myrcene	0.72	β -eudesmol	0.57	Terpinolene	0.34	Thymol methyl ether	0.82
α -thujene	0.71	α -eudesmol	0.55	α -terpinene	0.26	β -pinene	0.78
β -phellandrene	0.68	α -terpinene	0.54	Linalool	0.24	1,8-cineole	0.73
β -pinene	0.66	p-Cymene	0.43	Benzaldehyde	0.22	α -terpinene	0.49
Sabinene	0.47	Germacrene D	0.33	α -Caryophyllene	0.20	y-cadinene	0.47
α -phellandrene	0.37	α -phellandrene	0.26	β -selinene	0.20	δ -cadinene	0.44
Linalool	0.36	Borneol	0.25	y-cadinene	0.18	Terpinolene	0.41
Alloaromadendrene	0.32	Geranyl acetate	0.25	Camphene	0.18	Caryophyllene oxide	0.36
Globulol	0.29	α -thujene	0.20	δ -cadinene	0.16	Myrcene	0.35
α -gurgujene	0.25	Benzaldehyde	0.16	Isopulegol	0.13	Tricyclene	0.33
trans- β -Caryophyllene	0.25	10-epi- γ -eudesmol	0.10	α -phellandrene	0.11	α -thujene	0.25
Viridiflorol	0.24	α -Copaene	0.06	α -thujene	0.11	Ethanol	0.17
α -Copaene	0.10			Citronellol	0.07		
				Alcool fenchylique	0.07		
				Borneol	0.06		
				Patchoulene	0.06		
				Methyl benzoate	0.05		
				trans- β -ocimene	0.04		

The antifungal activity of EOs (white thyme, tea tree, cajeput and niaouli) against *C. auris* was also evaluated using the agar disc diffusion method and the results are summarized in Figure 10. As a negative control, 70 % ethanol (v/ v) was used, resulting in a 9 mm halo. Regarding the effect of EOs, both white thyme (21.4 ± 0.5 mm) and tea tree (20.0 ± 1.9 mm) oils demonstrated stronger antifungal activity against the tested strains when compared with cajeput (13.8 ± 1.1 mm) and niaouli (13.3 ± 1.1 mm)

oils. Furthermore, despite similar diameters between the tea tree and white thyme oil it, there was a marked decrease in the *C. auris* biomass all over the petri dish with the application of white thyme EOs (Figure 10).

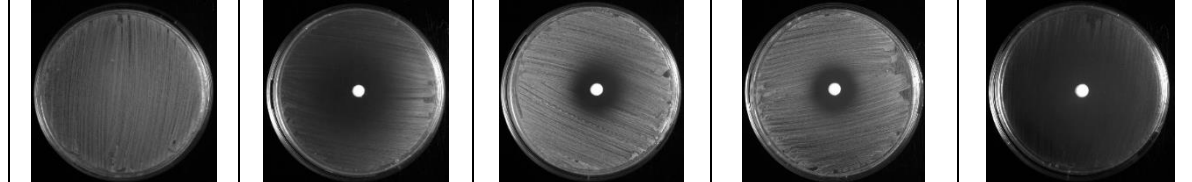
	Essential Oil Inhibition zone (mm)			
	Tea tree	Cajeput	Niaouli	White thyme
<i>C. auris</i> NCPF 8971	20.0 ± 1.9****	13.8 ± 1.1****	13.3 ± 1.1****	21.4 ± 0.5****
				

Figure 10. Antifungal activity of essential oils on *Candida auris* NCPF 8971 evaluated using the disk-diffusion assay.

**** $p < 0.0001$ indicates a statistically different reduction in comparison with the control (Tukey's multiple comparison test).

These results are in line with several reports of high antifungal activity of thyme and tea tree oils against both susceptible and drug-resistant strains of various *Candida* species [18,19]. Indeed, EOs and their constituents have been used against a wide range of fungal pathogens since they have the ability to hinder the growth and development of a diverse range of pathogens [10].

After these good preliminary results, the antifungal effect of white thyme, tea tree, cajeput and niaouli EOs in biofilm formation and 24 h-old biofilms of *C. auris* was evaluated (Figure 11). Direct application of two drops (2.4 % (v/v)) of EOs on biofilm formation (Figure 11.A) led to a total inhibition of biofilm growth. Moreover, the oils were also applied in pre-formed biofilms (Figure 11.B) and it was observed that the application of two drops (2.4 % (v/v)) of white thyme and cajeput oils induced a total eradication of the 24 h-old biofilms. In addition, tea tree and niaouli oils significantly reduced viable cells of those pre-formed biofilms ($p < 0.0001$), with reductions of 5 Log₁₀ CFU mL⁻¹ and 4 Log₁₀ CFU mL⁻¹ ($p < 0.001$), respectively.

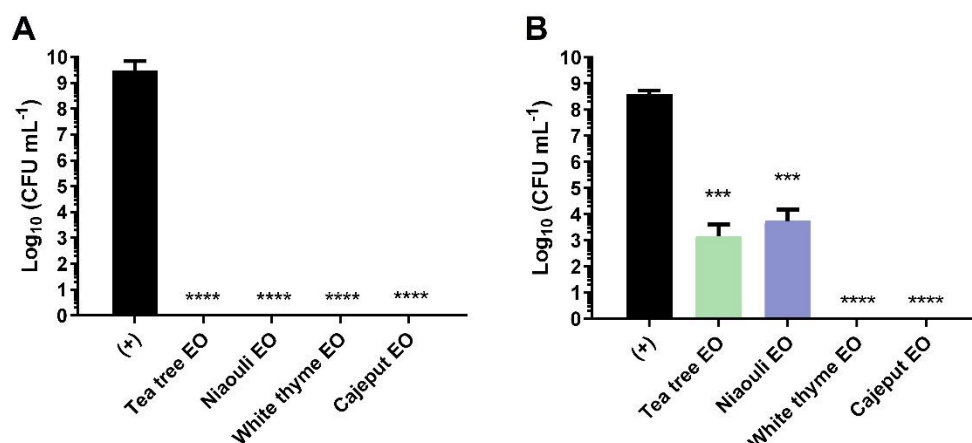


Figure 11. Antifungal effect of four essential oils (tea tree, niaouli, white thyme and cajeput) in (A) biofilm formation and (B) 24 h-old biofilms of *Candida auris* NCPF 8971 by quantification of CFUs per mL. Error bars indicate the respective standard derivation (SD). *** $p < 0.001$, **** $p < 0.0001$ indicates a statistically different reduction in comparison with the control (Tukey's multiple comparison test).

The results confirm the high ability of *C. auris* to form biofilms (Figure 11 – (+) control), as previously described by Horton *et al.* [20]. These authors demonstrated that the growth in synthetic sweat medium and in the skin of pigs allows *C. auris* to form dense biofilms that resist desiccation and thrive in conditions of evaporation. In addition, the draft genome that identifies several proteins involved in biofilm formation and recent descriptions of aggregative and non-aggregative phenotypes indicate the possibility of heterogeneous *C. auris* biofilm formation [7].

In relation to the application of EOs on biofilm formation (Figure 11.A), it was possible to verify that the application of only two drops of oil (white thyme, tea tree, cajeput or niaouli) induced to a total inhibition of biofilm growth after 24 h. Furthermore, the application of the same volume of white thyme and cajeput oils led to a total eradication of biofilms the 24 h-old (pre-formed biofilms) (Figure 11.B), showcasing their great efficacy. Besides, tea tree and niaouli oils significantly reduce viable cells of those pre-formed biofilms ($p < 0.0001$). In fact, the good anti-*Candida* activity of thyme was also verified in many reports [21]. Similarly, to the results obtained in this study, Asdadi *et al.* [21] showed that EO of *T. satureioides* (white thyme oil) was able to inhibit the growth of non-*Candida albicans* *Candida* (NCAC) species (*Candida krusei*, *Candida dubliniensis* and *C. glabrata*) resistant to conventional antifungal, such as FLC and amphotericine B.

In this study, the major compounds of the white thyme oil are borneol, α -terpineol and carvacrol (Table 2). It has been demonstrated that oxygenated terpene compounds, such as carvacrol, are often considered the main responsible for modifying membrane permeability by chemical reaction with amino

and hydroxylamine groups of membrane proteins [8]. Indeed, carvacrol has the highest antifungal activity, inhibiting the formation of hyphae and biofilms in *Candida* species [6,22]. Also, terpinen-4-ol and α -terpineol are recognized as potent compounds with a fungicidal effect [19]. Thus, the very good outcomes obtained in this work are justified by the important antifungal role of these compound.

In turn, the major compounds of the tea tree EO are terpinen-4-ol, γ -terpinene and α -terpinene (Table 2). Tea tree oil and their components increase yeast cell permeability and membrane fluidity, and become embedded in the lipid bilayer membrane, eventually disrupting its structure [23]. Besides that, tea tree oil also inhibits the formation of germ tubes or mycelial conversion and inhibits respiration in *C. albicans* [23]. In addition, Mondello *et al.* [19] suggested that terpinen-4-ol is a likely mediator of the *in vitro* and *in vivo* activity of tea tree oil and that could control *C. albicans* vaginal infection. The EO of *M. quinquenervia* (niaouli oil) was found to be effective against bacteria as well as a range of fungi, including *C. albicans* [24]. Additionally, Keereedach *et al.* [25] found that *M. cajuputi* (cajeput oil) had a potent antifungal activity against clinical isolates of *C. albicans* resistant to FLC, where it was able to reduce the MIC of FLC and reduce the expression level of *MDR1* (an important gene that plays a role in resistance to azole drugs in *C. albicans*). However, so far there are few studies regarding the effect of niaouli and cajeput oils in *Candida* species, being this the first study to use both oils in *Candida* biofilms. The major compounds of these two oils are cineole, α -terpineol and linalool in cajeput oil and *p*-cymene, limonene and 1,8-cineole in niaouli oil (Table 2). 1,8-Cineole showed some anti-*Candida* activity with a fungicidal effect [26]. In addition, linalool exerts antifungal activity by disrupting the membrane integrity and interrupting the cell cycle of planktonic *C. albicans*, inhibits germ tube formation and exhibits antifungal activity against *C. albicans* cells in biofilms. The inhibition of hyphae induction by limonene at low concentration may be responsible for the inhibition of biofilm formation. However, *p*-cymene, a thymol precursor, is generally defined as inactive [27,28].

Nevertheless, it is complicated to point the antifungal activity of a complex mixture (EOs) to specific constituents. In fact, it is believed that the antifungal activity most probably results from the combined effect of different compounds on several cellular targets [29]. In this study, the composition of the tested EOs suggests that each oil is featured by components common to the four EOs (27 %- 35 % of the total composition) and specific components (19 %- 33 %) (Table 2). So, it is reasonable to speculate that the antifungal activity of these four EOs can also be related to the presence of specific compounds, such as terpinen-4-ol, carvacrol, α -terpineol, linalool and limonene. Studies report that terpinen-4-ol, major component of the tea tree oil (42 %), have a lipophilic characteristic, probable presenting a direct action on the cell membrane structure and associated enzymes [30]. Carvacrol, present in white thyme oil (9

%), and linalool, present in the four EOs under study in a range between 0.2- 3 %, are associated with modification of membrane permeability and inhibition of hyphae and biofilm formation [8]. The antifungal activity of α - terpineol, present in the four EOs (3- 16 %), also occurs through the disruption of cell walls and cytoplasm, resulting in abnormal hyphae [31]. Limonene, constituent of the tea tree, niaouli and white thyme oils, acts on the genetic material yeast, leading to damage to the cell wall and intracellular structures, including nuclear alterations (condensation of genetic material and specific changes in mitochondria). This compound also induces dramatic structural changes in organelles, accompanied by cell wall disruption [32]. However, to better understand the antifungal effect of these EOs, the synergistic or antagonistic effect between the most abundant compounds and also those present in a smaller percentage in the mixture should be investigated [33]. Thus, more studies are needed in order to understand the interactions between components and consequently the mechanisms of action of EOs.

4. Conclusions

C. auris is recognized as a notorious nosocomial pathogen that requires urgent efforts to develop more efficient and safer alternative treatments, due to high transmissibility, incorrect use of antifungal drugs, identification challenges and consequent treatment failures. In this sense, EOs are an alternative therapy for this important pathogen. The results of this study demonstrate a high efficacy of the four EOs tested to inhibit the planktonic growth and completely inhibiting *C. auris* biofilm formation. Altogether, the data suggest that the application of these four EOs, and particularly white thyme and cajeput, as an efficient alternative both for preventing and treating infections caused by *C. auris* biofilms, it also reduces dependence on existing conventional antimicrobials.

CHAPTER II.2

COMPARING THE EFFECT OF *THYMUS* SPECIES ESSENTIAL OILS ON *CANDIDA AURIS*

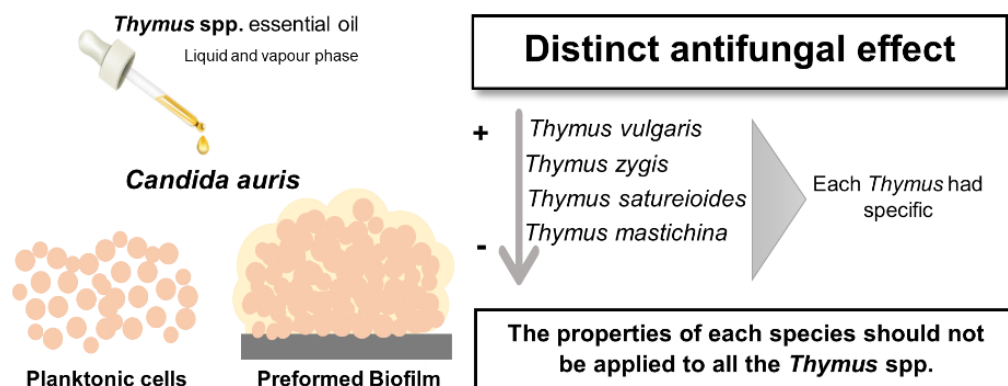


Figure 12. Subchapter II.2 workflow.

The effectiveness of essential oils can differ significantly, even when considering species within the same genus. So, the main goal of this subchapter was to compare the effect of several *Thymus* species-derived essential oils, namely *Thymus vulgaris*, *Thymus zygis*, *Thymus satureioides* and *Thymus mastichina* on both planktonic cells and biofilm of *Candida auris*, while also examining the relationship between their activities and chemical compositions.

This subchapter is based on the following publication: Ribeiro R, Fernandes L, Costa R, Cavaleiro C, Salgueiro L, Henriques M, Rodrigues M.E. Comparing the effect of *Thymus* spp. essential oils on *Candida auris*. Ind Crops Prod 2022; 178:114667. <https://doi.org/10.1016/J.INDCROP.2022.114667>.

1. Introduction

In the last decade, *Candida auris* has emerged as fungal human pathogen that causes hospital outbreaks, especially in intensive care units (ICU) [34,35]. Since its discovery in 2009, more than 30 countries have already identified this *Candida* species as a frightening pathogen [36,37]. The virulence factors of *C. auris* include surface's adhesion, thermo and salt tolerance, and the ability to develop biofilms, which are responsible to the enhancement of antifungal resistance [38–41]. The persistence of *C. auris* on several equipment and medical device's surfaces increases the risk of transmission [42]. In hospital environment, the fungal spread may occur between patients or via contact of health care workers with patients [35,43]. The ability of *C. auris* to persist on the patients' skin and its complex identification make the outbreaks more difficult to contain [34,44–46].

The conventional treatment of fungal infections consists of the administration or application of antifungal drugs. However, these agents have some disadvantages, such as lack of selectivity, toxicity and high antimicrobial resistance, mainly against azoles [47,48]. Nowadays, it is well known that some plant products, such as essential oils (EOs), are effective against fungal and bacterial infections, presenting good bioactivity and low toxicity [7]. EOs are complex mixtures of several volatile compounds produced by aromatic plants that can be used as an alternative antifungal therapy. However, their antifungal properties are difficult to associate to a specific compound [49]. Generally, the antifungal activity could be a result of the principal bioactive compounds of the EOs or due to a synergistic action between the major and the minor compounds [49]. EOs of several *Thymus* species are recognized by their antifungal activity [49,50]. Some of these oils are widely used, such as *Thymus vulgaris* and *Thymus zygis* [1]. The mechanism of action of EOs can include disruption of the wall or membrane of fungi cells, enhancing the permeability of these structures, and inhibition of the synthesis of DNA, RNA, proteins and polysaccharides [1]. Although there are several studies on the antifungal activity of *Thymus* species oils, there are still no reports of the effect of these oils against *C. auris*. Thus, this study evaluated the antifungal properties of EOs from *T. vulgaris* L., *T. zygis* subsp. *sylvestris* Loebl. ex L., *Thymus satureioides* Coss. and *Thymus mastichina* L. against *C. auris* biofilms and related the activities with their composition.

2. Materials and Methods

2.1 Microorganisms and culture conditions

C. auris NCPF 8971 was stored in broth medium with 20 % (v/ v) glycerol at -80 ± 2 °C until required, as mentioned in subchapter II.1. Before testing, cells were activated by thawing at room

temperature. Then they were subcultured into Sabouraud Dextrose Agar (SDA; Liofilchem) and incubated at 37 °C for 24 h. The inoculum was prepared on Sabouraud Dextrose Broth (SDB; Liofilchem) and was incubated at 37 °C for 18 h with agitation. After, the inoculum was centrifuged (6000 rpm, 10 min, 4 °C) and washed twice with Phosphate Buffered Saline (PBS 0.1 M; pH 7.5). The concentration was adjusted in PBS to obtain 1×10^8 cells mL⁻¹.

2.2 Essential oils

This study evaluated and compared the antifungal properties of EOs from *T. vulgaris* (florame®, France), *T. satureioides* (florame®, France), *T. mastichina* (florame®, France), and *T. zygis* subsp. *sylvestris* (*Parque Natural das Serras de Aire e Candeeiros*, Central Portugal), all with 100 % purity. All EOs samples were stored in the dark at room temperature. EO of *T. zygis* subsp. *sylvestris* was isolated by hydrodistillation during 3 h using a Clevenger-type apparatus, from flowering serial parts of the plants collected in *Parque Natural das Serras de Aire e Candeeiros* (Central Portugal). The rest of EOs in study were isolated by florame® (Saint-Rémy-de-Provence, France), through steam distillation of flower cups from Spain (origin of *T. vulgaris* and *T. mastichina*), and Morocco (origin of *T. satureioides*).

2.2.1 Chromatographic analysis of essential oils

The compositions of the EOs were determined by combination of gas chromatography with FID detectors (GC-FID) and gas chromatography-mass spectroscopy (GC/ MS) analysis. GC-FID analysis was performed in a Hewlett-Packard 6890 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) set with a single injector and two flame ionization detectors (FID). A divider (Agilent Technologies, part no. 5021-7148) was used for simultaneous sampling on two fused silica capillary columns: a SPB-1 (polydimethylsiloxane 30 m × 0.20 mm i.d., film thickness 0.20 µm) and a SupelcoWax-10 (polyethyleneglycol 30 m × 0.20 mm i.d., film thickness 0.20 µm). Oven temperature program: 70–220 °C (3 °C/ min), 220 °C (15 min); injector temperature: 250 °C; mobile phase: helium, with flow adjusted to maintain a linear velocity of 30 cm/ s; split ratio 1: 40; detectors temperature: 250 °C. For as chromatography–mass spectrometry (GC-MS) analysis was used a Hewlett-Packard 6890 gas chromatograph interfaced with a Hewlett-Packard mass selective detector MSD 5973 (Agilent Technologies). An HP1 (Agilent Technologies) fused silica column (polydimethylsiloxane 30 m × 0.25 mm i.d., film thickness 0.25 µm) was used. GC parameters as described above; interface temperature: 250 °C; MSD parameters: interface temperature: 250 °C; MS source temperature: 230 °C; MS quadrupole temperature: 150 °C; ionization energy: 70 eV; ionization current: 60 µA; scan range: 35–350 units;

scans/ s: 4.51. The compounds were identified by considering, concurrently: 1) the acquired retention indices on both SPB-1 and SupelcoWax-10 columns determined by linear interpolation relative to the retention times of C8–C23 of n-alkanes and compared with reference data from authentic products (available in the laboratory database of the Faculty of Pharmacy, University of Coimbra) and literature data (Wallace WE); 2) the acquired mass spectra compared with reference data from the laboratory database, the Wiley / NIST library [52] and literature [53]. Relative amount of each component was calculated from GC peaks areas.

2.3 Agar disk diffusion assay

The agar disk diffusion assay was performed based on European Committee on Antimicrobial Susceptibility Testing (EUCAST E.DEF 7.3.2)[54]. The *C. auris* suspension was spread onto SDA plates in three different directions. After the inoculum was dry, a sterile filter paper disk (6 mm) was impregnated with 10 μ L of each EO from *T. vulgaris*, *T. zygis*, *T. satureioides* and *T. mastichina*. One plate with cell suspension without EO was used as a positive control. The SDA culture plates were incubated at 37 °C for 24 h. The diameter of the inhibition zone was measured in mm. All experiments were performed in triplicate, in three independent assays.

2.4 Antifungal activity of essential oil from *Thymus* species

The antifungal activity of EOs from four *Thymus* species against preformed biofilms (24 h old biofilms) of *C. auris* NCPF 8971 was assessed. *C. auris* biofilms were developed according to the method described by Silva *et al.* [55], with some adaptations. The *C. auris* cell concentration, from liquid culture, was adjusted to 1×10^5 cells mL⁻¹ in SDB. Then, under aseptic conditions, 1 mL of cell suspension was transferred to glass wells inside a glass petri plates, as referred to in subchapter II.1. The biofilm culture was incubated aerobically for 24 h at 37 °C, under agitation at 120 rpm. After 24 h of incubation, medium of the biofilm culture was renewed. The effect of the EOs (100 % or 50 %, diluted in almond oil) was evaluated directly and indirectly. For this, 10 μ L of *T. vulgaris*, *T. satureioides*, *T. zygis* and *T. mastichina* EO (100 % or 50 %) were added to the glass wells in two different ways, direct application by addition in the medium (1 % or 0.5 %) and indirect application by placement, in paper discs which was placed near the wells (the set was kept inside a glass plate), respectively. Positive (biofilm without treatment) and negative (SBD medium) controls were included, as well as almond oil control. All glass petri plates were incubated for 24 h, at 37 °C and 120 rpm. All experiments were performed in triplicate, in three independent assays.

Cell viability was determined by colony-forming units (CFU). The biofilms were washed with PBS, then scraped from the wells with PBS (1 mL), serially diluted in PBS and plated onto SDA. After incubated for 24 h, at 37 °C, the number of grown colonies was counted. The results were presented as CFU per milliliter (Log_{10} (CFU mL⁻¹)).

2.5 Statistical analysis

Statistical evaluation was realized using GraphPad Software (version 6.01 for Macintosh). One-way ANOVA (analysis of variance) test was applied, and multiple comparisons of means of each treatment with EOs were done by Tukey test. The measures of central tendency and dispersion used were the mean and standard deviation, respectively. A p-value < 0.05 was considered significative.

3. Results and discussion

The main compounds of the oils are shown in Table 3 (Annex II - Table 22), where the compounds are listed by order of their elution on a polydimethylsiloxane column. Briefly, *T. vulgaris* and *T. zygis* oils are mainly composed by phenolic compounds, being thymol the main compound of *T. vulgaris* (63.1 %) and thymol (26.5 %) and carvacrol (22.7 %) the main compounds of *T. zygis* oil, whereas *T. satureioides* oil contains high amounts of borneol (29.3 %) and α -terpineol (15.9 %), and *T. mastichina* is characterized by high amounts of linalool (31.9 %).

Table 3. Chromatography analysis of the composition of essential oils from *Thymus vulgaris*, *Thymus zygis*, *Thymus satureioides* and *Thymus mastichina*, here shown in percentage (%)

RI ^a	RI ^b	Compound	Percent (%) in oil samples			
			<i>T. satureioides</i>	<i>T. mastichina</i>	<i>T. vulgaris</i>	<i>T. zygis</i>
1020	1215	1,8-cineole	2.7	8.5	10.0	7.2
1082	1543	Linalool	4.5	31.9	7.2	6.6
1145	1695	Borneol	29.3	5.8	3.4	4.9
1169	1692	α -Terpineol	15.9	10.0	0.5	1.2
1263	2183	Thymol	1.7	4.4	63.1	26.5
1277	2205	Carvacrol	7.0	0.5	5.5	22.7

Compounds listed in order of their elution on the SPB-1 column.

^aRetention index on the SPB-1 column relative to C8 - C24 *n*-alkanes.

^bRetention indices on the SupelcoWax-10 column relative to C8 - C24 *n*-alkanes.

The *in vitro* antifungal activity of EOs from *T. vulgaris*, *T. zygis*, *T. satureioides* and *T. mastichina* on *C. auris* planktonic cells was evaluated by zones of inhibition (D_{halo}) using the agar disk diffusion method. The analysis of the results obtained shows that all oils, regardless of the *Thymus* species, had inhibitory effects (Table 4).

Table 4. Diameter of the inhibition zone, in mm, of the essential oils from different plants of *Thymus* genus (10 μ L) for *Candida auris* NCPF 8971

Inhibition zone diameter, mm			
<i>Thymus vulgaris</i>	<i>Thymus zygis</i>	<i>Thymus satureioides</i>	<i>Thymus mastichina</i>
42.33 $\pm$ 3.77	28.25 $\pm$ 1.09***	20.00 $\pm$ 0.63****	13.60 $\pm$ 1.36****

P < 0.01 when compared with *T. mastichina*, and **P < 0.0001 when compared with *T. vulgaris*.

As previously demonstrated, *Thymus* species are potential antifungal agents because they have a strong action against fungal pathogenic microorganisms [48,56]. Although the EOs belong to same plant genus, their effect in *C. auris* is significantly different. *T. vulgaris* had the largest inhibition areas (42.33 $\pm$ 3.77 mm), being the tested EO with the highest activity. Some of major components of this oil are thymol (63.1 %), 1,8-cineole (10.0 %), linalool (7.2 %), and carvacrol (5.5 %) (Table 3), which could be the reason for its high antifungal activity [48]. In fact, several researchers evaluated the biological properties of *T. vulgaris* and verified that EO from this plant could inhibit fungal development [57,58]. These authors sustained that the remarkably antifungal potential was related to the high percentage of thymol that was the main active component of *T. vulgaris* EO. Thus, thymol can be the principal responsible compound for activity of *T. vulgaris* against *C. auris*. In fact, *T. zygis* was the second most potent oil and also contains a significant percentage of thymol (26.5 %), besides carvacrol (22.7 %), 1,8-cineole (7.2 %) and linalool (6.6 %). Previous investigations have claimed that antifungal activity of *T. zygis* could be related to the effect of their main compounds, thymol and p-cymene [56,59].

Even though *T. satureioides* and *T. mastichina* EOs have presented inhibition zones of 20.00 $\pm$ 0.63 mm and 13.60 $\pm$ 1.36 mm, respectively, their action against *C. auris* cells was not as significant as that of the other oils tested. The percentage of thymol of these oils (1.7 % and 4.4 %, respectively) is significantly lower compared to the *T. vulgaris* and *T. zygis* EOs (63.1 % and 26.5 %, respectively). The slight antifungal action may also be related to the high amount of the borneol, α -terpineol, and camphene in case of *T. satureioides*, and linalool, α -terpineol, and 1,8-cineole regarding *T. mastichina*. Although the activity of linalool, and other non-phenolic compounds is known [60], the interactions between the various

constituents of the EOs may increase or decrease the action of these agents, due to synergistic or antagonistic effects [61]. Faleiro *et al.* [62] studied *T. mastichina* antifungal properties and demonstrated that *Candida albicans* was not susceptible to 1,8-cineole, but the mixture of this compound plus linalool slightly increased the antifungal activity. Research carried out by Pina-Vaz *et al.* [63] validates the results obtained in this work. They investigated the effect of *Thymus* oils (*T. vulgaris*, *T. zygis* and *T. mastichina*) against *Candida* species fungal cells (*C. albicans*, *Candida glabrata*, *Candida krusei*, *Candida guilliermondii*, *Candida parapsilosis*, and *Candida tropicalis*) and found that *T. vulgaris* and *T. zygis* displayed better antifungal activity than *T. mastichina*.

Due to the positive effect found in the halo test, the activity of *T. vulgaris*, *T. zygis*, *T. saturoioides* and *T. mastichina* against preformed biofilms of *C. auris* was also assessed (Figure 13). Each EO was tested in both direct or indirect application and in different concentrations.

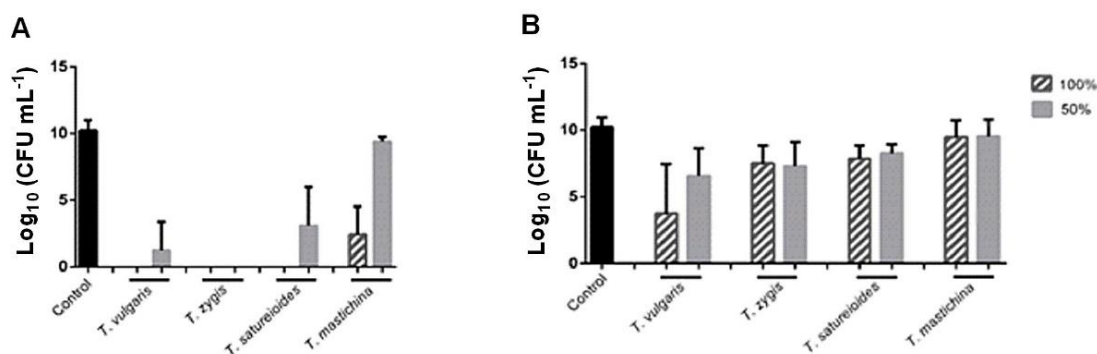


Figure 13. Assessment of the susceptibility of *Candida auris* NCPF 8971 24 h old biofilms to different essential oils from *Thymus* genus by quantification of CFUs per mL. Each essential oil was tested at 100 % and at 50 % with both (A) direct and (B) indirect application. Error bars indicate the respective standard derivation (SD). Differences in mean Log₁₀ CFU mL⁻¹ of the different treatments were compared to the control using one-way ANOVA with a post hoc Tukey test (significance at $P < 0.05$), with ** $P < 0.01$, *** $P < 0.001$ and **** $P < 0.0001$.

Overall, the direct application of *Thymus* EOs, on *C. auris* biofilms, had a higher effect than the indirect application, with statistically significant differences ($p < 0.001$) (except with 50 % of concentration of *T. mastichina*, not showing antifungal activity either when applied directly or indirectly). The antifungal effect of EOs at 100 % of concentration was equal or superior to action of EOs at 50 % of concentration, as expected.

The evaluation of indirect application had as main purpose to study the potential antifungal effect of vapor-phase of EOs (VP-EOs) from *Thymus* species, but it was noticed that *C. auris* biofilms were less

susceptible to VP-EOs than when EOs were applied directly. Differently, this research has demonstrated that VP-EOs possesses greater antifungal activity than their liquid phase [64,65]. According to Boukhatem *et al.* [65] that analysed the activity of EOs in liquid and vapor-phase, the vapor-phase of *T. vulgaris* (phenotype carvacrol) had greater action against planktonic cells of *Candida* species, namely *C. albicans*, *C. tropicalis*, and *C. parapsilosis*, than liquid phase. A possible explanation for the divergence between results can be related with testing conditions. In this study, the EOs were tested on biofilms that could be less susceptible to VP-EOs, contrasting with high susceptibility of the planktonic cells. Beside this, the *Candida* strain and the chemotype of *T. vulgaris* tested, in this study, are different from those previously studied by Boukhatem *et al.* [65]. All these factors can change the effectivity of antifungal activity of both liquid and VP-EOs [60].

Preformed biofilms of *C. auris* demonstrated higher susceptibility to direct application of *T. vulgaris* and *T. zygis* EOs than *T. satureioides* and *T. mastichina* for both concentrations tested (Figure 13). The constituents of EOs have different levels of lipophilicity according to the amount of polar functional groups in their constitution. EOs, due to their lipophilic character, can interact with lipid structures of the fungal cell membrane. This allows an easy penetration of EOs compound, modifying the cell membrane properties, such as permeability and fluidity. As result, fungal cells suffer structural damage that can result in cell death [66]. When added to a liquid medium, the distribution of the EO across medium depends on its hydrophilicity. Hence, the EOs with compounds with low solubility should have little distribution in aqueous medium and, therefore, should display poor activity on biofilms, which have an aqueous extracellular matrix. Thus, physical and chemical properties of the EOs, such as volatility and solubility, can be an important role on their activity [64]. Considering these properties, since linalool possesses low water solubility contrasting with thymol and carvacrol that have moderate solubility, the EOs with high amount of these last compounds, namely *T. vulgaris* and *T. zygis* EOs, presented great antifungal activity when applied directly, as observed in outcomes obtained [64,65]. The intrinsic activity of the constituents of EOs is also a factor to considerate in the evaluation of its efficacy. In this sense, the EOs with high amount of phenol monoterpenes or aldehyde are known to have high inhibitory effect on fungal cells, including in vapor-phase, followed by alcohol, ketone and ether [64]. In this context, the results obtained in this study (Figure 13.A) are in accordance with the expected, as *Thymus* EOs with major percentage of thymol and carvacrol (*T. vulgaris* and *T. zygis* EOs, respectively) demonstrated greater antifungal activity in both applications.

Among the tested *Thymus* oils, *T. vulgaris* was the one that showed significant action in the vapor-phase against *C. auris* biofilms at both concentrations (Figure 13.B). Comparing the percentage of these

compounds in each *Thymus*, approximately 70 % of *T. vulgaris* EO are phenolic compounds, namely thymol (63.1 %) and carvacrol (5.5 %). This rate is relatively superior to percentages verified for other *Thymus* tested, justifying the significant differences obtained between the activity of vapor-phase of *T. vulgaris* EO and the other EOs on *C. auris* biofilms. However, also the minor compounds of EOs may change, positive or negatively, the antifungal action of the main compounds due to synergistic and/or antagonistic effect resultant of their interactions [49].

According to the outcomes obtained, there are significant differences in antifungal activity of *Thymus* EOs on planktonic and biofilm cells of *C. auris* NCPF 8971, ranging considerably between species and according with their chemical profile. In this sense, the antifungal activity of *Thymus* EOs should not be widespread, and it is crucial specify the species as well as the main compounds of their EOs before attribute the antifungal potential.

4. Conclusions

EOs may be of future relevance to the treatment of multi-drug resistant fungi, including *Candida* infections. In this study, the antifungal effect of the EOs from *Thymus* species against planktonic and biofilms cells of *C. auris* was confirmed. However, the antifungal activity differs between the plant species, according to the chemical profile of their essential oils. Thus, the properties of each species should not be expended to all the *Thymus* species and the specification of species and chemotype is crucial. Among the *Thymus* species tested, *T. vulgaris* and *T. mastichina* had the highest and the slightest antifungal activity, respectively.

CHAPTER II.3

VAPOR-PHASE OF ESSENTIAL OILS AS A PROMISING SOLUTION TO PREVENT *CANDIDA* VAGINAL BIOFILMS CAUSED BY ANTIFUNGAL RESISTANT STRAINS

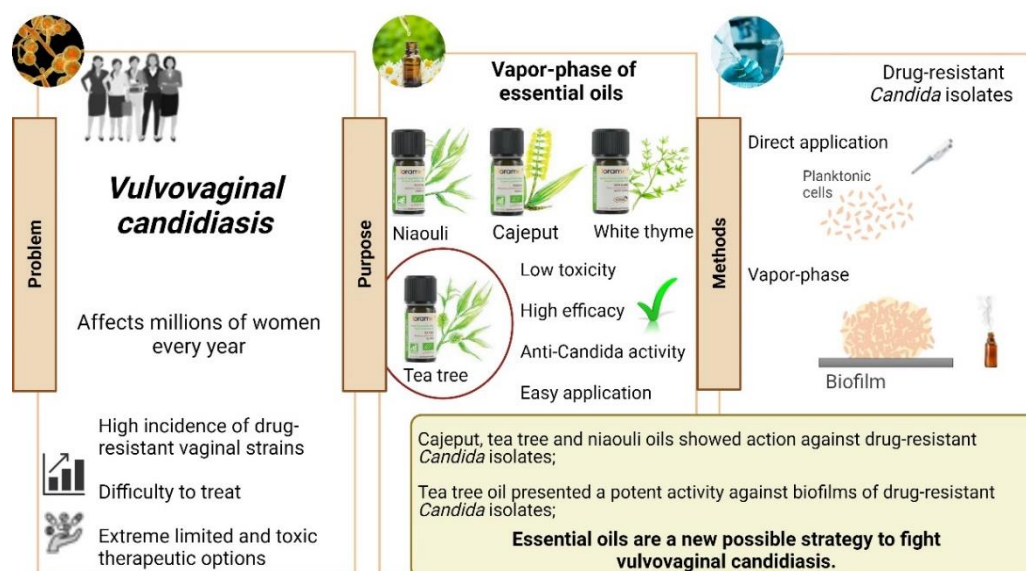


Figure 14. Subchapter II.3 workflow.

Vulvovaginal candidiasis is a high-incidence disease that represents a huge treatment challenge. So, the main goal of this subchapter was to investigate the antifungal potential of several essential oils, namely tea tree, niaouli, white thyme, and cajeput, against drug-resistant *Candida* vaginal isolates, including *Candida albicans*, *Candida glabrata*, *Candida krusei* (*Pichia kudriavzevii*) and *Candida parapsilosis*.

This subchapter is based on the following publication: Fernandes L, Gonçalves B, Costa R, Fernandes Â, Gomes A, Nogueira-Silva C, Silva S, Rodrigues ME, Henriques M. Vapor-Phase of Essential Oils as a Promising Solution to Prevent *Candida* Vaginal Biofilms Caused by Antifungal Resistant Strains. Healthcare 2022, Vol 10, Page 1649 2022;10:1649. <https://doi.org/10.3390/HEALTHCARE10091649>.

1. Introduction

Vulvovaginal candidiasis (VVC), an extremely common clinical condition that affects millions of women every year around the world, is an infection of the female reproductive tract mucosa with vulvar and/or intravaginal changes that consequently causes physical and mental suffering, involving considerable direct and indirect costs [67]. Furthermore, complicated episodes of VVC have been suggested to enhance the risk for acquisition of HIV and other complications including pelvic inflammatory disease, infertility, ectopic pregnancy, abortion and menstrual disorders [68]. During episodes of VVC the innate immune responses may differ, this fact suggests that the host environment performs a key role in the host-pathogen interaction [69]. *Candida* organisms are capable of colonizing the vaginal environment asymptomatically however, several factors may contribute to the development of infection, including pregnancy, immunosuppression (HIV/AIDS or diabetes mellitus), sexual activity, hormonal fluctuations, smoking, obesity and use of sodium glucose cotransporter 2 (SGLT2) inhibitors, broad spectrum antibiotics or oral contraceptives [70–73]. Furthermore, the vaginal pathogenicity of *Candida* organisms is facilitated by several virulence factors including the biofilm formation on vaginal walls and intrauterine devices (IUDs) [74]. Biofilms show high resilience to common antifungal agents and host immune responses, requiring long and intensive therapies and, in more severe cases, removal of the infected device in order to avoid relapses [75,76]. Of note, the increase of at-risk individuals and the widespread use of over-the-counter antifungals have been suggested to contribute to the increase/selection of drug-resistant strains [77,78]. Although *Candida albicans* is still the species most frequently causing CVV, there is growing concern about the incidence of non-*Candida albicans Candida* (NCAC) species. Among them, *Candida glabrata* or *Candida krusei*, which have shown low intrinsic susceptibility to common antifungals and, in addition, the ability to acquire resistance after exposure to the agent [70,79].

So, the increasing incidence of drug-resistant vaginal strains, the high difficulty to treat biofilm-related VVC and the extreme limited and toxic therapeutic options for this disease making crucial the development of more efficient and safer strategies to control it. In this sense therapies developed from natural products have emerged [80,81]. Honey, saponins, polyphenols (green tea), garlic extract, essential oils (EOs), usnic acid and peptides have shown to exert antimicrobial activity, both *in vivo* and *in vitro*, and therefore EOs became the most promising [82–84]. EOs can be a valuable alternative, since they present low toxicity and a wide range of proprieties, including anti-*Candida* activity [8,85–88] [Subchapter II.1 and II.2]. Nevertheless, EOs were shown to inhibit the growth of beneficial vaginal bacteria at high concentrations and may cause skin irritation, limiting their potential use in VVC [9]. Interestingly, EOs show vapor-phase (VP) antimicrobial activity, which has been shown to present higher

activity towards *Candida* growth that the liquid-phase and that would avoid direct contact of the EO with the skin [89]. Currently, although several approaches to the study of planktonic status have been reported, no standard assay has been established to evaluate microbial inhibition/ inactivation by EOs in the VP. Nevertheless, appropriate management of EOs during their use as an antimicrobial agent is very important, the system used, the environment and the package can interact [90].

So, this study investigated the ability of the VP of EOs (VP-EOs) to inhibit the biofilm formation and to destroy mature biofilms of antifungal-resistant vaginal isolates of *Candida* species, methodology performed on glassware, only, to avoid interferences. Therefore, the main aim is to contribute with the development of new, natural and effective strategies able to fight or prevent VVC biofilms.

2. Materials and Methods

2.1 Essential oils

This subchapter evaluated the antifungal activity of four EOs, the same ones examined in subchapter II.1, namely, tea tree (*Melaleuca alternifolia*; florame®, Portugal); niaouli (*Melaleuca quinquenervia*; florame®, Portugal); white thyme (*Thymus satureioides*; florame®, Portugal) and cajeput (*Melaleuca cajaputii*; florame®, Portugal) EOs (all with 100 % purity).

Analysis of the EOs was carried out by florame® (Saint-Rémy-de-Provence, France), using a Gas Chromatography (GC) with Flame-Ionization Detection (FID) (Annex I.1-4). The major compounds of the EOs are terpinen-4-ol (43 %), γ -terpinene (22 %) and α -terpinene (10 %) in tea tree EO, p-cymene, limonene and 1,8-cineole (57 %) in niaouli EO, α -terpineol, borneol (47 %) and carvacrol (9 %) in white thyme EO and 1,8-cineole (60 %), α -terpineol (12 %) and limonene (5 %) in cajeput EO. All EOs samples used in this study were stored in the dark, protected by aluminum foil, at room temperature.

2.1.1 Cytotoxicity of vapor-phase of essential oils

The effect of VP-EOs (tea tree, niaouli, white thyme and cajeput) on cell viability was evaluated by MTS (3-[4,5-carboxymethoxyphenyl]-2-(4-sulfophenyl)-2H-tetrazolium) test. For this, cells from human primary fibroblast cell lines (3T3-CCL 163-from the American Type Culture Collection) were first cultured in Dulbecco's modified eagle medium (DMEM) (Biochrom, Berlin, Germany) with 1 % streptomycin/penicillin-containing antibiotic (Biochrom, Berlin, Germany) and 10 % fetal bovine serum (FBS; Sigma-Aldrich) at 37 °C and 5 % CO₂ for 24 h. Then, the cells were trypsinized and seeded in glass wells (200 μ L per well) (Subchapter II.1) at the final concentration of 1×10^5 cells mL⁻¹, under the same

cultivation conditions described above. After 24 h of incubation, the cell cultures were treated with VP-EOs, 25 µL of each EOs (100 %) were discarded in a sterile white disk that was placed next to the wells, the set was kept inside a plate of glass for another 24 h at 37 °C and 5 % CO₂. After this time, the wells were washed twice with phosphate-buffered saline (PBS 1x) and then 80 µL of MTS (CellTiter 96 Aquoous One Solution Cell Proliferation Assay, Promega) with 1 % phenol-free DMEM was added to each glass well for 1 h. After this time, the absorbance (OD 490 nm) was measured in a microplate reader (HEALES “MB580”, Shenzhen, China). The VP-EOs cytotoxicity results were expressed as the percentage of viable cells in relation to the OD 490 nm of cells cultured without VP-EOs (100 % cell viability). These experiments were performed twice, and each analysis was performed in duplicate.

2.2 Microorganisms and initial culture conditions

For this study, *C. albicans* (n=3), *C. glabrata* (n=8), *C. krusei* (n=1) and *C. guilliermondii* (n=1) vaginal isolates belonging to a collection of yeasts created by the *Candida* Research Group of Centre of Biological Engineering of University of Minho, in the scope of a large-scale recovery of vaginal specimens of *Candida*, carried out in Health Centers and University Campus of North of Portugal, were used [73]. The identity of all isolates was obtained by PCR-based sequencing using specific primers (ITS1 and ITS4) against the 5.8S subunit gene [73,91]. Minimum inhibitory concentration (MIC) for each species was determined by the Epsilon-meter-test (E-test) methodology and according to the guidelines provided by the manufacturer. Fernandes *et al.* [73] also tested reference strains with MICs characterized by the CLSI19 microdilution method to ensure the reliability of the E-test results. In addition, a potential interpretation of MICs based on CLSI M6022 was also performed [73]. All the isolates used are at least resistant to one of the antifungal agents (fluconazole, ketoconazole or caspofungin) [92,93]. Table 5 presents the MIC of each antifungal and also the features reported by women at the moment of vaginal sample collection, including symptoms of vaginal infection, previous vaginal infections, use of over-the-counter antifungals and relevant health conditions. This study followed the Data Protection Legislation and was approved by the Portuguese Health Ethical Commissions (SECVS-UM 092/2017, CES-S Norte 49/218, CESH 151/2018, CES-USLAM 23/2018, CESH SOG 5/2018) [73].

Candida isolates were kept at -80 ± 2 °C in Sabouraud Dextrose Broth medium (SDB; Liofilchem) with 20 % (v/v) glycerol (Biochem). Prior to each assay, the isolates were subcultured on Sabouraud Dextrose Agar (SDA; Liofilchem) plates and incubated at 37 °C for 24 h. Then, 3-5 colonies of *Candida* isolates were inoculated onto SDB for 18 h at 37 °C under agitation (120 rev/ min). After that, centrifuged at 5000 g for 10 min at 4 °C and washed twice with PBS. The supernatants were discarded and the

pellets were suspended in SDB. For the subsequent analyses, the cellular density of the pre-inocula was adjusted to 1×10^8 or 1×10^5 cells mL⁻¹, depending on the intended analysis, using a Neubauer haemocytometer (Marienfeld, Lauda-Königshofen, Germany).

Table 5. List of vaginal isolates used in this study and respective data, including species, features of the women at the moment of sample collection and minimum inhibitory concentration (MIC) of fluconazole, ketoconazole and caspofungin for each isolate

Species	Isolate	Women's features			Relevant conditions	MIC ($\mu\text{g mL}^{-1}$)		
		Symptoms of infection	Previous infections	Use of over-the-counter antifungals		Fluconazole	Ketoconazole	Caspofungin
<i>C. glabrata</i>	Cg1		Yes	Yes		96 (R)	2 (R)	0.25 (I)
	Cg2		Yes	Yes		64 (R)	2 (R)	0.19 (I)
	Cg3		Yes		Diabetes	16 (SDD)	1.5 (R)	0.19 (I)
	Cg4		Yes			4 (SDD)	0.032 (S)	0.5 (R)
	Cg5		Yes	Yes		8 (SDD)	1.5 (R)	0.064 (S)
	Cg6	Yes	Yes			>256 (R)	1.5 (R)	0.032 (S)
	Cg7		Yes		Cancer	16 (SDD)	1.5 (R)	0.064 (S)
	Cg8	Yes	Yes		IUD	>256 (R)	6 (R)	0.047 (S)
<i>C. albicans</i>	Ca1	Yes	Yes	Yes		1 (S)	0.032 (S)	1 (R)
	Ca2	Yes	Yes		Pregnancy	64 (R)	32 (R)	0.064 (S)
	Ca3	Yes	Yes		Auto-immune disease	>256 (R)	0.38 (S)	0.19 (S)
<i>C. krusei</i>	Ck1	Yes	Yes			64 (R)	3(R)	0.38 (I)
<i>C. guilliermondii</i>	Cgi1					8	0.023	32 (R)

2.3 Evaluation of the antifungal activity of essential oils

2.3.1 Growth inhibition analysis

The inhibitory activity of the EOs selected in point 2.1.1 (tea tree, niaouli and cajepout) on the growth of the drug-resistant *Candida* isolates was evaluated using the disk-diffusion agar method [94]. Briefly, SDA plates were inoculated by using a swab dipped in cell suspensions (pre-inocula) adjusted 1×10^8 cells mL⁻¹. Then, 25 μ L of each EO (100 %) were discarded on sterile blank disks (Liofilchem®) and placed atop of the plates (disks without EO were also included as control). The SDA plates were incubated for 24 h (at 37 °C) and then, the inhibition zones induced by the EOs were measured (mm).

2.3.2 Effect of the vapor-phase of essential oils on biofilms

The effect of the VP-EOs on biofilm formation and on mature biofilms (24 h-old) of one strain of each *Candida* species (*C. glabrata*, *C. albicans*, *C. krusei* and *C. guilliermondii*) was evaluated. The strains and the EO for this assay were selected based on the results of the evaluation of the antimicrobial activity of the EOs in point 2.3.1. Biofilms were developed as described by Stepanović *et al.* [15], with some modifications due to the use of volatile compounds. In order to determine the effect of Tea Tree VP-EO (VP-TTEO) on biofilm formation, *Candida* cellular suspensions adjusted to 1×10^5 cells mL⁻¹ were transferred to glass wells (1 mL per well) and 25 μ L of tea tree EO (100 %) was discarded on a sterile blank disk which was placed near the wells (the set was kept inside a glass plate, as demonstrated in subchapter II.1). Plates were incubated for 24 h at 37 °C under agitation in an orbital shaker (120 rev min⁻¹). Additionally, biofilms were pre-formed during 24 h and, after this time, incubated in the presence of 25 μ L of tea tree EO discarded on a sterile blank disk, for additional 24 h. As control, biofilms were formed without any contact with the VP-TTEO during 24 h and 48 h.

Biofilms were analyzed by the following parameters: determination of the *Candida* cultivable cells number through the colony-forming units (CFU) counting methodology, quantification of biofilm biomass by staining with crystal violet (CV), determination of metabolic activity by XTT reduction assay and evaluation of biofilm cell morphology by scanning electron microscope (SEM) [95,55].

2.3.2.1 Quantification of *Candida* biofilm cultivable cells

The number of cultivable cells in the biofilms was estimated using the CFU counting methodology. Briefly, the biofilms were washed with PBS, to remove non-adherent cells, and then scraped from the wells with 1 mL of PBS. The suspensions obtained were serially diluted to 10^8 in PBS and then plated on

SDA. SDA plates were incubated (24 h at 37 °C) and the number of colonies grown was counted and translated into CFU per milliliter (Log (CFU mL⁻¹)) [95].

2.3.2.2 Quantification of *Candida* biofilm biomass

To quantify the biomass resulting from the EO assay, the biofilms treated with VP-TTEO and the respective controls were fixed with 1 mL of methanol, which was removed after 15 min and dried at room temperature. After, 1 mL of CV (1 %) were added to each well and incubated for 5 min. The glass wells were then gently washed with sterile, ultra-pure water and 1 mL of acetic acid (33 %) added to release and dissolve the stain. Thus, 200 µL of the solution obtained from each glass well was immediately transferred to a microtiter plate and the absorbance of each condition was read at 570 nm in triplicate, on a microtiter plate reader (Thermo Scientific™ Multiskan™ FC, Finland)[55].

2.3.2.3 Quantification of metabolic activity of *Candida* biofilms cells

An XTT reduction assay was used to determine the biofilm metabolic activity after contact with VP-TTEO. So, the culture medium was aspirated after 24 h or 48 h, and non-adherent cells were removed by washing with PBS. Then, 200 µL of a solution containing 100 µg µL⁻¹ of XTT (2,3-(2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium ydroxide) (Sigma–Aldrich, USA) and 10 µg µL⁻¹ of phenazine methosulphate (PMS) (Sigma–Aldrich, USA) was added to each well and incubated at 37 °C (120 rev min⁻¹) for 3 h in the dark, protected by aluminum foil. Thus, 150 µL were transferred from each glass well to a microtiter plate and the colorimetric changes were measured at 490 nm using a microtiter plate reader (HEALES “MB580”, Shenzhen, China) [95,96].

2.3.2.4 Scanning electron microscopy (SEM)

To examine the morphology of the biofilm cells after contact with VP-EOs, the biofilms treated with VP-TTEO and the respective controls were analyzed by SEM. For this, biofilms formed on glass coupons, under the same conditions and as described above, for 24 h were dehydrated with ethanol (using 70 % ethanol for 10 min, 95 % ethanol for 10 min and 100 % ethanol for 20 min). The samples were kept in a desiccator for at least 48 h. Prior to observation, coupons were sputtered with gold and observed with Phenom Desktop SEM [95].

2.4 Statistical analysis

The Prism software package (GraphPad Software version 6.01) was used to perform the statistical analysis of the results obtained in this study. For that, the cell cultivability of biofilms treated with VP-TTEO was compared with that of untreated biofilms using one-way ANOVA (analysis of variance) and Tukey's multiple comparisons test (confidence level of 95 % and statistical significance was assumed at $p < 0.05$). For all assays, three independent experiments were carried out (independent pre-inocula) and each analysis was performed in duplicate.

3. Results and Discussion

This study evaluated the antifungal activity of EOs and of their VP against antifungal-resistant vaginal isolates of various *Candida* species. Approximately 50 % of the isolates were collected from women with symptoms of vaginal infection (with VVC) and the remaining from asymptomatic women (colonized with *Candida* organisms) (Table 5). Importantly, almost all women reported one or more vaginal infections prior to the collection of the drug-resistant strains, suggesting that previous antifungal treatments may have contributed to the acquisition of resistance [77,78]. Additionally, the use of over-the-counter antifungals to treat self-diagnosed VVC, which was reported by 30 % of the women of this study (Table 5), can also be suggested to contribute to the selection of low-susceptible strains [93,97]. Moreover, some women reported IUD use, pregnancy, or immunocompromised conditions (Table 5), so these women are at greater risk of developing VVC and, consequently, are more likely to fail treatment [98,99]. Of note, that all the clinical vaginal isolates used during this study are at least resistant to one of antifungal agents (fluconazole, ketoconazole and caspofungin) (Table 5). Indeed, the extremely limited options to treat VVC caused by drug-resistant strains make the development of new strategies, to meet the drug-resistance challenge, crucial [100]. EOs appear as a potential alternative, presenting low toxicity and the main advantage of natural antimicrobial agents of avoiding the development of antifungal resistance [101–103].

The cytotoxic effect of the tea tree, niaouli, cajeput and white thyme VP-EOs was determined against the 3T3 cell line (Figure 15). Viability of the unexposed to VP-EOs cell cultures (control) was set at 100 % to compare with the responses of the cell cultures exposed to VP-EOs. In cultures exposed to VP-EOs of tea tree, cajeput and niaouli, the viability was superior to 70 %. However, the viability of cultures incubated with VP of white thyme EOs was inferior to 70 %. So, the results (Figure 15) show that VP of tea tree,

cajeput and niaouli EOs were not cytotoxic, since the relative cell viability is lower than 70 % of the control (no EOs), based on ISO 10993-5:2009 [104].

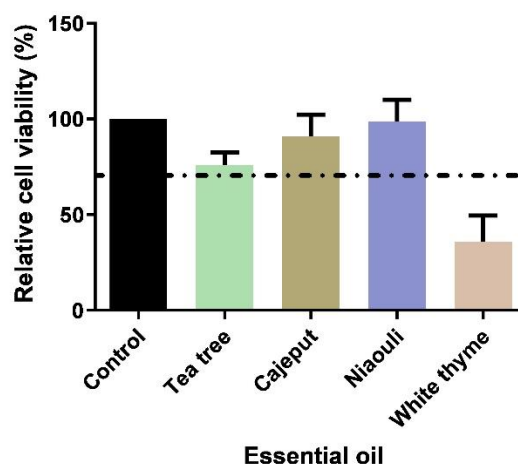


Figure 15. Vapor-phase of essential oils (VP-EOs) cytotoxicity, expressed as the percentage of viable cells in relation to the absorbance values (OD 490 nm) of cells cultured without VP-EOs (100 % cell viability). The dashed line stands for the normative limit of 70 % metabolic activity (ISO 10993-5:2009).

An initial screening of the non-cytotoxic EOs in test was performed, for this the antifungal activity of tea tree, cajeput and niaouli EOs against antifungal-resistant isolates of *C. albicans*, *C. glabrata*, *C. guilliermondii* and *C. krusei* was evaluated, using the disk-diffusion agar method and the results are summarized in Table 6. The results revealed that all EOs were able to inhibit the growth of the tested isolates, although with different impacts (Table 6). Tea tree EO had the greater inhibitory effect (20- 39 mm of inhibition zone), followed by cajeput (10- 21 mm) and niaouli (10- 17 mm) EOs (Table 6). Among the isolates, the highest inhibition was found in *C. glabrata* Cg1 and the lowest in *C. krusei* Ck1 (Table 6).

Previous studies have also reported high antifungal activity of tea tree EOs against susceptible and drug-resistant strains of various *Candida* species, including *C. albicans*, *C. glabrata* and *C. krusei* [18,105,106]. In fact, tea tree EO was found to increase yeast cell permeability and membrane fluidity and to inhibit the acidification of the medium [107]. Important, the antifungal activity of EOs has been shown to be influenced by several factors such as, original species, geographic and climatic conditions, biological and physical-chemical properties of the soil and storage conditions [108]. As such, an adequate control of these factors may allow the maximization of the antifungal activity of EOs.

Table 6. Anti-*Candida* activity of essential oils on drug-resistant isolates evaluated through the disk-diffusion method

Species	Isolate	Essential Oil Inhibition zone (mm)		
		Tea tree	Cajeput	Niaouli
<i>C. glabrata</i>	Cg1	39.0 ± 1.7	21.0 ± 2.6	13.0 ± 2.0
	Cg2	29.7 ± 0.6	18.8 ± 1.5	12.7 ± 1.2
	Cg3	28.0 ± 0.0	10.3 ± 0.5	10.4 ± 0.9
	Cg4	34.0 ± 1.0	14.8 ± 0.5	12.8 ± 0.5
	Cg5	26.3 ± 4.9	19.3 ± 3.8	16.0 ± 1.0
	Cg6	32.8 ± 3.2	19.0 ± 1.7	15.8 ± 2.2
	Cg7	20.5 ± 1.0	10.5 ± 1.0	10.5 ± 1.0
	Cg8	20.0 ± 0.8	10.3 ± 0.5	10.0 ± 0.0
<i>C. albicans</i>	Ca1	22.5 ± 2.4	25.5 ± 3.5	10.8 ± 1.0
	Ca2	24.8 ± 2.4	21.5 ± 1.3	12.8 ± 1.3
	Ca3	23.8 ± 0.5	18.7 ± 1.5	10.8 ± 0.5
<i>C. krusei</i>	Ck1	21.8 ± 2.1	12.3 ± 0.6	12.0 ± 1.3
<i>C. guilliermondii</i>	Cgi1	28.0 ± 8.9	20.3 ± 2.5	17.3 ± 2.9

One of the most important virulence factors of *Candida* species is their ability to form biofilms, which promote the development of VVC and make its treatment extremely difficult and often ineffective [105]. Therefore, there is an urgent need for more effective, low-toxic and not expensive solutions to treat biofilm-related VVC. EOs have been suggested as a promising solution due their ability to inhibit the biofilm formation and reduce pre-formed biofilms of various *Candida* species, including *C. albicans*, *C. glabrata*, *C. parapsilosis* and *C. krusei* [106]. Furthermore, EOs present high volatility and their VP was shown to possess higher antimicrobial activity than the liquid phase [89,107]. Inouye *et al.* [108] suggested that EOs in their aqueous state, lipophilic molecules associate to form micelles and thus suppress their binding to organisms, while those in the vapor-phase allow free binding. As such, this study investigated, for the first time, the effect of the VP-EO on *Candida* biofilm formation and on pre-formed biofilms of antifungal-resistant strains (*C. albicans* Ca2, *C. guilliermondii* Cgi1, *C. glabrata* Cg7 and *C. krusei* Ck1). Importantly, *C. albicans* Ca2 and *C. krusei* Ck1 present an extremely high resistance to fluconazole, the most prescribed antifungal agent, *C. glabrata* Cg7 is resistant to ketoconazole, a less common azole, and *C. guilliermondii* Cgi1 present resistance to caspofungin, a highly toxic antifungal (Table 5). Tea tree EO was selected for the assays with biofilms, due their high antifungal activity against these drug-resistant isolates (Table 6).

In order to study the effect of VP-TTEO on the biofilm formation (Figure 16) and pre-formed biofilms (Figure 17) of antifungal-resistant *Candida* isolates (*C. albicans* Ca2, *C. guilliermondii* Cgi1, *C. glabrata* Cg7 and *C. krusei* Ck1), the number of cultivable cells (Figure 16.A and Figure 17.A), the total biomass (Figure 16.B and Figure 17.B), metabolic activity (Figure 16.C and Figure 17.C) and cells morphology (Figure 16.D and Figure 17.D) were evaluated.

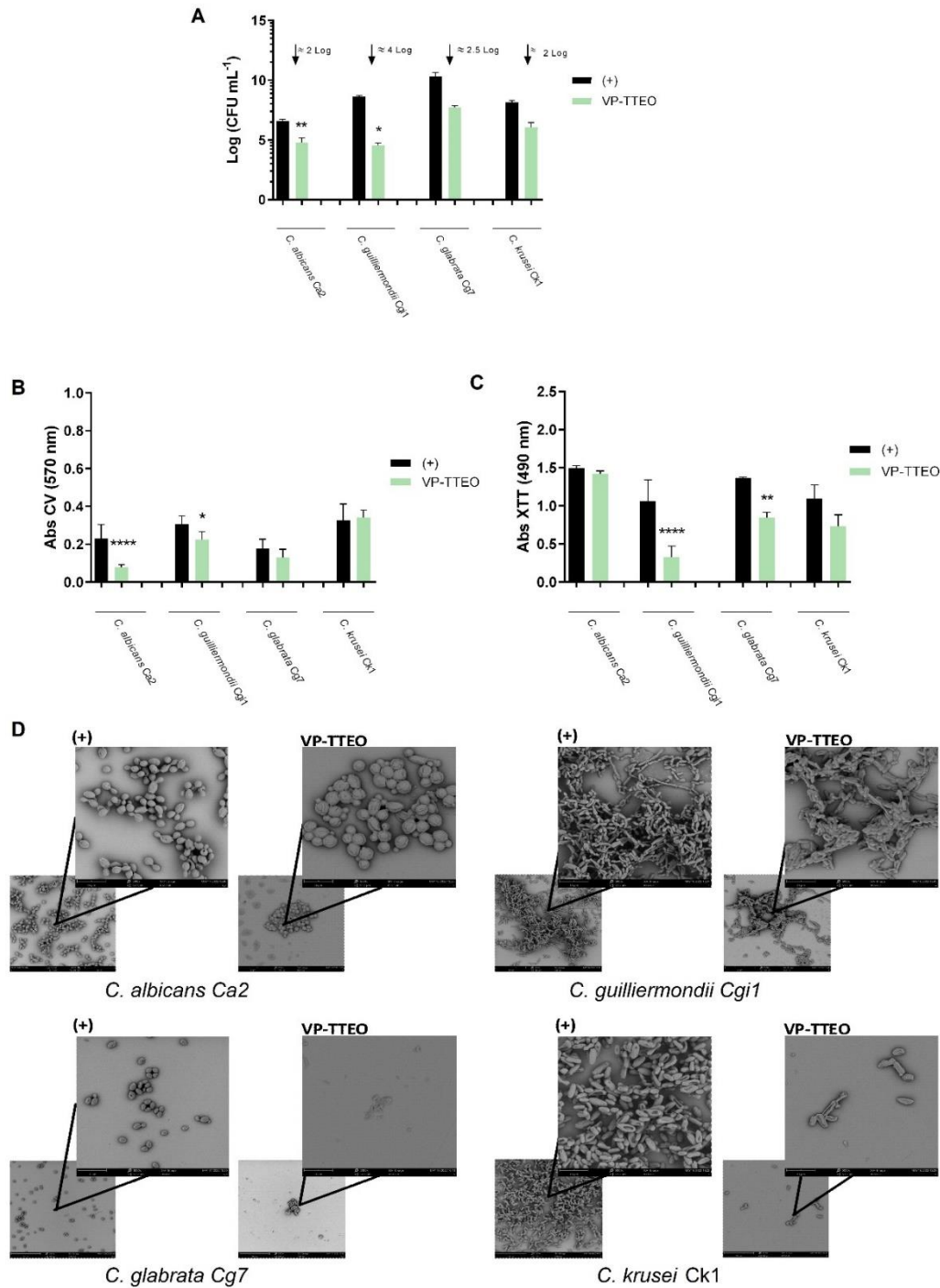


Figure 16. Effect of the vapor-phase of tea tree essential oil (VP-TTEO) on the biofilm formation of antifungal-resistant *Candida* isolates. Biofilms of *Candida albicans* Ca2, *Candida guilliermondii* Cgi1, *Candida glabrata* Cg7 and *Candida krusei* Ck1 were developed in the absence (control) and presence of the VP-TTEO. **(A)** Number of cultivable cells (Log CFUs mL⁻¹); **(B)** Absorbance values of Crystal Violet solutions (Abs CV) and **(C)** Absorbance values of XTT solutions (Abs XTT). * indicate statistical reduction of biofilms cell cultivability in comparison with the respective control (* p< 0.1, ** p< 0.01, *** p< 0.001, **** p< 0.0001). **(D)** Scanning electron microscope (SEM) images. The bar represents 30 μ m (lower magnification image) or 10 μ m (higher magnification image).

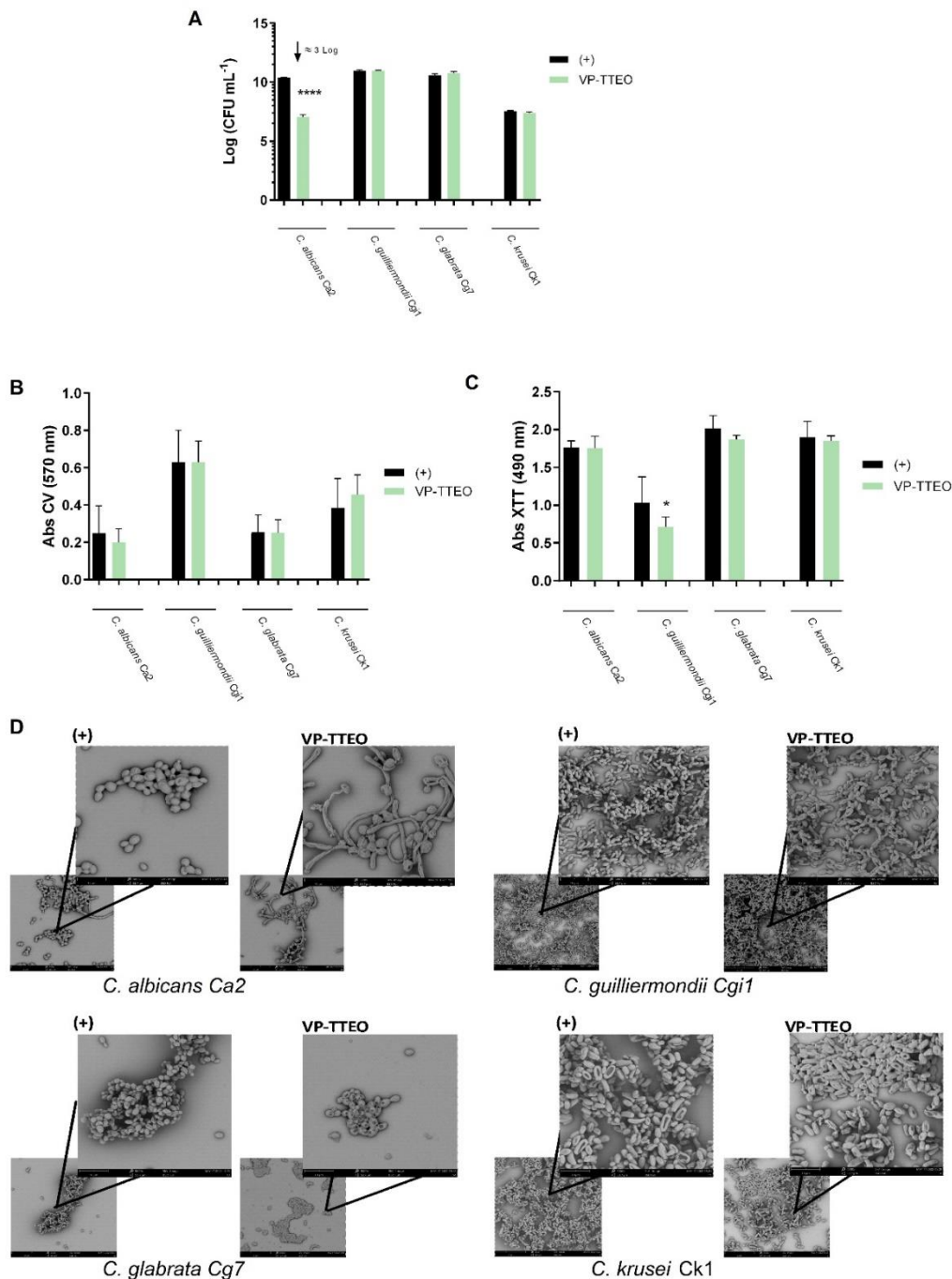


Figure 17. Effect of the vapor-phase of tea tree essential oil (VP-TTEO) on pre-formed biofilms of antifungal-resistant *Candida* isolates. Biofilms of *Candida albicans* Ca2, *Candida guilliermondii* Cgi1, *Candida glabrata* Cg7 and *Candida krusei* Ck1 were developed in the absence (control) and presence of the VP-TTEO. **(A)** Number of cultivable cells (Log CFUs mL⁻¹); **(B)** Absorbance values of Crystal Violet solutions (Abs CV) and **(C)** Absorbance values of XTT solutions (Abs XTT). * indicate statistical reduction of biofilms cell cultivability in comparison with the respective control (* p< 0.1, ** p< 0.01, *** p< 0.001, **** p< 0.0001). **(D)** Scanning electron microscope (SEM) images. The bar represents 30 µm (lower magnification image) or 10 µm (higher magnification image).

The results of cultivable cells number revealed that tea tree EO had high inhibitory effect on the biofilm formation of all the isolates tested (Figure 16.A). Indeed, the VP-TTEO resulted in a significant reduction of 2 to 4 orders of magnitude (Log CFU mL⁻¹) in the cell cultivability of the biofilm in all the isolates tested (Figure 16.A). VP-TTEO reduced 2 Log CFU mL⁻¹ in *C. albicans* ca2 and *C. krusei* Ck1 and the highest inhibition was obtained in *C. guilliermondii* Cgi1 biofilms followed by those of *C. glabrata* Cg7 (2.5-4 Log CFU mL⁻¹). In addition, the biomass quantification revealed significant alteration of two species, *C. albicans* Ca2 and *C. guilliermondii* Cgi1, a statistically lower amount of biomass was found on biofilm formation of *C. albicans* Ca2 (p< 0.0001) and *C. guilliermondii* Cgi1 (p< 0.1) (Figure 16.B). Although CV staining quantifies both dead and live cells and biofilm matrix, it is possible that while the biofilm was not completely removed, a large part of the cells were dead [109]. The effect of VP-TTEO on the metabolic activity of biofilm formation cells was also evaluated (Figure 16.C) and a decrease was observed in the four *Candida* species tested, with greater emphasis on *C. guilliermondii* Cgi1 (p< 0.0001), followed by *C. glabrata* Cg7 (p< 0.01). In agreement with the results in Figure 16.A and Figure 16.B, *C. guilliermondii* Cgi1 showed a greater decrease in metabolic activity. The results of these three parameters are confirmed by the SEM images (Figure 16.D). In fact, when comparing the image of the untreated biofilm cells (control) with the images after contact with VP-TTEO an increase in the number of damaged cells (membrane change, no hyphae or burst cells) is evident for the four *Candida* strains tested.

Additionally, the VP-TTEO were also able to reduce (4 Log CFU mL⁻¹) the number of viable cells on mature biofilms (24 h-old) of *C. albicans* Ca2 (Figure 17.A) and change their morphology (Figure 17.D). Indeed, due to cellular stress, the *C. albicans* can transition from yeast to hypha [110]. Concerning the quantification of biomass, no significant differences were observed between the VP-TTEO and the control (Figure 17.B). Instead, there was a significant decrease in metabolic activity in *C. guilliermondii* Cgi1 (p< 0.1) and a slight decrease in metabolic activity in *C. krusei* Ck1 and *C. glabrata* Cg7 cultivated in the presence of VP-TTEO (Figure 17.C), with morphology alteration of the *C. glabrata* biofilm cells, compared

to the absence of the EOs (Figure 17.D). Indeed, from the SEM images (Figure 17.D) of the treatment with VP-TTEO, alterations in the cell morphology of the *C. albicans* Ca2 and *C. glabrata* Cg7 biofilms are detected. In fact, *C. albicans* Ca2 changed from yeast to hypha after exposure to VP-TTEO. Interestingly, in *C. albicans* strain and *C. guilliermondii* strain there is an opposite effect between metabolic activity and the biomass quantification both in the biofilm formation and in mature biofilms, this curious fact may be due to the unique structure of each biofilm and the biomass quantification assay did not differentiate between living and dead cells. These results, together with those obtained with CV (Figure 16.B), cultivable cells number (Figure 16.A) and SEM (Figure 16.D) suggest a more promising effect of VP-TTEO on biofilm formation.

Overall, the results show a significant inhibitory effect of the VP-TTEO on the development of *Candida* biofilms of antifungal-resistant isolates. However, the induced effect by VP-TTEO may differ according to the *Candida* species, due to the differences that each species presents both in its planktonic state and in the biofilm structure. In this sense, it would be important to determine the mode of action of VP-TTEO on different *Candida* species. Furthermore, a possible factor that can influence this effect is the distance of the EO from *Candida* cells and the atmospheric dimension of the VP-EO.

4. Conclusions

This subchapter suggests a promising use of the VP-EOs to prevent biofilm-related VVC caused by drug-resistant strains. Interestingly, a VP-mediated treatment has additional advantages, including high efficacy without requiring direct contact of the liquid phase with the skin and easy mode of application (for instance on women's underwear). Of note, some antifungal-resistant isolates used in this study were collected from women (symptomatic or not) presenting clinically relevant conditions such as pregnancy and immunodepression, in which prevention and quick treatment of VVC are imperative. However, the prophylactic treatment of VVC with common antifungals, besides not always effective, may contribute to the selection of strains with low antifungal susceptibility. By contrast, the VP-EOs seems to be a safe and effective solution to prevent biofilm-related VVC in at-risk women such those using IUDs, immunosuppressed and pregnant. In this particularly study, tea tree, cajeput and niaouli EOs present an important high antifungal activity and VP-TTEO presents an important preventive effect in biofilm-related VVC caused by antifungal-resistant strains.

References

- [1] Karpiński TM. Essential Oils of *Lamiaceae* Family Plants as Antifungals. *Biomolecules* 2020;10:103. <https://doi.org/10.3390/biom10010103>.
- [2] Chowdhary A, Voss A, Meis JF. Multidrug-resistant *Candida auris*: 'new kid on the block' in hospital-associated infections? *J. Hosp Infect* 2016;94:209–12. <https://doi.org/10.1016/j.jhin.2016.08.004>.
- [3] Chow NA, Gade L, Tsay S, *et al.* Multiple introductions and subsequent transmission of multidrug-resistant *Candida auris* in the USA: a molecular epidemiological survey. *Lancet Infect Dis* 2018;18:1377–84. [https://doi.org/10.1016/S1473-3099\(18\)30597-8](https://doi.org/10.1016/S1473-3099(18)30597-8).
- [4] Aldejohann AM, Wiese-Posselt M, Gastmeier P, *et al.* Expert recommendations for prevention and management of *Candida auris* transmission. *Mycoses* 2022. <https://doi.org/10.1111/MYC.13445>.
- [5] Jeffery-Smith A, Taori SK, Schelenz S, *et al.* *Candida auris*: A review of the literature. *Clin Microbiol Rev* 2018;31:e00029-17. <https://doi.org/10.1128/CMR.00029-17>.
- [6] Shaban S, Patel M, Ahmad A. Improved efficacy of antifungal drugs in combination with monoterpene phenols against *Candida auris*. *Sci Rep* 2020;10:1162. <https://doi.org/10.1038/s41598-020-58203-3>.
- [7] Borman AM, Szekely A, Johnson EM. Comparative Pathogenicity of United Kingdom Isolates of the Emerging Pathogen *Candida auris* and Other Key Pathogenic *Candida* Species. *MSphere* 2016;1:e00189-16. <https://doi.org/10.1128/msphere.00189-16>.
- [8] Palmeira-de-Oliveira A, Salgueiro L, Palmeira-de-Oliveira R, *et al.* Anti-*Candida* Activity of Essential Oils. *Mini-Rev Med Chem* 2009;9:1292–305. <https://doi.org/10.2174/138955709789878150>.
- [9] Di Vito M, Mattarelli P, Modesto M, *et al.* *In Vitro* Activity of Tea Tree Oil Vaginal Suppositories against *Candida* spp. and Probiotic Vaginal Microbiota. *Phytother Res* 2015;29:1628–33. <https://doi.org/10.1002/ptr.5422>.
- [10] Swamy MK, Akhtar MS, Sinniah UR. Antimicrobial properties of plant essential oils against human pathogens and their mode of action: An updated review. *Evid based Complement Altern Med* 2016. <https://doi.org/10.1155/2016/3012462>.
- [11] Tran HNH, Graham L, Adukwu EC. *In vitro* antifungal activity of *Cinnamomum zeylanicum* bark and leaf essential oils against *Candida albicans* and *Candida auris*. *Appl Microbiol Biotechnol* 2020;104:8911–24. <https://doi.org/10.1007/s00253-020-10829-z>.
- [12] Cosmétiques et Huiles Essentielles Bio Florame (accessed November 2, 2021).
- [13] CLSI. Reference Method for Broth Dilution Antifungal Susceptibility Testing of Filamentous Fungi. Clinical and Laboratory Standards Institute 2017.
- [14] Touati I, Ruiz N, Thomas O, *et al.* Hyporientalin A, an anti-*Candida* peptaibol from a marine *Trichoderma orientale*. *World J Microbiol Biotechnol* 2018;34:1–12. <https://doi.org/10.1007/S11274-018-2482-Z/TABLES/5>.
- [15] Stepanović S, Vuković D, Dakić I, *et al.* A modified microtiter-plate test for quantification of *staphylococcal* biofilm formation. *J Microbiol Methods* 2000;40:175–9. [https://doi.org/10.1016/S0167-7012\(00\)00122-6](https://doi.org/10.1016/S0167-7012(00)00122-6).
- [16] Antifungal Susceptibility Testing and Interpretation | *Candida auris* | Fungal Diseases | CDC. <https://www.cdc.gov/fungal/candida-auris/c-auris-antifungal.html> (accessed November 2, 2021).
- [17] Chatterjee S, Alampalli SV, Nageshan RK, *et al.* Draft genome of a commonly misdiagnosed multidrug resistant pathogen *Candida auris*. *BMC Genomics* 2015;16:686. <https://doi.org/10.1186/s12864-015-1863-z>.
- [18] Kozics K, Bučková M, Puškárová A, *et al.* The effect of ten essential oils on several cutaneous drug-resistant microorganisms and their cyto/genotoxic and antioxidant properties. *Molecules* 2019;24:pii: E4570. <https://doi.org/10.3390/molecules24244570>.
- [19] Mondello F, De Bernardis F, Girolamo A, *et al.* *In vivo* activity of terpinen-4-ol, the main bioactive component of *Melaleuca alternifolia* Cheel (tea tree) oil against azole-susceptible and -resistant human pathogenic *Candida* species. *BMC Infect Dis* 2006;6. <https://doi.org/10.1186/1471-2334-6-158>.

- [20] Horton M V., Johnson CJ, Kernien JF, *et al.* *Candida auris* Forms High-Burden Biofilms in Skin Niche Conditions and on Porcine Skin. *MSphere* 2020;5:e00910-19. <https://doi.org/10.1128/msphere.00910-19>.
- [21] Asdadi A, Alilou H, Akssira M, *et al.* Chemical Composition and Anticandidal Effect of Three *Thymus* Species Essential Oils from Southwest of Morocco against the Emerging Nosocomial Fluconazole-Resistant Strains. *J Biol Agric Healthc* 2014;4:16–26.
- [22] Salgueiro LR, Cavaleiro C, Gonçalves MJ, *et al.* Antimicrobial activity and chemical composition of the essential oil of *Lippia graveolens* from Guatemala. *Planta Med* 2003;69:80–3. <https://doi.org/10.1055/s-2003-37032>.
- [23] Carson CF, Hammer KA, Riley T V. *Melaleuca alternifolia* (tea tree) oil: A review of antimicrobial and other medicinal properties. *Clin Microbiol Rev* 2006;19:50–62. <https://doi.org/10.1128/CMR.19.1.50-62.2006>.
- [24] Wilkinson JM, Cavanagh HMA. Antibacterial activity of essential oils from Australian native plants. *Phytother. Res.* 2005;19:643–6. <https://doi.org/10.1002/ptr.1716>.
- [25] Keereedach P, Hrimpeng K, Boonbumrung K. Antifungal Activity of Thai Cajuput Oil and Its Effect on Efflux-Pump Gene Expression in Fluconazole-Resistant *Candida albicans* Clinical Isolates. *Int J Microbiol* 2020;2020. <https://doi.org/10.1155/2020/5989206>.
- [26] Oliva B, Piccirilli E, Ceddia T, *et al.* Antimycotic activity of *Melaleuca alternifolia* essential oil and its major components. *Lett Appl Microbiol* 2003;37:185–7. <https://doi.org/10.1046/j.1472-765X.2003.01375.x>.
- [27] Zore GB, Thakre AD, Jadhav S, *et al.* Terpenoids inhibit *Candida albicans* growth by affecting membrane integrity and arrest of cell cycle. *Phytomedicine* 2011;18:1181–90. <https://doi.org/10.1016/j.phymed.2011.03.008>.
- [28] Tampieri MP, Galuppi R, MacChioni F, *et al.* The inhibition of *Candida albicans* by selected essential oils and their major components. *Mycopathologia* 2005;159:339–45. <https://doi.org/10.1007/s11046-003-4790-5>.
- [29] Cox SD, Mann CM, Markham JL. Interactions between components of the essential oil of *Melaleuca alternifolia*. *J Appl Microbiol* 2001;91:492–7. <https://doi.org/10.1046/j.1365-2672.2001.01406.x>.
- [30] Brilhante RSN, Caetano ÉP, Lima RAC de, *et al.* Terpinen-4-ol, tyrosol, and β -lapachone as potential antifungals against dimorphic fungi. *Braz J Microbiol* 2016;47:917. <https://doi.org/10.1016/J.BJM.2016.07.015>.
- [31] Kong Q, Zhang L, An P, *et al.* Antifungal mechanisms of α -terpineol and terpene-4-alcohol as the critical components of *Melaleuca alternifolia* oil in the inhibition of rot disease caused by *Aspergillus ochraceus* in postharvest grapes. *J Appl Microbiol* 2019;126:1161–74. <https://doi.org/10.1111/JAM.14193>.
- [32] Muñoz JE, Rossi DCP, Jabes DL, *et al.* *In Vitro* and *In Vivo* Inhibitory Activity of Limonene against Different Isolates of *Candida* spp. *Journal of Fungi* 2020, Vol 6, Page 183 2020;6:183. <https://doi.org/10.3390/JOF6030183>.
- [33] Souza EL, Stamford TLM, Lima EO, *et al.* Effectiveness of *Origanum vulgare* L. essential oil to inhibit the growth of food spoiling yeasts. *Food Control* 2007;18:409–13. <https://doi.org/10.1016/j.foodcont.2005.11.008>.
- [34] Ruiz-Gaitán A, Moret AM, Tacias-Pitarch M, *et al.* An outbreak due to *Candida auris* with prolonged colonisation and candidaemia in a tertiary care European hospital. *Mycoses* 2018;61:498–505. <https://doi.org/10.1111/myc.12781>.
- [35] Shastri PS, Shankarnarayan SA, Oberoi J, *et al.* *Candida auris* candidaemia in an intensive care unit – Prospective observational study to evaluate epidemiology, risk factors, and outcome. *J Crit Care* 2020;57:42–8. <https://doi.org/10.1016/j.jcrc.2020.01.004>.
- [36] Welsh RM, Sexton DJ, Forsberg K, *et al.* Insights into the unique nature of the east asian clade of the emerging pathogenic yeast *candida auris*. *J Clin Microbiol* 2018;57:1–5. https://doi.org/10.1128/JCM.00007-19_rfseq1.

- [37] Wang TZ, White KN, Scarr JV, *et al.* Preparing your healthcare facility for the new fungus among us: An infection preventionist's guide to *Candida auris*. *Am J Infect Control* 2020;48:825–7. <https://doi.org/10.1016/j.ajic.2020.01.021>.
- [38] Oh M, Heyl J, Babu MBA BA. *Candida auris* in the Age of Resistance. *Cureus* 2020;12:1–5. <https://doi.org/10.7759/cureus.10334>.
- [39] Bravo Ruiz G, Lorenz A. What do we know about the biology of the emerging fungal pathogen of humans *Candida auris*? *Microbiol Res* 2021;242:126621. <https://doi.org/10.1016/j.micres.2020.126621>.
- [40] Shyni UK, Lavanya R. A study on transmission dynamics of the emerging *Candida Auris* infections in Intensive Care Units: Optimal control analysis and numerical computations. *Physica A: Statistical Mechanics and Its Applications* 2021;561:125253. <https://doi.org/10.1016/j.physa.2020.125253>.
- [41] Chow NA, Muñoz JF, Gade L, *et al.* Tracing the Evolutionary History and Global Expansion of *Candida auris* Using Population Genomic Analyses. *MBio* 2020;11. <https://doi.org/10.1128/mBio.03364-19>.
- [42] Horton M V., Nett JE. *Candida auris* Infection and Biofilm Formation: Going Beyond the Surface. *Curr Clin Microbiol Rep* 2020;7:51–6. <https://doi.org/10.1007/s40588-020-00143-7>.
- [43] de Cássia Orlandi Sardi J, Silva DR, Soares Mendes-Giannini MJ, *et al.* *Candida auris*: Epidemiology, risk factors, virulence, resistance, and therapeutic options. *Microb Pathog* 2018;125:116–21. <https://doi.org/10.1016/j.micpath.2018.09.014>.
- [44] Barreto TL, Rossato L, de Freitas ALD, *et al.* Miltefosine as an alternative strategy in the treatment of the emerging fungus *Candida auris*. *Int J Antimicrob Agents* 2020;56. <https://doi.org/10.1016/j.ijantimicag.2020.106049>.
- [45] Lockhart SR. *Candida auris* and multidrug resistance: Defining the new normal. *Fungal Genetics and Biology* 2019;131. <https://doi.org/10.1016/j.fgb.2019.103243>.
- [46] Chaabane F, Graf A, Jequier L, *et al.* Review on Antifungal Resistance Mechanisms in the Emerging Pathogen *Candida auris*. *Front Microbiol* 2019;10:1–8. <https://doi.org/10.3389/fmicb.2019.02788>.
- [47] Bona E, Cantamessa S, Pavan M, *et al.* Sensitivity of *Candida albicans* to essential oils: are they an alternative to antifungal agents? *J Appl Microbiol* 2016;121:1530–45. <https://doi.org/10.1111/jam.13282>.
- [48] Leal F, Taghouti M, Nunes F, *et al.* Thymus Plants: A Review—Micropropagation, Molecular and Antifungal Activity. *Active Ingredients from Aromatic and Medicinal Plants*, InTech; 2017, p. 1–15. <https://doi.org/10.5772/66623>.
- [49] Ballester-Costa C, Sendra E, Fernández-López J, Pérez-Álvarez JA, Viuda-Martos M. Chemical composition and in vitro antibacterial properties of essential oils of four Thymus species from organic growth. *Ind Crops Prod* 2013;50:304–11. <https://doi.org/10.1016/j.indcrop.2013.07.052>.
- [50] Salehi B, Abu-Darwish MS, Tarawneh AH, *et al.* *Thymus* spp. plants - Food applications and phytopharmacy properties. *Trends Food Sci Technol* 2019;85:287–306. <https://doi.org/10.1016/j.tifs.2019.01.020>.
- [51] Wallace WE. “Retention Indices” in in NIST Chemistry WebBook, NIST Standard Reference Database Number 69, Eds. P.J. Linstrom and W.G. Mallard, National Institute of Standards and Technology, Gaithersburg MD, 20899,. <https://doi.org/https://doi.org/10.18434/T4D303>.
- [52] Mc Lafferty H. ‘Wiley registry of mass spectral data 9th/NIST 08.’ Mass Spectral Library 2009.
- [53] Adams RP. Identification of essential oil components by gas chromatography/mass spectrometry. 4th ed. Allured publishing; 2007.
- [54] Arendrup MC, Meletiadis J, Mouton JW, *et al.* EUCAST antifungal MIC method for yeasts: Method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for yeasts. *Eucast* 2020;1–21.
- [55] Silva S, Henriques M, Martins A, *et al.* Biofilms of non-*Candida albicans* *Candida* species: Quantification, structure and matrix composition. *Med Mycol* 2009;47:681–9. <https://doi.org/10.3109/13693780802549594>.

- [56] De Lira Mota KS, De Oliveira Pereira F, De Oliveira WA, *et al.* Antifungal activity of *thymus vulgaris* l. essential oil and its constituent phytochemicals against *rhizopus oryzae*: Interaction with ergosterol. *Molecules* 2012;17:14418–33. <https://doi.org/10.3390/molecules171214418>.
- [57] Rajkowska K, Nowicka-Krawczyk P, Kunicka-Styczynska A. Effect of clove and thyme essential oils on *Candida* biofilm formation and the oil distribution in yeast cells. *Molecules* 2019;24:1–12. <https://doi.org/10.3390/molecules24101954>.
- [58] Sharifzadeh A, Jebeli Javan A, Shokri H, *et al.* Evaluation of antioxidant and antifungal properties of the traditional plants against foodborne fungal pathogens. *J Mycol Med* 2016;26:e11–7. <https://doi.org/10.1016/j.mycmed.2015.11.002>.
- [59] Gonçalves MJ, Cruz MT, Cavaleiro C, *et al.* Chemical, antifungal and cytotoxic evaluation of the essential oil of *Thymus zygis* subsp. *sylvestris*. *Ind Crops Prod* 2010;32:70–5. <https://doi.org/10.1016/j.indcrop.2010.03.005>.
- [60] Dias IJ, Trajano ERIS, Castro RD, *et al.* Antifungal activity of linalool in cases of *Candida* spp. isolated from individuals with oral candidiasis. *Braz. J. Microbiol.* 2018;78:368–74. <https://doi.org/10.1590/1519-6984.171054>.
- [61] Rodrigues M, Lopes AC, Vaz F, *et al.* *Thymus mastichina*: Composition and biological properties with a focus on antimicrobial activity. *Pharmaceuticals* 2020;13:1–26. <https://doi.org/10.3390/ph13120479>.
- [62] Faleiro M, Miguel M, Ladeiro F, *et al.* Antimicrobial activity of essential oils isolated from Portuguese endemic species of *Thymus*. *Lett Appl Microbiol* 2003;36:35–40. <https://doi.org/10.1046/J.1472-765X.2003.01259.X>.
- [63] Pina-Vaz C, Rodrigues AG, Pinto E, *et al.* Antifungal activity of Thymus oils and their major compounds. *J. Eur. Acad. Dermatol. Venereol.* 2004;18:73–8. <https://doi.org/10.1111/J.1468-3083.2004.00886.X>.
- [64] Tullio V, Nostro A, Mandras N, *et al.* Antifungal activity of essential oils against filamentous fungi determined by broth microdilution and vapour contact methods. *J Appl Microbiol* 2007;102:1544–50. <https://doi.org/10.1111/J.1365-2672.2006.03191.X>.
- [65] Boukhatem MN, Darwish NHE, Sudha T, *et al.* *In Vitro* Antifungal and Topical Anti-Inflammatory Properties of Essential Oil from Wild-Growing *Thymus vulgaris* (*Lamiaceae*) Used for Medicinal Purposes in Algeria: A New Source of Carvacrol. *Scientia Pharmaceutica* 2020, Vol 88, Page 33 2020;88:33. <https://doi.org/10.3390/SCIPHARM88030033>.
- [66] Ferreira EDS, Rosalen PL, Benso B, *et al.* The Use of Essential Oils and Their Isolated Compounds for the Treatment of Oral Candidiasis: A Literature Review. *Evid based Complement Altern Med* 2021;2021. <https://doi.org/10.1155/2021/1059274>.
- [67] Denning DW, Kneale M, Sobel JD, *et al.* Global burden of recurrent vulvovaginal candidiasis: a systematic review. *Lancet Infect Dis* 2018;18:e339–47. [https://doi.org/10.1016/S1473-3099\(18\)30103-8](https://doi.org/10.1016/S1473-3099(18)30103-8).
- [68] Nwadioha SI, Egah DZ, Alao OO, *et al.* Risk factors for vaginal candidiasis among women attending primary health care centers of Jos, Nigeria. *Journal of Clinical Medicine and Research* 2010;2:110–3.
- [69] Bojang E, Ghuman H, Kumwenda P, *et al.* Immune Sensing of *Candida albicans*. *Journal of Fungi* 2021, Vol 7, Page 119 2021;7:119. <https://doi.org/10.3390/JOF7020119>.
- [70] Gonçalves B, Ferreira C, Alves CT, *et al.* Vulvovaginal candidiasis: epidemiology, microbiology and risk factors. *Crit Rev Microbiol* 2016;42:905–27. <https://doi.org/10.3109/1040841X.2015.1091805>.
- [71] Willems HME, Ahmed SS, Liu J, *et al.* Vulvovaginal Candidiasis: A Current Understanding and Burning Questions. *J Fungi* 2020, Vol 6, Page 27 2020;6:27. <https://doi.org/10.3390/JOF6010027>.
- [72] Venugopal D, Husain K, Mustafa SA, *et al.* Epidemiology, risk factors and antimicrobial profile of Vulvovaginal Candidiasis (VVC): A study among women in the central region of Saudi Arabia. *J Mycol Med* 2021;31:101049. <https://doi.org/10.1016/J.MYCMED.2020.101049>.
- [73] Fernandes Â, Azevedo N, Valente A, *et al.* Vulvovaginal candidiasis and asymptomatic vaginal colonization in Portugal: epidemiology, risk factors and antifungal pattern. *Med Mycol* 2022;60. <https://doi.org/10.1093/MMY/MYAC029>.

- [74] Harriott MM, Lilly EA, Rodriguez TE, *et al.* *Candida albicans* forms biofilms on the vaginal mucosa. Microbiology (Reading) 2010;156:3635–44. <https://doi.org/10.1099/mic.0.039354-0>.
- [75] Auler ME, Morreira D, Rodrigues FFO, *et al.* Biofilm formation on intrauterine devices in patients with recurrent vulvovaginal candidiasis. Med Mycol 2010;48:211–6. <https://doi.org/10.3109/13693780902856626>.
- [76] Ramage G, Rajendran R, Sherry L, *et al.* Fungal biofilm resistance. Int J Microbiol 2012;2012:1–14. <https://doi.org/10.1155/2012/528521>.
- [77] Kalaivasan K, Singh R, Chaturvedula L. Fungal profile of vulvovaginal candidiasis in a tertiary care hospital. Journal of Clinical and Diagnostic Research 2017;11:DC06–9. <https://doi.org/10.7860/JCDR/2017/23578.9475>.
- [78] Marchaim D, Lemanek L, Bheemreddy S, *et al.* Fluconazole-resistant *Candida albicans* vulvovaginitis. Obstet. Gynecol. 2012;120:1407–14. <https://doi.org/10.1097/AOG.0b013e31827307b2>.
- [79] Nagashima M, Yamagishi Y, Mikamo H. Antifungal susceptibilities of *Candida* species isolated from the patients with vaginal candidiasis. J. Infect. Chemother 2016;22:124–6. <https://doi.org/10.1016/j.jiac.2015.08.008>.
- [80] Rodrigues ME, Silva S, Azeredo J, *et al.* Novel strategies to fight *Candida* species infection. Crit Rev Microbiol 2014;7828:1–13. <https://doi.org/10.3109/1040841X.2014.974500>.
- [81] Cowan MM. Plant products as antimicrobial agents. Clin Microbiol Rev 1999;12:564–582. <https://doi.org/10.1128/cmr.12.4.564>.
- [82] Sardi JCO, Almeida AMF, Mendes Giannini MJS. New antimicrobial therapies used against fungi present in subgingival sites - A brief review. Arch Oral Biol 2011;56:951–9. <https://doi.org/10.1016/j.archoralbio.2011.03.007>.
- [83] Doddanna SJ, Patel S, Sundarrao MA, *et al.* Antimicrobial activity of plant extracts on *Candida albicans*: an *in vitro* study. Indian J Dent Res 2013;24:401–5. <https://doi.org/10.4103/0970-9290.118358>.
- [84] Sardi JCO, Scorzoni L, Bernardi T, *et al.* *Candida* species: Current epidemiology, pathogenicity, biofilm formation, natural antifungal products and new therapeutic options. J Med Microbiol 2013;62:10–24. <https://doi.org/10.1099/jmm.0.045054-0>.
- [85] Soliman S, Alnajdy D, El-Keblawy AA, *et al.* Plants' natural products as alternative promising anti-*Candida* drugs. Pharmacogn Rev 2017;11:104–122. https://doi.org/10.4103/phrev.phrev_8_17.
- [86] Sharifi-Rad J, Sureda A, Tenore GC, *et al.* Biological activities of essential oils: From plant chemoecology to traditional healing systems. Molecules 2017;22:pii:E70. <https://doi.org/10.3390/molecules22010070>.
- [87] Hammer KA, Carson CF, Riley T V. Antifungal activity of the components of *Melaleuca alternifolia* (tea tree) oil. J Appl Microbiol 2003;95:853–60. <https://doi.org/10.1046/j.1365-2672.2003.02059.x>.
- [88] Cannas S, Usai D, Tardugno R, *et al.* Chemical composition, cytotoxicity, antimicrobial and antifungal activity of several essential oils. Nat Prod Res 2016;30:332–9. <https://doi.org/10.1080/14786419.2015.1060592>.
- [89] Mandras N, Nostro A, Roana J, *et al.* Liquid and vapour-phase antifungal activities of essential oils against *Candida albicans* and non-*albicans Candida*. BMC Complement Altern Med 2016;16. <https://doi.org/10.1186/s12906-016-1316-5>.
- [90] Reyes-Jurado F, Navarro-Cruz AR, Ochoa-Velasco CE, *et al.* Essential oils in vapor phase as alternative antimicrobials: A review. Crit Rev Food Sci Nutr 2019;60:1641–50. <https://doi.org/10.1080/10408398.2019.1586641>.
- [91] Williams DW, Wilson MJ, Lewis MAO, *et al.* Identification of *Candida* species by PCR and restriction fragment length polymorphism analysis of intergenic spacer regions of ribosomal DNA. J Clin Microbiol 1995;33:2476–2479. <https://doi.org/10.1128/jcm.33.9.2476-2479.1995>.
- [92] Clinical and Laboratory Standards Institute (CLSI). M38 Reference method for broth dilution antifungal susceptibility testing of yeasts. Third. 2017.

- [93] Richter SS, Galask RP, Messer SA, *et al.* Antifungal susceptibilities of *Candida* species causing vulvovaginitis and epidemiology of recurrent cases. J Clin Microbiol 2005;43:2155–62. <https://doi.org/10.1128/JCM.43.5.2155-2162.2005>.
- [94] Barry AL, Brown SD. Fluconazole disk diffusion procedure for determining susceptibility of *Candida* species. J Clin Microbiol 1996;34:2154–7. <https://doi.org/10.1128/jcm.34.9.2154-2157.1996>.
- [95] Silva S, Henriques M, Oliveira R, *et al.* *In vitro* biofilm activity of non-*Candida albicans* *Candida* species. Curr Microbiol 2010;61:534–40. <https://doi.org/10.1007/s00284-010-9649-7>.
- [96] Hawser S. Comparisons of the susceptibilities of planktonic and adherent *Candida albicans* to antifungal agents: a modified XTT tetrazolium assay using synchronised *C. albicans* cells. J. Med. Vet. Mycol. 1996;34:149–52.
- [97] Cross EW, Park S, Perlin DS. Cross-Resistance of clinical isolates of *Candida albicans* and *Candida glabrata* to over-the-counter azoles used in the treatment of vaginitis. Microb. Drug Resist. 2000;6:155–61. <https://doi.org/10.1089/107662900419474>.
- [98] Zitvogel L, Apetoh L, Ghiringhelli F, *et al.* Immunological aspects of cancer chemotherapy. Nat Rev Immunol 2008;8:59–73. <https://doi.org/10.1038/nri2216>.
- [99] Aguin TJ, Sobel JD. Vulvovaginal candidiasis in pregnancy. Curr Infect Dis Rep 2015;17:30. <https://doi.org/10.1007/s11908-015-0462-0>.
- [100] Sobel JD, Sobel R. Current treatment options for vulvovaginal candidiasis caused by azole-resistant *Candida* species. Expert Opin Pharmacother 2018;19:971–7. <https://doi.org/10.1080/14656566.2018.1476490>.
- [101] Vukovic N, Milosevic T, Sukdolak S, *et al.* Antimicrobial activities of essential oil and methanol extract of *Teucrium montanum*. Evid based Complement Altern Med 2007;4:17–20. <https://doi.org/10.1093/ecam/nem108>.
- [102] Nenoff P, Haustein UF, Brandt W. Antifungal activity of the essential oil of *melaleuca alternifolia* (tea tree oil) against pathogenic fungi *in vitro*. Skin Pharmacol Physiol 1996;9:388–94. <https://doi.org/10.1159/000211450>.
- [103] Scalas D, Mandras N, Roana J, *et al.* Use of *Pinus sylvestris* L. (*Pinaceae*), *Origanum vulgare* L. (*Lamiaceae*), and *Thymus vulgaris* L. (*Lamiaceae*) essential oils and their main components to enhance itraconazole activity against azole susceptible/not-susceptible *Cryptococcus neoformans* strains. BMC Complement Altern Med 2018;18. <https://doi.org/10.1186/S12906-018-2219-4>.
- [104] International standard: ISO 10993-5 Biological evaluation of medical devices (Part 5): Tests for *in vitro* cytotoxicity, 2009.
- [105] Calderone RA, Fonzi WA. Virulence factors of *Candida albicans*. Trends Microbiol 2001;9:327–335. [https://doi.org/10.1016/S0966-842X\(01\)02094-7](https://doi.org/10.1016/S0966-842X(01)02094-7).
- [106] Jafri H, Ahmad I. *Thymus vulgaris* essential oil and thymol inhibit biofilms and interact synergistically with antifungal drugs against drug resistant strains of *Candida albicans* and *Candida tropicalis*. J Mycol Med 2020;30:100911. <https://doi.org/10.1016/j.mycmed.2019.100911>.
- [107] Tyagi AK, Malik A. Liquid and vapour-phase antifungal activities of selected essential oils against *Candida albicans*: Microscopic observations and chemical characterization of *cymbopogon citratus*. BMC Complement Altern Med 2010;10:65. <https://doi.org/10.1186/1472-6882-10-65>.
- [108] Inouye S, Abe S, Yamaguchi H, *et al.* Comparative study of antimicrobial and cytotoxic effects of selected essential oils by gaseous and solution contacts. International Journal of Aromatherapy 2003. [https://doi.org/10.1016/S0962-4562\(03\)00057-2](https://doi.org/10.1016/S0962-4562(03)00057-2).
- [109] Pantanella F, Valenti P, Natalizi T, *et al.* Analytical techniques to study microbial biofilm on abiotic surfaces: pros and cons of the main techniques currently in use. Ann Ig 2013;25:31–42. <https://doi.org/10.7416/AI.2013.1904>.
- [110] Haynes K. Virulence in *Candida* species. Trends Microbiol 2001;9:591–6. [https://doi.org/10.1016/S0966-842X\(01\)02237-5](https://doi.org/10.1016/S0966-842X(01)02237-5).

CHAPTER III

VAGINAL MICROBIOTA CONDITIONS

Vulvovaginal candidiasis is a disease that affects millions of women worldwide. Conventional treatments often involve oral formulations, topical creams, or ointments, with an increase in drug administration through vaginal canal. The use of simulated biological fluids (e.g. vaginal fluid) in the evaluation of antifungal therapies may better replicate real biological environments, providing a better understanding of the behavior of the antifungal agents in the complex context of the female reproductive system.

An essential consideration in the development of new therapies is the impact on the vaginal microbiome, a diverse community of microorganisms that naturally colonizes the vaginal mucosa. This microbiome plays a crucial role in maintaining the overall health and homeostasis of the vaginal environment. Particularly remarkable are species like *Lactobacillus*, which are believed to be beneficial for preserving this delicate balance. In fact, the balance of the vaginal ecosystem results from synergistic and complex interactions established between the different microorganisms that colonize the vaginal mucosa. A healthy vaginal microbiota predominantly comprises *Lactobacillus* species.

Therefore, any therapeutic intervention, including antifungal therapies, must carefully consider not only their efficacy against *Candida* species but also their potential impact on the beneficial members of the microbiome. Achieving this balance is essential for women's overall well-being.

CHAPTER III.1

SIMULATED VAGINAL FLUID: *CANDIDA* RESISTANT STRAINS' BIOFILM CHARACTERIZATION AND VAPOR-PHASE OF ESSENTIAL OIL EFFECT

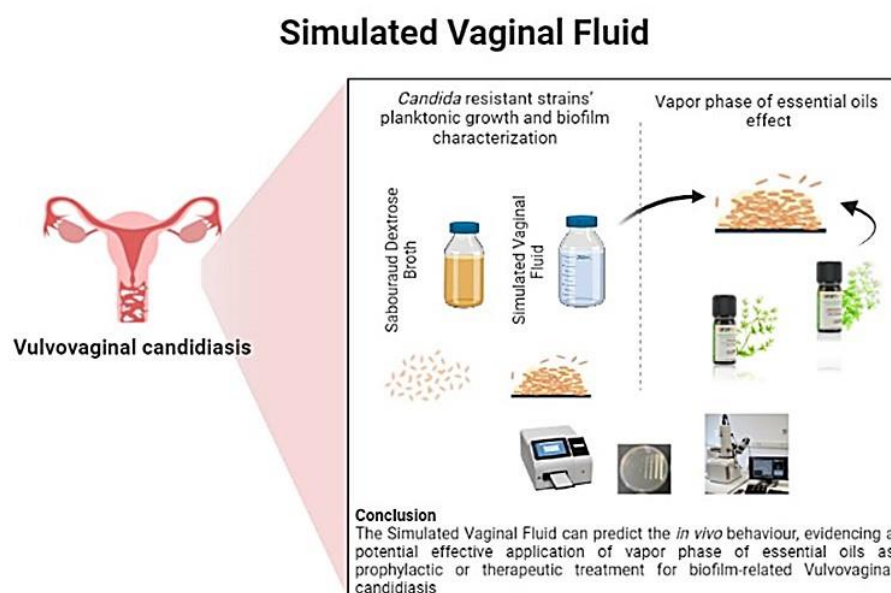


Figure 18. Subchapter III.1 workflow.

The use of simulated biological fluids, such as vaginal fluid, in the assessment of antifungal therapies could offer a more realistic representation of biological environments. This approach may contribute to a better understanding of the antifungal's behavior. So, the main objective of this subchapter was to compare planktonic growth and biofilm formation of *Candida* species, on common growth medium and on vaginal simulation conditions, simulated vaginal fluid. Furthermore, the essential oils effect against biofilms of these *Candida* species was also evaluated.

This subchapter is based on the following publication: Fernandes L, Costa R, Henriques M, Rodrigues ME. Simulated Vaginal Fluid: *Candida* resistant strains' biofilm characterization and vapor phase of essential oil effect. J Mycol Med 2022;101329. <https://doi.org/10.1016/J.MYCMED.2022.101329>.

1. Introduction

Vulvovaginal candidiasis (VVC) is a very common disease, which affects about 70 % of women at least once during their childbearing years [1,2]. In fact, approximately 138 million women worldwide suffer up to 4 episodes of recurrent VVC (RVVC) per year, due to treatment failure [3]. The most common pathogen is *Candida albicans*; however, the abundance of non-*Candida albicans Candida* (NCAC) has increased over time in VVC infections, particularly in RVVC [4,5]. Among the available antifungals, azoles are the most frequently used drugs in the treatment of VVC. Nevertheless, increasing rates of resistance to azoles class threaten the effectiveness of these agents, in both *C. albicans* and NCAC species [6]. Of the NCAC species, *Candida glabrata* has shown higher rates of resistance to azoles agents, *Candida krusei* (teleomorph *Pichia kudriavzevii*) is intrinsically resistant to azoles and *Candida guilliermondii*, despite the low incidence of candidemia caused by this organism, has particular clinical significance as it exhibits increased resistance to antifungal agents, compared to other *Candida* species [7,8]. In fact, the biofilm formation, characteristic of this species, leads to high levels of resilience to common antifungal agents, requiring long and intensive therapies [9,10].

Oral formulations, topical creams or ointments are the most conventional pharmaceutical forms [11]. Indeed, drug administration to the vaginal via has gained increasing attention in past decades [11,12]. The vaginal epithelium is usually coated with a surface film of moisture, the vaginal fluid. Vaginal fluid is composed of cervical fluid (contain epithelial glycogen carbohydrates, amino acids, aliphatic acids, and proteins) and small amounts of the secretion from Bartholin's glands (contains a variety of antimicrobial substances, including lysozyme, lactoferrin, fibronectin, polyamines such as spermine and IgA) in the vaginal wall, which serve as a protective barrier against infections [11]. So, the use of simulated biological fluids in *in vitro* evaluation of antifungal therapies is a promising technique which can allow a better understanding of the antifungal's behavior within biological environments, helping to determine mechanisms of action and potentially predict *in vivo* behavior [13].

Essential oils (EOs) have been suggested as potential alternative treatment, with strong anti-*Candida* effect reported combined with lower side effects, lower toxicity and better biodegradability when compared to available antimicrobial agents [14,15]. So, the administration of EOs through their vapor-phase (VP-EOs) can be used for the local release of volatile and bioactive compounds from EOs directly at the local of action (vaginal epithelium). In fact, in Chapter II and Mandras *et al.* [16] had already shown greater activity for the growth of *Candida* species, avoiding direct contact of the EO with the skin. In this study, two EOs (oregano and white thyme) were selected, due to their characteristics. White thyme EO is recognized for respiratory tract cleansing and calming properties, furthermore, it is described in the

literature that it was able to inhibit the growth of NCAC species resistant to conventional antifungals [17–19]. In turn, Oregano EO has purifying and stimulating properties and it is, also, described in the literature that it was able to inhibit, *in vitro*, the germination and development of the filamentous form of *C. albicans* and to induce a significant reduction in biofilm formation [19,20].

The most important objective of this subchapter was to compare planktonic growth and biofilm formation of drug-resistant *Candida* species between common growth medium and under vaginal simulation conditions. For this purpose, a synthetic medium with physical and chemical properties very similar to those of vaginal secretions from health women was used. In addition, under the same conditions this study also investigated the ability of VP-EOs to inhibit biofilm formation and to destroy mature biofilms of these vaginal isolates.

2. Materials and Methods

2.1 Microorganisms and initial culture conditions

For this study, *C. albicans*, *C. glabrata*, *C. krusei* and *C. guilliermondii* vaginal isolates, recovered from patients and identified as resistant to antifungal agents, belonging to a collection of yeasts created by the *Candida* Research Group of Centre o Biological Engineering were used [21] (Subchapter II.3). *Candida* isolates were stored at -80 ± 2 °C in Sabouraud Dextrose Broth (SDB; Liofilchem) medium with 20 % (v/v) glycerol. The isolates were subcultured from the frozen stock onto Sabouraud Dextrose Agar (SDA; Liofilchem) plates and incubated during 24 h. Then, colonies from SDA plates were used to inoculate SDB for 18 h at 37 °C under agitation (120 rev min⁻¹). The cellular suspensions were centrifuged at 5000 g for 10 min at 4 °C and washed twice with Phosphate Buffered Saline (PBS x1).

This study evaluated the antifungal activity of two EOs (oregano EO (*Origanum compactum*, florame®, Portugal) and white thyme EO (*Thymus satureioides*; florame®, Portugal)) with 100 % purity (chemical composition in Annex I.4 and I.5). EO's products were stored in the dark at room temperature.

2.2 Simulated Vaginal Fluid

Simulated Vaginal Fluid (SVF) was modified from Sosinska *et al.* and Owen & Katz [22,23], and consisted of 58 mM NaCl (Biochem), 18 mM KOH (AppliChem), 2 mM Ca(OH)₂ (Frilabo), 1.75 mM glycerol (Biochem), 6.7 mM urea (Frilabo), 33 mM glucose (Biochem), and 6.7 g L⁻¹ yeast nitrogen base (YNB) (Difco). In addition, natural compounds in the vaginal fluid as acetic acid (17 mM; pKa 54.76) and lactic acid (22 mM; pKa 53.85) were added to maintain the pH at 4.2.

2.3 Effect of Simulated Vaginal Fluid and Sabouraud Dextrose Broth on vaginal *Candida* isolates

2.3.1 Planktonic growth

Vaginal *Candida* isolates cells from the pre-inoculum were cultivated in SVF or SDB (pH at 5.6 measured by digital pH meter (edge®, Hanna)). Cultures of *Candida* cells (1×10^5 cells mL⁻¹) were placed in 25 mL Erlenmeyer flasks, maintained at 37 °C with agitation (120 rev min⁻¹) and the increase in optical density, at 620 nm, was measured over time using a microtiter plate reader (Thermo Scientific™ Multiskan™ FC, Finland). The results were presented as optical density along 24 h of growth, measured at every 3 h [24].

2.3.2 Biofilm formation

The biofilm's growth and structure of the vaginal *Candida* isolates (*C. glabrata*, *C. albicans*, *C. krusei* and *C. guilliermondii*) was evaluated. For this, the biofilms were developed as described in the previous Chapter and by Stepanović *et al.* [25], with some modifications. Briefly, *Candida* cellular suspensions were adjusted to 1×10^5 cells mL⁻¹, in SDB or SVF, and transferred, under aseptic conditions, to 96-well flat tissue culture plates (polystyrene, Orange Scientific, Braine-L'Alleud, Belgium) (200 µL per well). Culture plates were incubated for 24 h at 37 °C under agitation in an orbital shaker (120 rev min⁻¹) conditions, during 24 h and 48 h. Biofilms were analysed through the colony-forming units (CFUs) counting methodology and observed under scanning electron microscopy (SEM) [26,27].

The number of cultivable cells in the biofilms formed in SDB or SVF was assessed using the CFUs counting methodology. Briefly, after 24 h or 48 h, the biofilms were washed with PBS, to remove non-adherent cells, and then scraped from the wells with 1 mL of PBS. The obtained suspensions were serially diluted in PBS and plated onto SDA. The SDA plates were incubated for 24 h at 37 °C and then, the number of grown colonies was counted. The results were presented as colony-forming units per square centimeters (Log (CFU cm⁻²)).

In order to examine and compare the biofilm structure of the drug-resistant *Candida* isolates formed in SDB or in SVF, they were observed by SEM. For that, biofilms formed in steel coupons, as described above, for 24 h, were dehydrated with ethanol (using 70 % ethanol for 10 min, 95 % ethanol for 10 min and 100 % ethanol for 20 min) and air dried for 20 min. The samples were kept in a desiccator for at least 24 h. Prior to observation, the coupons were subjected to sputter coated with gold and observed with an S-360 SEM (Leo, Cambridge, MA, USA) [28].

2.4 Effect of the vapor-phase of essential oils on biofilms

The effect of the VP-EOs (oregano and white thyme EOs) on biofilm formation and on mature biofilms (24 h-old) of four clinical isolates of *Candida* species (*C. glabrata*, *C. albicans*, *C. krusei* and *C. guilliermondii*) was evaluated. Biofilms were developed as described above, with some modifications due to the use of volatile compounds (Chapter II). In order to determine the effect of VP-EOs on biofilm formation, *Candida* cellular suspensions adjusted to 1×10^5 cells mL⁻¹ were transferred to glass wells (1 mL per well) and 25 µL of each EOs (100 %) was discarded on a sterile blank disk, which was placed near the wells (the set was kept inside a glass plate). Plates were incubated for 24 h at 37 °C under agitation in an orbital shaker (120 rev/ min). Additionally, biofilms were pre-formed during 24 h and, after this time, they were incubated in the presence of 25 µL of EOs placed on a sterile blank disk, for an additional 24 h. As control, biofilms were formed without any contact with the VP-EOs during 24 h and 48 h. Biofilms were analyzed through determining the number of *Candida* cultivable cells through CFUs counting methodology, as described above.

2.5 Statistical analysis

The results obtained in this study were statistically analyzed using the Prism software package (GraphPad Software version 6.01). For that, the cell cultivability of biofilms formed in SVF or in SDB, or treated with VP-EOs was compared with that of untreated biofilms using one-way ANOVA and Dunnett's multiple comparisons test. All tests were performed with a confidence level of 95 %. For all assays, three independent experiments were carried out (independent pre-inocula) and each analysis was performed in duplicate or triplicate. Statistical significance was assumed at $p < 0.05$.

3. Results and Discussion

The administration of drugs to the vaginal via has gained increasing attention, highlighting the need for reliable *in vitro* methods to evaluate the performance of new formulations or therapies [29]. Thus, in a first step, the planktonic growth of four *Candida* vaginal isolates under simulating vaginal environment conditions (SVF at pH 4.2) and SDB medium was monitored and compared along 24 h (Figure 19). The four *Candida* species used (*C. albicans* Ca2, *C. guilliermondii* Cgi1, *C. glabrata* Cg7 and *C. krusei* Ck1) were selected from a previous work (Subchapter II.3), as antifungal-resistant strains. The SDB complex medium (pH 5.6 ± 0.2), which contains pancreatic digest of casein, peptic digest of animal tissue and dextrose, is recommended for yeast isolation, qualitative procedures and for the culture or subculture of

fungi from clinical and non-clinical species [30]. The low pH and high concentration of dextrose promotes the growth of yeast while inhibiting bacterial growth [30]. On the other hand, the SVF proposed by Sosinska *et al.* and Owen & Katz [22,23] (with some modification), which was used in this study has been used to simulate the composition of vaginal fluids and to test the diffusion and permeation of new drug delivery systems [23,29]. SVF used as the growth medium consists of YNB with modified concentrations of calcium, potassium, chloride and sodium ions, in agreement with concentrations in the vaginal fluid of healthy premenopausal women during the non-menstrual phase [23,29]. The medium also contained glycerol, urea and glucose, which served as the main source of carbon. SVF was buffered at pH 4.2 using the physiological buffers found in the vaginal fluid, i.e lactic acid and acetic acid [23,29]. In healthy women, *Lactobacillus* species produces lactic acid, which act as buffer and maintains the pH of vagina between 4.0 and 5.0 (acidic), depending on a woman's menstrual cycle. Hydrogen peroxide (H_2O_2) and bacteriocins are also produced, which resist the overgrowth of pathogenic microbes. During pregnancy, the pH drops (3.8- 4.4) and in post-menopause the pH increases (7.0-7.4) [11].

In planktonic conditions, all *Candida* isolates had a similar growth rate between both culture medium (SDB and SVF) along 24 h. Despite, the SVF had only a slight effect on the growth rate of *C. albicans* Ca2 (Figure 19.A), *C. guilliermondii* Cgi1 (Figure 19.B) and *C. glabrata* Cg 7 (Figure 19.C) cells. Moreover, the growth rate of *C. krusei* Ck1 with SDB and SVF was parallel, and after 24 h the same growth rate was observed (Figure 19.D). This led us to conclude that the SVF is also a good medium to isolate and culture or subculture the *Candida* species.

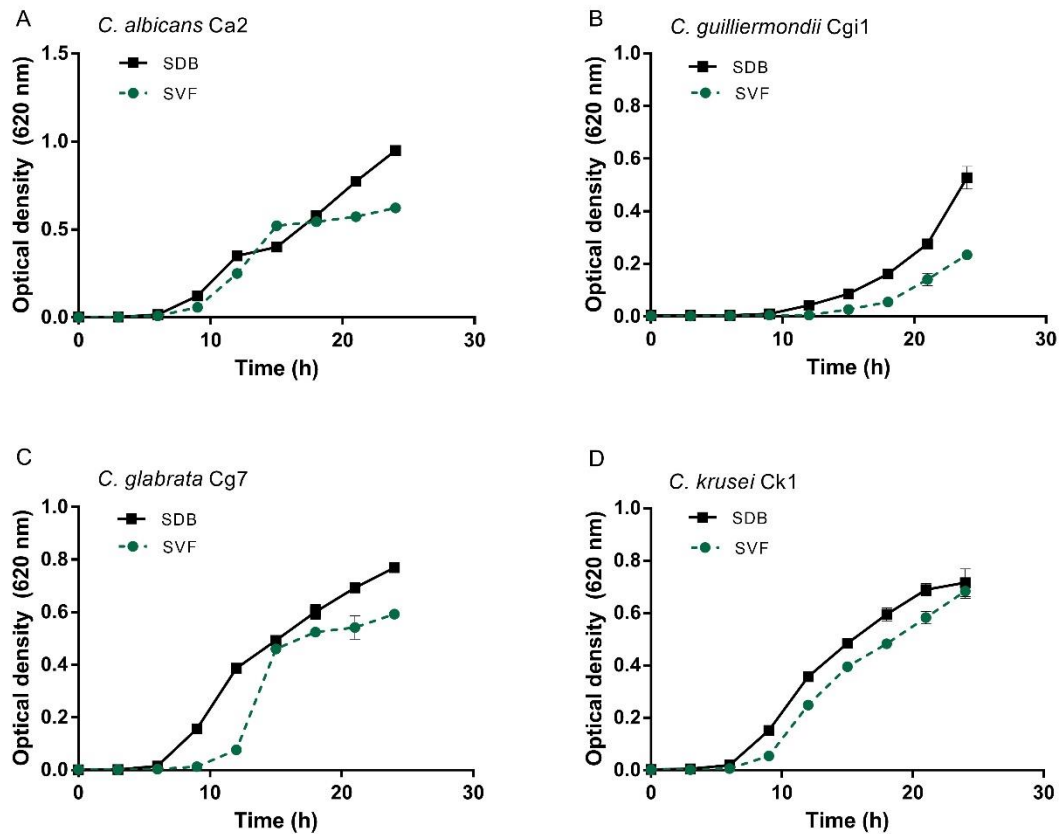


Figure 19. Simulated Vaginal Fluid (SVF) and Sabouraud Dextrose Broth (SDB) on vaginal *Candida* isolates planktonic cells. Planktonic growth curves of (A) *Candida albicans* Ca2, (B) *Candida guilliermondii* Cgi1, (C) *Candida glabrata* Cg7 and (D) *Candida krusei* Ck1 cells cultivated in SVF and SDB over 24 h.

After the overgrowth of the *Candida* species, the colonization of the vaginal epithelium occurs and consequently the transformation from asymptomatic to symptomatic, leading to biofilm formation [31]. So, the biofilm formation (24 h and 48 h) of *Candida* isolates under conditions simulating the vaginal environment (SVF at pH 4.2) and SDB medium was compared (Figure 20 and Figure 21). Biofilms were analyzed through determining the CFUs counting methodology (Figure 20) and by SEM (Figure 21).

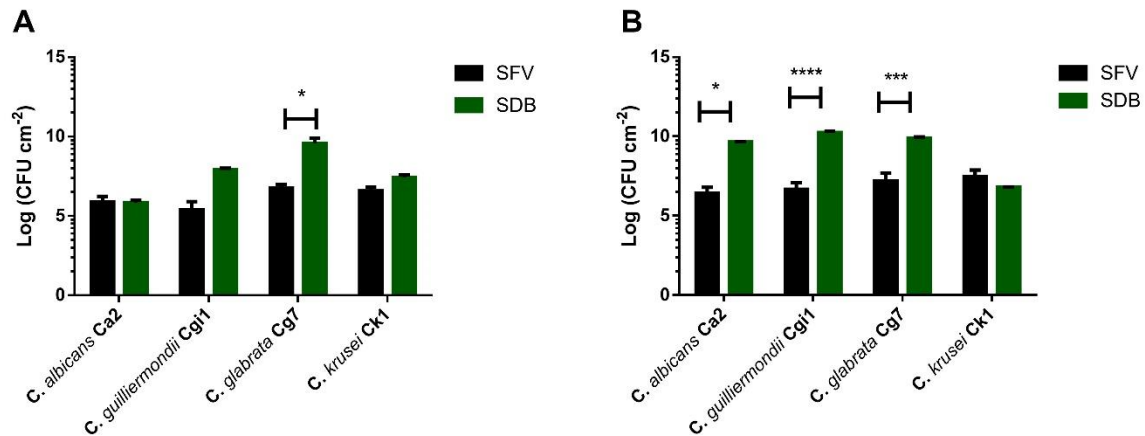


Figure 20. Biofilm formation of antifungal-resistant *Candida* isolates in Simulated Vaginal Fluid (SVF) and Sabouraud Dextrose Broth (SDB). Number of cultivable cells (Log CFUs cm⁻²) for (A) 24 h and (B) 48 h. * indicate statistical reduction of biofilms cell cultivability in comparison with the respective control (* p < 0.1, ** p < 0.01, *** p < 0.001, **** p < 0.0001) (Dunnett's multiple comparisons test).

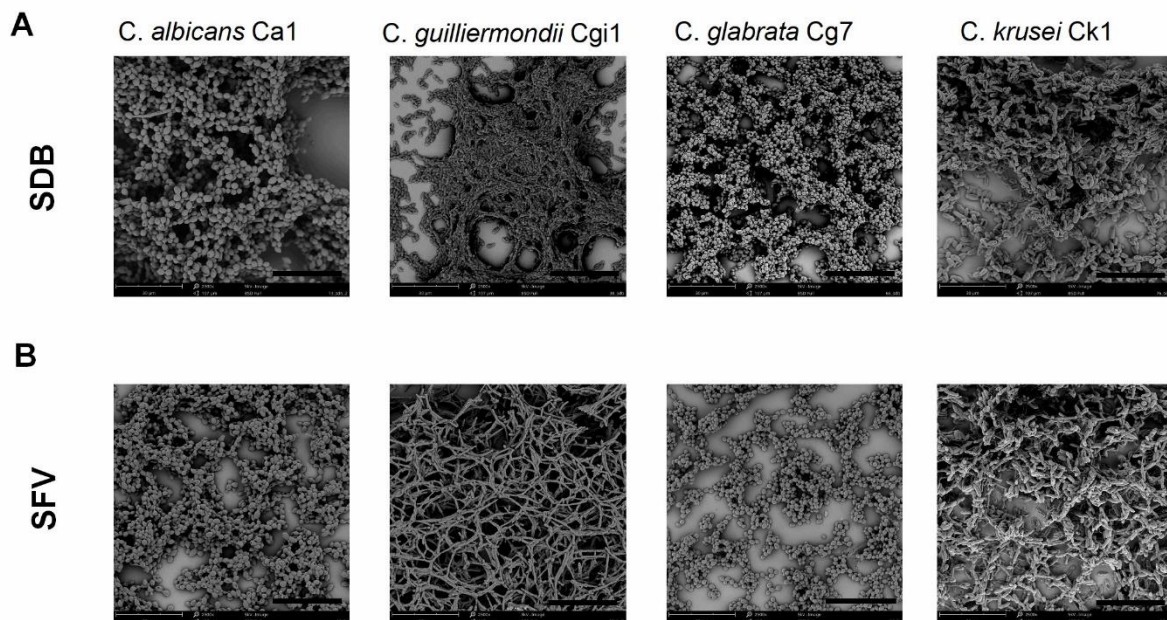


Figure 21. Scanning Electron Microscope (SEM) observations of biofilms formation structure (24 h) of antifungal-resistant *Candida* isolates in (A) Sabouraud Dextrose Broth (SDB) and (B) Simulated Vaginal Fluid (SVF). Magnification: 5,000×. Scale bar=30 μm.

The biofilm formation capacity of the *Candida* isolates after 24 h is similar between both medium (SDB and SVF), except for *C. glabrata* Cg7 which had a lower capacity to form biofilm in SVF (Figure 20.A). Interestingly, in the SVF medium, all *Candida* strains have the same capacity of biofilm formation, while in the SDB medium, some species stand out with greater biofilm formation. Thus, the structure and morphological characteristics of the biofilms formed for 24 h, with SVF and SDB, was observed by SEM (Figure 21). In general, *Candida* isolates maintained the same morphology formed in SVF and SDB, i.e., the species that had more tendency to filament in SDB (Figure 21.A) also filamented in SVF (Figure 21.B), such as *C. guilliermondii* or *C. krusei*. However, in the SDB *Candida* isolates cells formed a multilayer and compact biofilm that covered the entire surface, constituted by network of yeast or hyphal forms (Figure 21.A). Differently, in the SVF, the biofilm consisted of a discontinuous multilayer with a lower number of cells. Indeed, the overall architecture of the biofilm can vary depending on the substrate on which it is formed and growth conditions, such as medium composition, pH and oxygen availability, as well as matrix composition [27,32,33]. Although the biofilm formation is a process present in *Candida* species, it differs significantly from strain to strain, depending on the ability of each strain to present certain characteristics, such as dimorphic growth or producing extracellular polymeric substances [33]. For example, in the case of *C. albicans*, the biofilm characteristic of this strain consists of a two-layer structure composed of a heterogeneous mixture of yeasts, pseudohyphae and hyphae embedded in extracellular polymeric substances, composed mainly of carbohydrates, proteins, phosphorus and hexosamines. On the other hand, the typical biofilm formation of *C. glabrata* is a compact monolayer or multilayer structure of only blastospores, where the cells are embedded within an extracellular matrix composed of high levels of carbohydrates, proteins and hexosamines [27].

Regarding the number of cultivable cells of biofilms after 48 h (Figure 20.B), the SVF showed lower number of cells in three *Candida* isolates (*C. albicans* Ca2, *C. guilliermondii* Cgi1 and *C. glabrata* Cg7), resulting in a statistically significant decrease on cell cultivability ($p \leq 0.0001$) compared to biofilms formed in the SDB. Despite this, it is considered that the differences found are not very significant, as cell formation and morphology were maintained.

The bioavailability of vaginally administered therapies depends on the effective dissolution of solid drug particles, if present, in the vaginal fluid prior to absorption, and the degree of deactivation by enzymes present in the fluids [11]. So, to optimize formulations for the vaginal via, it is important to consider several points, specifically the effective dissolution in the vaginal fluid [11,29]. Vaginal fluid is, in fact, the first physical barrier that the administered formulations encounter, and it has been demonstrated that its composition, pH and osmolarity can significantly affect the delivery of drugs and

efficacy [34]. Indeed, vaginal pH affects the degree of ionization of drugs, which can affect their absorption [11]. Considering the characteristics of these fluids and their impact on the effectiveness of the drug formulation, it is crucial to evaluate the effect of therapies with medium that mimics the vaginal environment. Therefore, in this work the effect of VP-EOs on *Candida* species in SVF was evaluated. This approach may allow the reduction of the drug dose needed to produce the pharmacological effect, possibly minimizing systemic side effects and eliminating problems related to first-pass metabolism [35]. So, in order to study the effect of the vapor-phase of two EOs (oregano (VP-OEO) and white thyme (VP-WTEO)) on the biofilm formation and pre-formed biofilms of antifungal-resistant *Candida* isolates (*C. albicans* Ca2, *C. guilliermondii* Cgi1, *C. glabrata* Cg7 and *C. krusei* Ck1) in SVF, the number of cultivable cells (Figure 22) was evaluated.

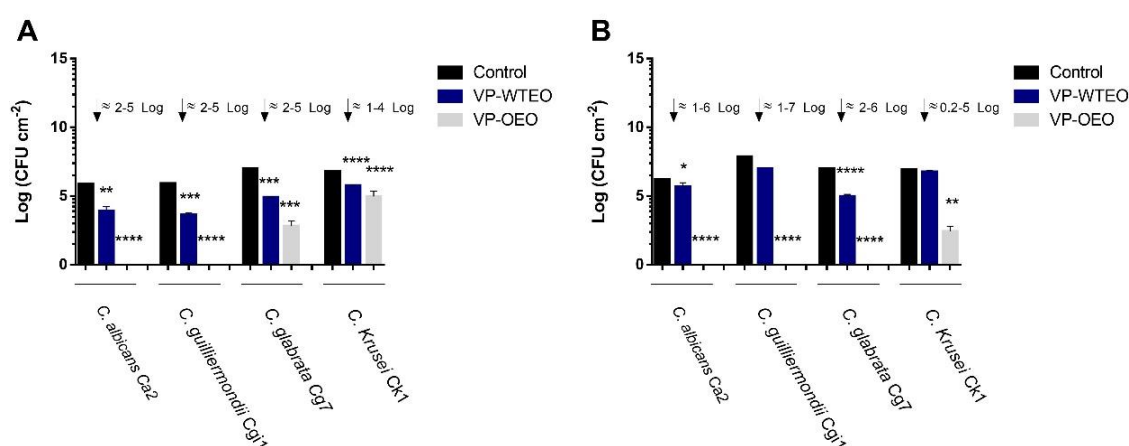


Figure 22. Effect of the vapor-phase of essential oils (oregano (VP-OEO) and white thyme (VP-WTEO)) on (A) biofilm formation and (B) pre-formed biofilms of antifungal-resistant *Candida* isolates. Biofilms of *Candida albicans* Ca2, *Candida guilliermondii* Cgi1, *Candida glabrata* Cg7 and *Candida krusei* Ck1 were developed in the absence (control) and presence of the VP-EOs. * indicate statistical reduction of biofilms cell cultivability in comparison with the respective control (* $p < 0.1$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

Generally, the VP-EOs of both oils led to inhibition of biofilm formation and reduction of mature biofilm. The results related to biofilm formation revealed that both EOs had a high inhibitory effect on all tested isolates (Figure 22.A). In fact, VP-OEO was able to completely inhibit the formation of *C. albicans* Ca2 and *C. guilliermondii* Cgi1 biofilms. In addition, a significant reduction, ranging from 1-5 orders of magnitude (Log CFU cm⁻²), in comparison to the absence of VP-EOs was observed in the formation of *C. glabrata* and *C. krusei* Ck1. Moreover, the VP-OEO was also able to completely reduce mature biofilms (24 h) of *C. albicans* Ca2, *C. glabrata* Cg7 and *C. guilliermondii* Cgi1 (Figure 22.B). However, with VP-

WTEO only a small reduction was observed to the different species, ranging from 0.2- 2 orders of magnitude (Log CFU cm⁻²), both in the formation of biofilms and in mature biofilms (Figure 22). The significant difference observed in the antifungal activity of the two EOs may be a result of the main bioactive compounds of the EOs or due to a synergistic action between the minor and major compounds [36]. In fact, the antifungal activity of EOs can differ greatly even within the same plant species, according to their origin and chemical profile (Subchapter II.2).

Curiously and according to previous results when the VP-EOs was able to reduce mature biofilms, its impact was higher than on biofilm formation (Figure 22). The results obtained in this work agree with those results obtained in Chapter II, where SDB was the medium used, this means that the effect of VP-EOs is not affected by SVF as growth medium and that this fluid allows the dissolution of the volatile and bioactive compounds. Inclusive, the results obtained with SVF predict *in vivo* behavior, suggesting a potential effective application of VP-EOs as prophylactic or therapeutic treatment for biofilm-related VVC. In fact, also Wang *et al.* and Pietrella *et al.* [37,38] verified that EO was highly effective in accelerating the elimination of *C. albicans in vivo*, from the vagina of experimentally infected mice, by prophylaxis and therapeutic treatments.

4. Conclusions

In the present study, was compared the planktonic growth and biofilm formation of *Candida* species, between SDB and under simulated fluid vaginal (SVF) conditions in order to validate an *in vitro* model. SVF was evaluated as reliable and capable of mimic the vaginal environment and allow the assessment of drug/compound permeation, in this case VP-EOs, intended for vaginal administration. Indeed, this study demonstrates that the SVF medium offers a reliable and predictive tool for mimicking these environments and allow, in fact, to evaluate the behaviour of VP-EOs intended for vaginal administration. Thus, a potential application of VP-EOs as a prophylactic or therapeutic treatment for VVC is suggested. Of the two EOs tested (oregano and white thyme), VP-OEOs showed the highest antifungal activity.

CHAPTER III.2

VAPOR-PHASE OF WHITE THYME ESSENTIAL OIL EFFECT ON *CANDIDA ALBICANS* AND *LACTOBACILLUS GASSERI* COLONIZATION IN A RECONSTITUTED HUMAN VAGINAL EPITHELIUM

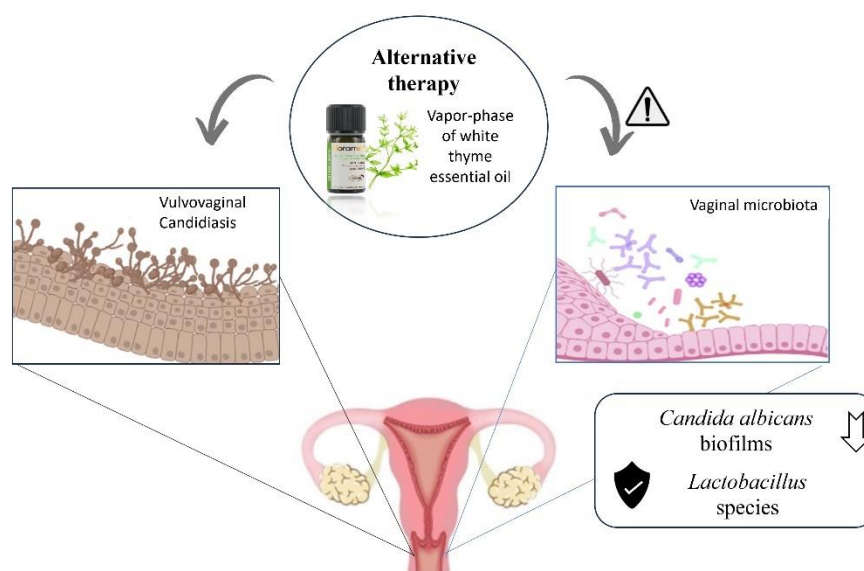


Figure 23. Subchapter III.2 workflow.

The development of new therapies for vulvovaginal candidiasis must take into consideration the potential impact on other colonizing species. Thus, the main goal of this subchapter was to evaluate the effect of the vapor-phase of white thyme essential oil, on *Candida albicans* biofilm formation and on co-colonization of a reconstituted human vaginal epithelium by *C. albicans* together with the vaginal species *Lactobacillus gasseri*.

This subchapter is based on the following publication: Fernandes L, Silva I, Costa R, Silva S, Mira Pereira N, Costa-de-Oliveira S, Henriques M, Rodrigues ME. Vapor-phase of white thyme essential oil effect on *Candida albicans* and *Lactobacillus gasseri* colonization in a reconstituted human vaginal epithelium (In submission)

Presented as Video abstract: Fernandes L, Costa R, Silva S, Henriques M, Rodrigues ME. *Efeito da fase de vapor de óleos essenciais em Candida albicans e Lactobacillus gasseri aderidos a epitélio vaginal humano reconstituído. Congresso internacional em língua portuguesa Microbiologia 2022, Online, 2022.*

And oral presentation: Fernandes L, Costa R, Silva S, Mira Pereira N, Costa-de-Oliveira S, Henriques M, Rodrigues ME. Vapor-phase of white thyme essential oil effect on *Candida albicans* and *Lactobacillus gasseri* colonization in a reconstituted human vaginal epithelium. 5th Congress of Baltic Microbiologists (FEMs grant), 2023, Vilnius, Lithuania.

1. Introduction

The balance of the vaginal ecosystem is the result of synergy and complex interactions between the host and different microorganisms present in the microbiome that colonizes the vaginal mucosa [39,40]. Indeed, vaginal microbiota includes a diverse range of beneficial microorganisms, predominantly constituted by the *Lactobacillus* species. Such beneficial bacteria include *Lactobacillus crispatus*, *Lactobacillus jensenii*, *Lactobacillus iners* and *Lactobacillus gasseri*, that coexist with the human host in mutualism, with the host providing nutrients and the bacteria protecting the vaginal environment from the colonization of pathogenic microorganisms [41]. Indeed, *Lactobacillus* species play a key protective role in preventing gynecological diseases [42,43]. Vulvovaginal Candidiasis (VVC) is a prevalent infection of the vaginal mucosa caused by *Candida* species [2,44]. To note, *Lactobacillus* species were reported to inhibit various virulence traits of *Candida* species including their adherence to epithelial cells, suppressing the possible attachment of fungal cells [45]. In addition, these bacterial cells secrete biosurfactants and release hydrogen peroxide and lactic acid that inhibit growth of *Candida* species and also formation of invasive hyphae [45].

Likewise, *Lactobacillus* species, *Candida* species are also present in the vaginal microbiota as a commensal microorganism, however, certain factors such as broad-spectrum antibiotics, pregnancy, immunosuppression or contraceptives can lead to its transition to a pathogenic microorganism [2]. *Candida albicans* is the most frequent species that causes VVC [21]. This infectious disease is often treated with antifungal drugs, especially azoles. However, these agents are fungistatic and cells exposed repeatedly to these antifungals lead to adaptation and consequent resistance [39,46]. Thus, the increase of drug resistance combined with the high incidence of VVC highlights the need to develop new therapeutic approaches, more effective, without the risk of inducing resistance, for the prevention and treatment of this gynecological infection. However, it is imperative that the development of these new therapies do not disrupt the vaginal microbial homeostasis, in particular the difference naturally colonizing *Lactobacillus* species. Thus, the present study aims to evaluate the induced effect of a previously developed therapy (Subchapter III.1) in the vaginal species *L. gasseri*, and also on *C. albicans*. This therapy consists of exposure to the vapor-phase of white thyme essential oil (VP-WTEO), after evaluation the toxicity of this therapy in a toxicological model of *Galleria mellonella* larvae. Previous work has demonstrated that VP-EOs exhibit strong antifungal activity against drug-resistant *Candida* species thus emerging as an interesting alternative for VVC treatment (Subchapter II.3 and III.1). Furthermore, after exposure to VP-EO, significant inhibition of biofilm formation and the ability to destroy mature biofilms of drug resistant vaginal *Candida* isolates was observed (Subchapter II.3).

2. Materials and Methods

2.1 Microorganisms and culture conditions

In this study, 3 *C. albicans* clinical isolates resistant to one of antifungal agents (fluconazole, ketoconazole and caspofungin) belonging to a collection of yeasts created by the *Candida* Research Group of the Centre of Biological Engineering of University of Minho [21], and a reference strain of *Lactobacillus gasseri* ATCC 33323 (acquired from DSMZ), were used. The *Candida* isolates and *L. gasseri* were kept in Sabouraud Dextrose Broth (SDB; Liofilchem, Italy) and in Mann Rogosa and Sharpe broth (MRS, Liofilchem) broth, respectively, both with 20 % (v/v) glycerol (Biochem Chemopharma, France), at -80 ± 2 °C. These species were sub-cultured from the frozen stock onto Sabouraud Dextrose Agar (SDA; Liofilchem) (*Candida* isolates) and MRS agar (*L. gasseri*) plates and incubated for 24 h at 37 °C, *L. gasseri* was incubated in a 5 % CO₂ environment in saturated humidity. Before the test, the pre-inoculum of *C. albicans* was prepared in SDB with colonies grown on SDA plates (18 h at 37 °C and under agitation at 120 rev/ min) and *L. gasseri* was pre-cultured in MRS Broth twice with 1 % (v/v) taken directly from the cryovial (24 h at 37 °C in an environment of 5 % CO₂ in saturated humidity). So, the cellular suspensions were centrifuged and washed twice with Phosphate Buffered Saline (PBS) (5000 g for 10 min, at 4 °C).

The assays of this work were carried out in simulated vaginal fluid (SVF). SVF consisted of 58 mM NaCl (Biochem Chemopharma, France), 33 mM glucose (Biochem), 18 mM KOH (AppliChem), 2 mM Ca(OH)₂ (Frilabo), 1.75 mM glycerol (Biochem), 6.7 g L⁻¹ yeast nitrogen base (YNB) (Difco), 6.7 mM urea (Frilabo) and acetic acid (17 mM; pKa 54.76) and lactic acid (22 mM; pKa 53.85) were added to maintain the pH at 4.2, as described in subchapter III.1.

2.2 Essential oils

The antifungal activity of VP-WTEO (*Thymus satureioides*; florame®, France) was evaluated. White thyme EO (WTEO) was tested 100 % pure and stored in the dark at room temperature. From the analysis carried out by the company florame®, it became known that the major compounds in the WTEO are borneol (31 %), α-terpineol (16 %) and carvacrol (9 %) (Subchapter II.1). For this analysis, the company used Gas Chromatography (GC) with Flame-Ionisation Detection (FID): the hydrogen carrier gas; the polar Elite-WAX column (100 % polyethylene glycol) (60 m/ 0.25 mm/ 0.25 µm) and the non-polar Elite-5 columns (5 % diphenyl, 95 % dimethylpolysiloxane) (60 m/ 0.25 mm/ 0.25 µm) (Annex I.4).

2.2.1 Essential oil cytotoxicity

The cytotoxicity of the WTEO was tested using the *in vivo* *Galleria mellonella* larvae model [47]. For that, a group of 10 *G. mellonella* larvae was exposed to VP-WTEO for 72 h. Initially, *G. mellonella* larvae were raised in the dark at 25 °C, with a diet based on pollen grains. Afterward, the larvae selected according to the development stage, approximately 250 mg, were placed in glass Petri dishes in the presence of the WTEO (25 µL-placed in a glass well), allowing the diffusion of the oil without direct contact with the larvae, in a controlled atmosphere with a volume of $1.5 \times 10^6 \text{ cm}^3$. After that, larvae were kept at 37 °C in the dark, without nutrition. As a negative control, a set of larvae was maintained under the same conditions, except for exposure to VP-WTEO. The survival and morphology of the larvae were monitored, and survival curves were constructed, as described by Araújo *et al.* [47]. In this type of test, larvae are considered dead when they show no movement after touch.

2.3 Susceptibility of *Candida albicans* to white thyme essential oil

The disk diffusion method based on Barry and Brown [48] and as described in the previous chapter, was used to evaluate the effect of WTEO on drug-resistant clinical isolates of *C. albicans* (Ca1, Ca2, Ca3). Swabs dipped in the cell solution adjusted to $1 \times 10^8 \text{ cells mL}^{-1}$ were used to inoculate SDA plates. After the plates were completely dry, 25 µL of the WTEO (100 %) was applied in sterile white disks (Liofilchem®) on the plates. Negative (vegetable oil) and positive (cell suspension) controls were performed. The plates were incubated at 37 °C for 24 h and inhibition zone diameters were measured (mm).

2.4 Antifungal activity of vapor-phase of white thyme essential oil on biofilms

The effect of the VP-WTEO on biofilm formation and on mature biofilms (24 h-old) of *C. albicans* (Ca2) was evaluated. Biofilms were developed as described in Chapter II in order to allow the diffusion of volatile compounds and their interaction with microorganisms. To determine the effect of VP-WTEO on biofilm formation, 1 mL of *Candida* cell suspension adjusted to $1 \times 10^5 \text{ cells mL}^{-1}$ was transferred to a glass well and 25 µL of WTEO (100 %) was discarded on a sterile blank disk. The set was kept inside a glass plate and the plates were incubated (24 h at 37 °C, 120 rev/ min). Once formed, the biofilms were incubated in the presence of VP-WTEO for another 24 h. As a control, biofilms were formed without exposure to VP-WTEO for 24 h or 48 h.

The VP-WTEO effect on biofilms was analysed by (2.4.1) quantification of biofilm biomass by staining with crystal violet (CV) and (2.4.2) determination of metabolic activity by XTT reduction assay [26,49].

2.4.1 Quantification of *Candida* biomass

For biomass quantification, biofilms were fixed with 1 mL of methanol, after 15 min was removed and allowed to dry at room temperature. After drying, 1 mL of CV (1 %) was added to each well and incubated for 5 min. Then, the biofilms were washed with sterile ultrapure water and completely dry, and afterwards 1 mL of acetic acid (33 %) was added. Thus, 200 μ L of this solution, from each glass well, was transferred to a microtiter plate and the absorbance of each condition was read at 570 nm in triplicate, using a microtiter plate reader (Thermo Scientific™ Multiskan™ FC, Finland).

2.4.2 Quantification of metabolic activity of *Candida* cells

Biofilm metabolic activity was determined by the XTT (2,3-(2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide) reduction assay. For this, the culture medium was removed, and biofilms washed with PBS. Then, 200 μ L of a solution containing 10 μ g μ L⁻¹ of phenazine methosulfate (PMS, Sigma–Aldrich, USA) plus 100 μ g μ L⁻¹ of XTT (Sigma–Aldrich) was added to each well and incubated for 3 h at 37 °C (130 rpm) in the dark. Then, similar to the previous procedure, 150 μ L from each glass well was transferred to a microtiter plate and the colorimetric changes were measured at 490 nm using a microtiter plate reader (HEALES “MB580”, Shenzhen, China).

2.5 Effect of vapor-phase of white thyme essential oil in colonization of *Candida albicans* and *Lactobacillus gasseri* of a reconstituted human vaginal epithelium

The commercially reconstituted human vaginal epithelium (RHVE) (0.5 cm²; SkinEthic Laboratories, France) was used as a mean to mimic an *in vitro* model of vaginal candidiasis to study the effect of the VP-WTEO on colonization prompted by *C. albicans* and/or *L. gasseri* [50,51]. For that, RHVE tissue inserts (0.5 cm²) were placed in glass wells. To study single species infection, 4 RHVE tissues were infected with 1 mL of standardized suspension (1x 10⁷ cells mL⁻¹) of *C. albicans* or *L. gasseri* strains separately, prepared as previously described in the SVF. In dual species studies, *C. albicans* suspension (500 μ L; 2x 10⁷ cells mL⁻¹) was combined with *L. gasseri* suspension (500 μ L; 2x 10⁷ cells mL⁻¹) and used to infected two RHVE tissues. To determine the effect of VP-WTEO on microorganisms RHVE colonization, two infected tissues of each condition were incubated in the presence of 25 μ L of WTEO (100 %) on glass petri dishes. As negative control, two infected tissues of each condition were tested in the absence of the WTEO. All tissues were incubated at 37 °C in a 5 % CO₂ environment in saturated humidity, with agitation of 120 rpm for 24 h.

After the incubation, the tissues were washed with PBS to remove non-adherent microorganism's cells. Subsequently, the tissues were used for (2.5.1) histological analysis and for (2.5.2) molecular studies. Additionally, the activity of lactate dehydrogenase (LDH) was also evaluated to evaluate indirectly the damage of microorganisms caused on the epithelium (2.5.3) [51].

2.5.1 Histological analysis and microscopic observation

The part of the RHVE tissue intended for histology processing was initially fixed in 2 % (v/v) paraformaldehyde and stored at room temperature. Using standard histological techniques, tissue was embedded in paraffin wax and 20 µm sections were cut and placed on HistoBond+ coated microscope slides. Afterwards, the sections were dewaxed by processing through xylene, immersed in ethanol and then in water and stained with the periodic acid Schiff method (staining of keratinocytes and *Candida* or *Lactobacillus* cells). Then, the sections were analyzed in bright field using an Olympus BX51 epifluorescence microscope coupled to a DP72 digital camera (Olympus Portugal SA, Portugal) and the images were acquired using Olympus Cell-B software.

2.5.2 Quantification of *Candida* and *Lactobacillus* cells in RHVE

Candida's and *Lactobacillus*' DNA present in tissues under different conditions was extracted using the DNA extraction kit (DNeasy Blood & Tissue Kit, Qiagen). The DNA from each tissue was quantified using the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, USA). Both species were quantified using qRT-PCR (quantitative real-time PCR) employing a CF X96 qRT-PCR system (Bio-Rad, Berkeley, USA). For quantification, a final reaction volume of 10 µL was prepared, consisting of 2 µL of DNA, 5 µL of working concentration SsoFast EvaGreen Supermix, 0.1 µL of each primer (Table 7) and 2.8 µL of H₂O. Negative controls were performed, replacing the DNA with nuclease-free water.

Table 7. Primers for real time-PCR assay for quantification of *Candida albicans* and *Lactobacillus gasseri* cells in reconstituted human vaginal epithelium

Target	Primer	Sequence (5' -> 3')
<i>Candida albicans</i>	Forward	GAGCGTCGTTTCTCCCTCAAACCGCTGG
	Reverse	GGTGGACGTTACCGCCGCAAGCAATGTT
<i>Lactobacillus gasseri</i>	Forward	AGGCACTAGAAGCCGATGAA
	Reverse	CACTGCGTCTTCCTTCAACA

The PCR cycling conditions for the *L. gasseri* and *C. albicans* involved of an initial denaturation step at 98 °C (2 min) or 95 °C (3 min), then 40 cycles of denaturation at 98 °C (5 s) or 95 °C (10 s)

and primer annealing at 55 °C (5 s) or 55 °C (30 s), respectively. The same PCR protocols were used to construct calibration curves (threshold cycle (CT) values vs Log of cells) for both species (Figure 24), obtained from serial dilutions of known cell concentrations, as described by Alves *et al.* [51].

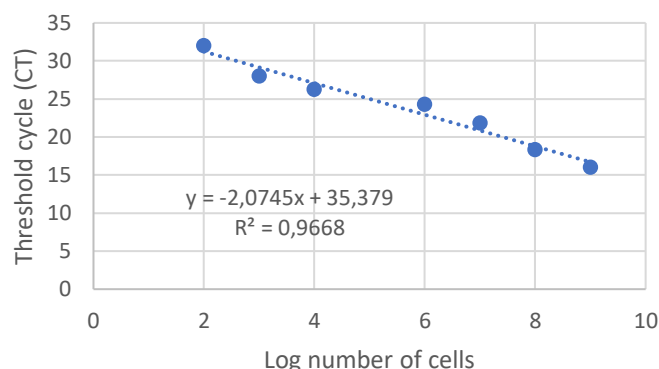


Figure 24. The standard calibration curve of threshold cycle (CT) values vs Log number of cells.

2.5.3 Lactate dehydrogenase (LDH) assay

To measure epithelial cell damage, the CytoTox-ONE Homogeneous Membrane Integrity Assay kit (Promega) was used to determine the release of LDH from the RHVE in the culture medium. LDH activity was analyzed in a spectrophotometer (FLUOstar OPTIMA; BMG Labtech, Ortenberg/Germany) at 560 nm excitation and 590 nm emission. The LDH released during infection or co-infection and treatment with VP-WTEO for both species was expressed as LDH activity in relation to the infected epithelium and the untreated epithelium. Also, the VP-WTEO effect on tissue expressed as LDH activity relative to tissue with SVF (control) was evaluated. All experiments were performed in triplicate.

2.6 Statistical analysis

The results were statistically analysed using the Prism software package (GraphPad Software version 8.01). One-way ANOVA (analysis of variance) or two-way ANOVA was performed, Tukey's multiple comparison test. Results were expressed as mean $\pm$ standard deviation (SD) and the statistical analyses performed were considered significant when $p < 0.05$.

3. Results and Discussion

Under healthy conditions, the vaginal mucosa is inhabited by several microorganisms, including bacteria and fungi, which normally coexist and interact with each other and with the host. However, several factors can upset this balance and lead to the development of diseases [41]. In fact, it is well established that the decreased of vaginal lactobacilli population is linked with the decrease in vaginal health. Thus, it is imperative the development of new therapeutic approaches for VVC, that do not cause an imbalance and disruption in the remaining microbiota.

According to previous work, EOs and especially the VP-EOs have shown great antifungal activity against drug-resistant *Candida* species (Subchapter II.3 and III.1). Therefore, it is crucial to understand the effect induced by this new alternative (VP-EOs) in vaginal *Lactobacillus* species as well. For this, in the first step, the cytotoxicity of VP-WTEO was evaluated. The model of *G. mellonella* larvae is widely used to study the effectiveness of new antimicrobial compounds, immune responses and, in this study, cytotoxicity. Among other advantages, the wax model is suitable for these toxicological studies due to its functional and structural similarity to the innate immune response of mammals [52]. Therefore, the *in vivo* toxicity of VP-WTEO was evaluated from *G. mellonella*, where it was found that after 72 h of exposure to VP-WTEO, there was no evidence of toxicity (Figure 25). In fact, 100 % of larvae remained alive with no significant differences in larvae exposed and not exposed to VP-WTEO.

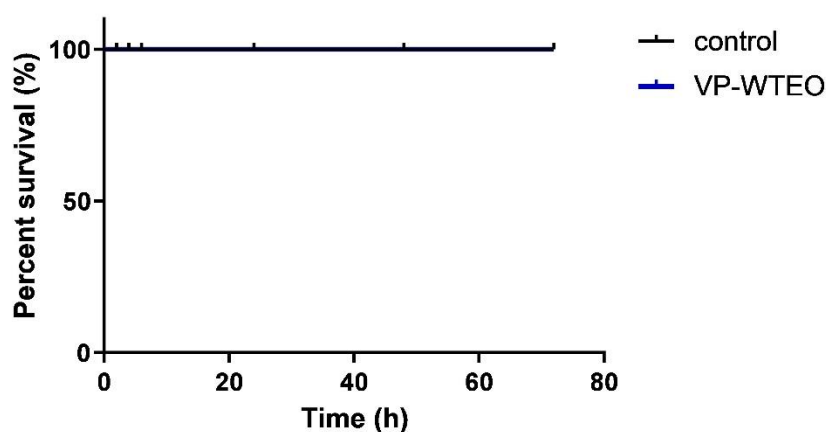


Figure 25. Vapor-phase of white thyme essential oil (VP-WTEO) toxicity measured in *in vivo* *Galleria mellonella* model. Survival curves of *G. mellonella* larvae exposed to VP-WTEO and respective control (without exposure to VP-WTEO). There was no significant difference between control and VP-WTEO exposure.

Then, the antifungal activity of WTEO against planktonic cells of resistant antifungal isolates of *C. albicans* (n=3) was evaluated (Table 8) through the agar disk diffusion method, evaluated by the halo diameter formed (D_{halo}).

Table 8. Anti-*Candida* activity of white thyme essential oil on drug-resistant vaginal isolates. Results obtained from the agar disk diffusion method and zones of inhibition measured through the diameter of the halo (D_{halo}) in millimeters (mm)

Species	Isolate	D_{halo} (mm)
<i>C. albicans</i>	Ca1	22.8 ± 0.5
	Ca2	22.5 ± 1.7
	Ca3	24.7 ± 0.6

The analysis of Table 8 shows that WTEO induced a large inhibitory effect in all clinical isolates, with D_{halo} ranging from 22.5 mm to 24.7 mm. These results agree with other studies that also reported high antifungal activity of WTEO against drug-resistant strains of various *Candida* species [53–57]. The anticandidal activity of thyme has been suggested to be related to morphological changes in *Candida* cells including shrinkage of the cell membrane, disruption of cell membrane permeability and leakage of intracellular material [58,59].

WTEO is complex mixture of compounds extracted by steam distillation of flowering tops, characterized by high concentrations of borneol, α -terpineol and carvacrol. Hammer *et al.* [60] observed that the components with lower values of Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC) in *Candida* species were terpinen-4-ol and α -terpineol. Indeed, the antimicrobial activity of terpenes, at higher concentrations has been attributed to their interactions with cell membranes and consequent total loss of homeostasis and severe membrane damage and death, and at relatively low concentrations, these interactions can result in changes such as inhibition of respiration and change in permeability [60–63]. Furthermore, these components also showed relatively rapid killing effects against *C. albicans* in time-of-kill assays. In addition to this compound, borneol was previously reported to disrupt *C. albicans* biofilm formation [63].

However, the diffusion method results are only an initial screening of the work, since the EOs effect can differ depending on the direct application or only exposure to the vapor-phase. The antifungal activity when applied directly depends on its diffusibility and solubility in the liquid phase, while the activity when only exposed to VP-EOs depends on its volatility [16, 64]. So, the VP-WTEO effect on *Candida* biofilm formation and on pre-formed biofilms of fluconazole and ketoconazole resistant strain (*C. albicans* Ca2)

was then evaluated, this strain was selected as it presented an extremely high level of resistance to fluconazole (Subchapter II.3). In this sense, some factors were quantified to determine the VP-WTEO effect, such as the number of cultivable cells (data presented in previous subchapter III.1), biofilm biomass (Figure 26.A), and the metabolic activity of biofilm cells (Figure 26.B).

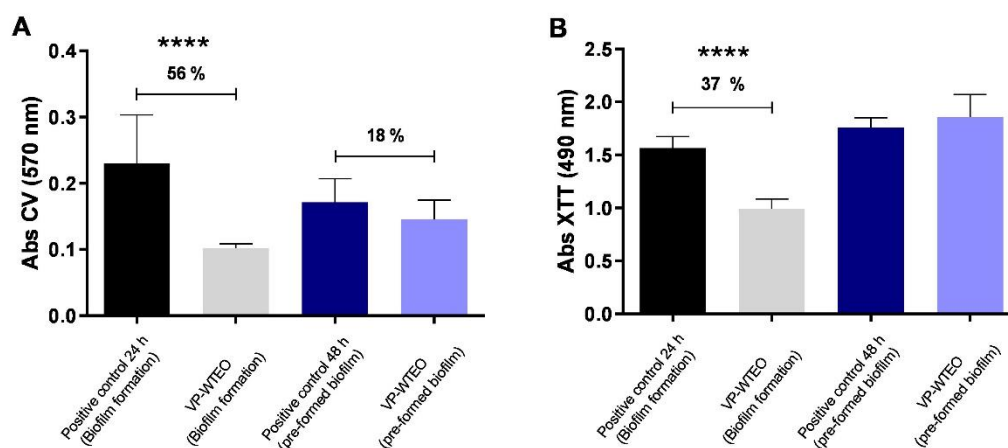


Figure 26. Effect of the vapor-phase of white thyme essential oils (VP-WTEO) on the biofilm formation and pre-formed biofilm of antifungal-resistant *Candida albicans* Ca2. **(A)** Biofilm biomass (Abs CV) and **(B)** metabolic activity (Abs XTT). * indicate statistical reduction of biofilms cell cultivability in comparison with the respective control (* $p < 0.1$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

Previous work demonstrated that VP-WTEO led to the inhibition of biofilm formation and reduction of mature biofilm of *C. albicans* Ca2 (Subchapter III.1). So, from this result previously obtained with the results of the biomass quantification and the metabolic activity of the biofilm cells, it was observed that the VP-WTEO has an effect against the drug resistant *C. albicans* biofilms. Indeed, the cell cultivability of biofilms developed in the presence of the VP-WTEO showed a significant reduction, ranging from 1 to 2 orders of magnitude (Log CFU cm⁻²), in comparison to the absence of VP-WTEO (Subchapter III.1). Regarding the quantification of biomass and metabolic activity, the results showed a significant reduction, ranging from 18 - 56 % (Figure 26.A) and 37 % (Figure 26.B), respectively. This set of results confirms the effectiveness of VP-WTEO in these two stages of biofilms, not only in inhibition biofilms formation, but also impacting already biofilms formed in an already organized structure. In fact, previous work has reported that the vapor generated by EOs has a greater antimicrobial impact compared to the EOs liquid phase when applied by direct contact. Furthermore, a study carried out by Wang *et al.* [65], demonstrated that the anti-*C. albicans* activity of borneol (major constituent of WTEO) in the vapor-phase was significantly

greater than in the liquid phase. The authors observed that concentrations lower than the MIC values of the liquid phase of borneol reduced (60 - 99 %) the germ tube formation of *C. albicans* ATCC 10231 [65]. Germ tube formation and biofilm integrity in *Candida* species, mainly *C. albicans*, are important virulence factors. The germ tube (filamentous form) allows penetration into deeper layers of the mucosa and confers greater resistance to phagocytosis, facilitating the transition from the state of harmless symbiosis to a pathogen [63]. The fact that the antifungal activity of EO molecules in the vapor-phase does not require direct contact, suggests that such EOs can be used in the prevention and treatment of several diseases, such as VVC.

The effect of new antifungal therapies on other colonizing species is extremely important. Therefore, in this work a RHVE was used and co-colonization by *C. albicans* and *L. gasseri* was induced. This procedure aimed to evaluate the effect of the developed therapy (VP-WTEO) on beneficial species of the vaginal microbiome, such as *Lactobacillus*. For this purpose, the species of *L. gasseri* was selected due to its predominance in vaginal environments and also because it was recently shown to inhibit growth of *C. albicans*, both in planktonic and in mixed biofilms, resulting in enhanced loss of yeast cell viability [66–68]. The infection, co-infection and subsequent VP-WTEO effect of RHVE by *C. albicans* and *L. gasseri* were evaluated after 24 h and the results confirm the efficacy of VP-WTEO against the *C. albicans* colonization (Figure 27 and 28).

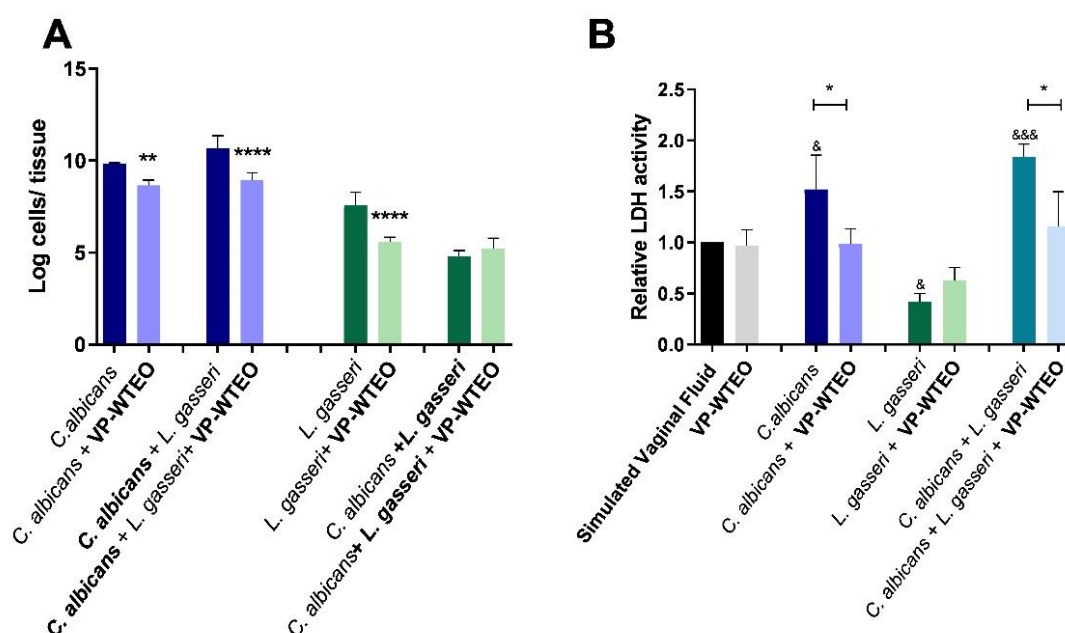


Figure 27. Effect of the vapor-phase of white thyme essential oil (VP-WTEO) on single and co-colonization of *Candida albicans* with *Lactobacillus gasseri* of the reconstituted human vaginal epithelium (RHVE) after 24 h. (A) Number

of cells (Log cells/ tissue) on single and mixed colonization in relation to the treated RHVE (VP-WTEO). * indicate statistical reduction of number of cells in comparison with the respective control (** $p < 0.001$, **** $p < 0.0001$).
(B) Relative lactate dehydrogenase (LDH) activity measured in the culture supernatants after 24 h of single and co-colonization in relation to simulated vaginal fluid (&) and VP-WTEO effect (*). */& indicate statistical reduction of LDH activity in comparison with the respective control (* $p < 0.1$, *** $p < 0.001$).

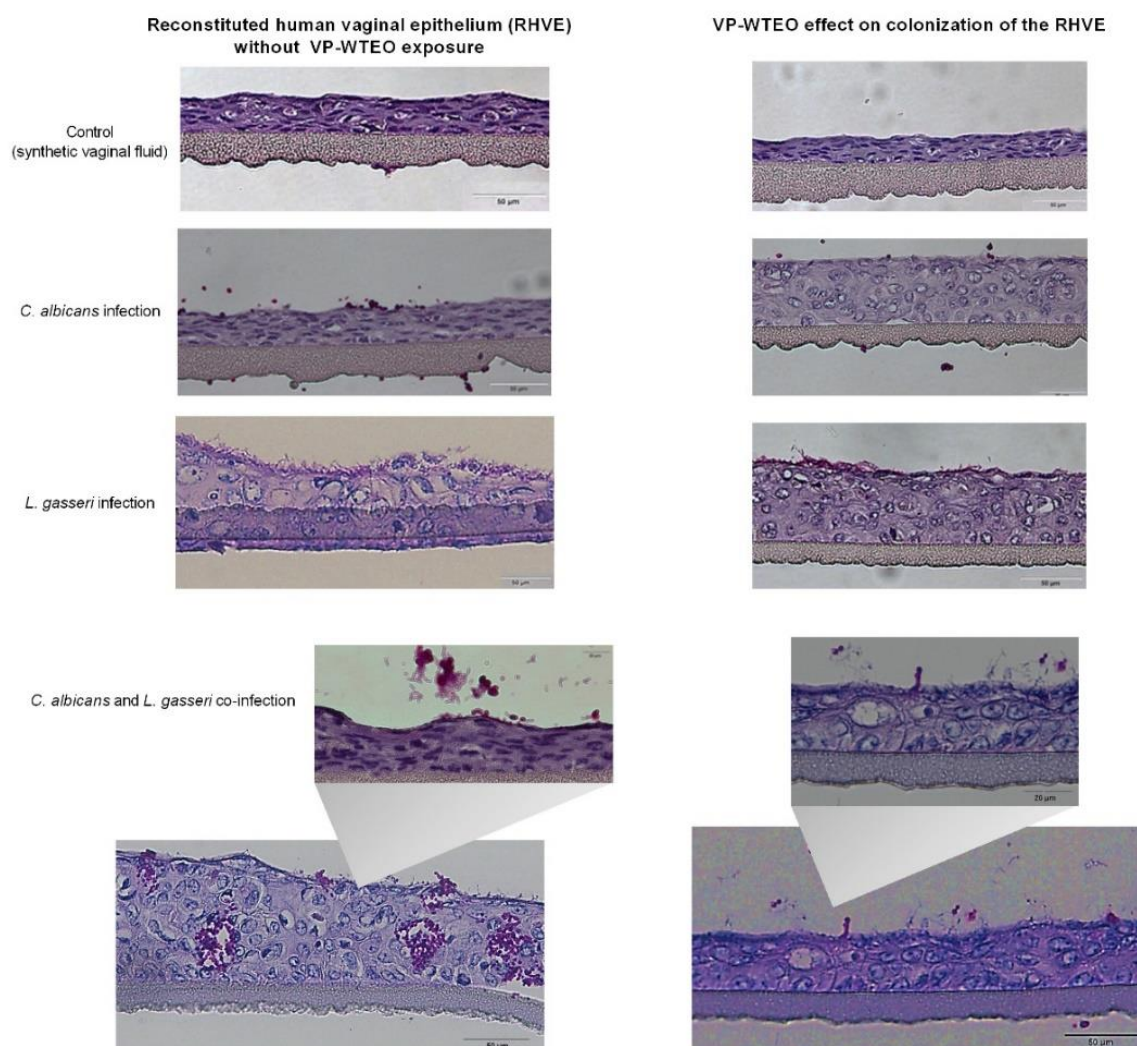


Figure 28. Effect of the vapor-phase of white thyme essential oils (VP-WTEO) on single and mixed colonization of *Candida albicans* and *Lactobacillus gasseri* in reconstituted human vaginal epithelium (RHVE) after 24 h. Image acquired by epifluorescence microscopy. The bar represents 50 μm and 20 μm.

The results obtained from the PCR analysis (Figure 27.A) show a significant decrease in the number of cells occurring both in simple colonization ($p < 0.001$) and in co-colonization with *L. gasseri* ($p < 0.0001$). Furthermore, this reduction on colonization, after VP-WTEO exposure is notably observed in

tissue images (Figure 28). Regarding the effect of exposure of *L. gasseri* to VP-WTEO, there is no interference in the number of cells when it is present in co-colonization with *C. albicans*.

These data can also be confirmed by microscopic imaging of the RHVE (Figure 28), where the *Lactobacillus* species remain in the tissue after exposure to therapy and, even when co-colonize with *C. albicans* occurs, only this last species is affected. These results are extremely important, as these data demonstrate the therapy affects *C. albicans* and does not interfere with *L. gasseri* cells. This fact is very important, because the decrease of *Lactobacillus* in the vaginal microflora would harm the host and enhance VVC. Indeed, the role of *Lactobacillus* species as general probiotics and as an inhibitor for VVC development and other gynaecological diseases has been previously demonstrated [66,69–75]. Zangl *et al.* [70] demonstrated that *Lactobacillus* species can improve the treatment of VVC. Furthermore, they observed that the organic acids produced by *Lactobacillus* species positively influence the effectiveness of antifungal treatment by increasing the permeability of the fungal plasma membrane structure. Also, Dos Santos *et al.* [75] showed that some *Lactobacillus* strains produced biosurfactants that exhibited considerable anti-adhesion and anti-biofilm activities against *C. albicans*.

The results obtained from the LDH assays are consistent with the last results (Figure 27.B). The LDH assay is used to assess the level of damage to the cell plasma membrane. When the plasma membrane is damaged, LDH is released into the cell culture medium [76]. LDH activity levels revealed no significant effects between VP-WTEO exposure in RHVE compared to control (SVF) (Figure 27.B). This result could also be confirmed by the microscopic image observation (Figure 28), where RHVE exposed to VP-WTEO did not undergo morphological changes. Therefore, it is possible to conclude that VP-OEO treatment does not cause relevant tissue damage and its application seems to be safe for a future application. Furthermore, these data confirm the results of the *in vivo* absence of cytotoxicity (Figure 25). A significant increase in LDH was observed when tissue was colonized by *C. albicans* in both conditions, single ($p < 0.1$) and mixed colonization ($p < 0.001$). In turn, a significant decrease ($p < 0.1$) of LDH was observed when only *L. gasseri* was present. As expected, *Candida* species are able to cause vaginal epithelium damage [51,77], while the *Lactobacillus* cells assumed a protector role. In fact, the effectiveness of applying *Lactobacillus* as a prophylaxis has been well-founded in long-term administration [41]. Reid *et al.* [74] demonstrated that the administration of *Lactobacillus* probiotics in recommended amounts can restore host-microbial and immune homeostasis. Regarding the applied treatment, an important fact can be drawn from the data obtained, VP-WTEO exposure allowed a significant decrease ($p < 0.1$) in epithelium damage when colonization by *C. albicans* occurs, in both conditions (Figure 27.B). Although an increase in LDH activity was verified when *L. gasseri* was exposed to VP-WTEO, together with

C. albicans (simulating to the vaginal flora), there was a decrease in the damage level. So, it can be concluded that VP-WTEO significantly reduced *Candida* species, controlling the growth of this fungus.

4. Conclusions

EOs have been suggested as potential sources of alternatives and new therapeutic products, with fewer side effects, lower toxicity and better biodegradability when compared to available antimicrobial agents. In this study, WTEO was selected, and its anti-*Candida* activity in the liquid phase and vapor-phase was demonstrated. Biofilm formation and biofilm integrity in *Candida* are important virulence factors, and overall, the VP-WTEO results demonstrated the antifungal potential of this therapy, as it was able to interfere with both forms. The possibility of interfering with these more resistant structures, such as biofilms, is a promising therapeutic strategy.

References

- [1] Dvornik A, Golle A, Novak D, *et al.* Treatment of vulvovaginal candidiasis: a review of the literature 2015;24:5–7. <https://doi.org/10.15570/actaapa.2015.2>.
- [2] Gonçalves B, Ferreira C, Alves CT, *et al.* Vulvovaginal candidiasis: Epidemiology, microbiology and risk factors. *Crit Rev Microbiol* 2016;42:905–27. <https://doi.org/10.3109/1040841X.2015.1091805>.
- [3] Sherry L, Kean R, McKloud E, *et al.* Biofilms formed by isolates from recurrent vulvovaginal candidiasis patients are heterogeneous and insensitive to fluconazole. *Antimicrob Agents Chemother* 2017;61. <https://doi.org/10.1128/AAC.01065-17>
- [4] A Spinillo, E Capuzzo, R Gulminetti, Prevalence of and risk factors for fungal vaginitis caused by non-*albicans* species. *Am J Obstet Gynecol* 1997;176:138–41. [https://doi.org/10.1016/S0002-9378\(97\)80026-9](https://doi.org/10.1016/S0002-9378(97)80026-9).
- [5] Mintz JD, Martens MG, Mintz JD, *et al.* Prevalence of *Non-Albicans Candida* Infections in Women with Recurrent Vulvovaginal Symptomatology. *Adv Infect Dis* 2013;3:238–42. <https://doi.org/10.4236/AID.2013.34035>.
- [6] Marchaim D, Lemanek L, Bheemreddy S, *et al.* Fluconazole-resistant *Candida albicans* vulvovaginitis. *Obstetrics and Gynecology* 2012;120:1407–14. <https://doi.org/10.1097/AOG.0b013e31827307b2>.
- [7] Cheng J-W, Liao K, Kudinha T, *et al.* Molecular epidemiology and azole resistance mechanism study of *Candida guilliermondii* from a Chinese surveillance system. *Sci Rep* 2017;7. <https://doi.org/10.1038/S41598-017-01106-7>.
- [8] Berkow EL, Lockhart SR. Fluconazole resistance in *Candida* species: a current perspective. *Infect Drug Resist* 2017;10:237. <https://doi.org/10.2147/IDR.S118892>.
- [9] Auler ME, Morreira D, Rodrigues FFO, *et al.* Biofilm formation on intrauterine devices in patients with recurrent vulvovaginal candidiasis. *Med Mycol* 2010;48:211–6. <https://doi.org/10.3109/13693780902856626>.
- [10] Ramage G, Rajendran R, Sherry L, *et al.* Fungal biofilm resistance. *Int J Microbiol* 2012;2012:1–14. <https://doi.org/10.1155/2012/528521>.
- [11] Marques MRC, Loebenberg R, Almukainzi M. Simulated Biological Fluids with Possible Application in Dissolution Testing. *Dissolut Technol* 2011;18:15–28. <https://doi.org/10.14227/DT180311P15>.
- [12] Hillery AM, Lloyd AW, Swarbrick J. Drug delivery and targeting for pharmacists and pharmaceutical scientists 2001: (1st ed.). 475. <https://doi.org/10.1201/b12801>
- [13] Innes E, Yiu HHP, McLean P, *et al.* Simulated biological fluids – a systematic review of their biological relevance and use in relation to inhalation toxicology of particles and fibres. *Crit Rev Toxicol* 2021;51:217–48. <https://doi.org/10.1080/10408444.2021.1903386>.
- [14] Marino A, Nostro A, Mandras N, *et al.* Evaluation of antimicrobial activity of the hydrolate of *Coridothymus capitatus* (L.) *Reichenb. fil. (Lamiaceae)* alone and in combination with antimicrobial agents. *BMC Complement Med Ther* 2020;20:89. <https://doi.org/10.1186/s12906-020-2877-x>.
- [15] Bona E, Cantamessa S, Pavan M, *et al.* Sensitivity of *Candida albicans* to essential oils: are they an alternative to antifungal agents? *J Appl Microbiol* 2016;121:1530–45. <https://doi.org/10.1111/JAM.13282>.
- [16] Mandras N, Nostro A, Roana J, *et al.* Liquid and vapour-phase antifungal activities of essential oils against *Candida albicans* and non-*albicans Candida*. *BMC Complement Altern Med* 2016;16. <https://doi.org/10.1186/s12906-016-1316-5>.
- [17] Asdadi A, Alilou H, Akssira M, *et al.* Chemical Composition and Anticandidal Effect of Three *Thymus* Species Essential Oils from Southwest of Morocco against the Emerging Nosocomial Fluconazole-Resistant Strains. *J Biol Agric Healthc* 2014;4:16–26.
- [18] Fernandes L, Ribeiro R, Costa R, *et al.* Essential Oils as a Good Weapon against Drug-Resistant *Candida auris*. *Antibiotics* 2022, Vol 11, Page 977 2022;11:977. <https://doi.org/10.3390/ANTIBIOTICS11070977>.
- [19] Cosmétiques et Huiles Essentielles Bio Florame. <https://fr.florame.com/> (accessed July 26, 2022).

- [20] Hacıoglu M, Oyardi O, Kirinti A. Oregano essential oil inhibits *Candida* spp. biofilms. Zeitschrift Fur Naturforschung - Section C Journal of Biosciences 2021;76:443–50. <https://doi.org/10.1515/znc-2021-0002>.
- [21] Fernandes Â, Azevedo N, Valente A, *et al.* Vulvovaginal candidiasis and asymptomatic vaginal colonization in Portugal: epidemiology, risk factors and antifungal pattern. Med Mycol 2022;60. <https://doi.org/10.1093/MMY/MYAC029>.
- [22] Sosinska GJ, Groot PWJ, Matto MJT, *et al.* Hypoxic conditions and iron restriction affect the cell-wall proteome of *Candida albicans* grown under vagina-simulative conditions. Microbiology (Reading) 2008;154:510–20. <https://doi.org/10.1099/MIC.0.2007/012617-0>.
- [23] Owen DH, Katz DF. A vaginal fluid simulant. Contraception 1999;59:91–5. [https://doi.org/10.1016/S0010-7824\(99\)00010-4](https://doi.org/10.1016/S0010-7824(99)00010-4).
- [24] Gonçalves B, Bernardo R, Wang C, *et al.* Effect of progesterone on *Candida albicans* biofilm formation under acidic conditions: A transcriptomic analysis. Int. J. Med. Microbiol. 2020;310:151414. <https://doi.org/10.1016/J.IJMM.2020.151414>.
- [25] Stepanović S, Vuković D, Dakić I, *et al.* A modified microtiter-plate test for quantification of *staphylococcal* biofilm formation. J Microbiol Methods 2000;40:175–9. [https://doi.org/10.1016/S0167-7012\(00\)00122-6](https://doi.org/10.1016/S0167-7012(00)00122-6).
- [26] Silva S, Henriques M, Oliveira R, *et al.* *In vitro* biofilm activity of non-*Candida albicans* *Candida* species. Curr Microbiol 2010;61:534–40. <https://doi.org/10.1007/s00284-010-9649-7>.
- [27] Silva S, Henriques M, Martins A, *et al.* Biofilms of non-*Candida albicans* *Candida* species: quantification, structure and matrix composition. Med Mycol 2009;47:681–9. <https://doi.org/10.3109/13693780802549594>.
- [28] Rodrigues CF, Rodrigues ME, Henriques M. Susceptibility of *Candida glabrata* biofilms to echinocandins: alterations in the matrix composition. Biofouling 2018;34:569–78. <https://doi.org/10.1080/08927014.2018.1472244>.
- [29] Falavigna M, Pattacini M, Wibel R, *et al.* The Vaginal-PVPA: A Vaginal Mucosa-Mimicking *In Vitro* Permeation Tool for Evaluation of Mucoadhesive Formulations. Pharmaceutics 2020;12:1–15. <https://doi.org/10.3390/PHARMACEUTICS12060568>.
- [30] Sabouraud Dextrose Broth. Liofilchem® - Sabouraud Dextrose Broth - Rev01 / 09022016
- [31] Johal HS, Garg T, Rath G, *et al.* Advanced topical drug delivery system for the management of vaginal candidiasis. Drug Deliv 2014;23:550–63. <https://doi.org/10.3109/10717544.2014.928760>.
- [32] Li X, Yan Z, Xu J. Quantitative variation of biofilms among strains in natural populations of *Candida albicans*. Microbiology (N Y) 2003;149:353–62. <https://doi.org/10.1099/mic.0.25932-0>.
- [33] Chandra J, Kuhn DM, Mukherjee PK, *et al.* Biofilm formation by the fungal pathogen *Candida albicans*: Development, architecture, and drug resistance. J Bacteriol 2001;183:5385–94. <https://doi.org/10.1128/JB.183.18.5385-5394.2001>.
- [34] Neves J, Rocha CMR, Gonçalves MP, *et al.* Interactions of microbicide nanoparticles with a simulated vaginal fluid. Mol Pharm 2012;9:3347–56. <https://doi.org/10.1021/MP300408M>.
- [35] Wen H, Jung H, Li X. Drug Delivery Approaches in Addressing Clinical Pharmacology-Related Issues: Opportunities and Challenges. AAPS J 2015;17:1327. <https://doi.org/10.1208/S12248-015-9814-9>.
- [36] Ballester-Costa C, Sendra E, Fernández-López J, *et al.* Chemical composition and *in vitro* antibacterial properties of essential oils of four *Thymus* species from organic growth. Ind Crops Prod 2013;50:304–11. <https://doi.org/10.1016/J.INDCROP.2013.07.052>.
- [37] Wang Y, Zeng H, Tian J, *et al.* *In Vitro* and *In Vivo* Activities of Essential Oil from the Seed of *Anethum graveolens* L. against *Candida* spp. Evid Based Complement Alternat Med 2011;2011. <https://doi.org/10.1155/2011/659704>.
- [38] Pietrella D, Angiolella L, Vavala E, *et al.* Beneficial effect of *Mentha suaveolens* essential oil in the treatment of vaginal candidiasis assessed by real-time monitoring of infection. BMC Complement Altern Med 2011;11:1–10. <https://doi.org/10.1186/1472-6882-11-18/FIGURES/5>.

- [39] Larsen B, Monif GRG. Understanding the bacterial flora of the female genital tract. Clin Infect Dis 2001;32:e69-77. <https://doi.org/10.1086/318710>.
- [40] Sobel JD. Vaginitis. N Engl J Med 1997;337:1896–903. <https://doi.org/10.1056/NEJM199712253372607>.
- [41] Chee WJY, Chew SY, Than LTL. Vaginal microbiota and the potential of *Lactobacillus* derivatives in maintaining vaginal health. Microbial Cell Factories 2020 19:1 2020;19:1–24. <https://doi.org/10.1186/S12934-020-01464-4>.
- [42] Matsuda Y, Cho O, Sugita T, *et al.* Culture Supernatants of *Lactobacillus gasseri* and *L. crispatus* Inhibit *Candida albicans* Biofilm Formation and Adhesion to HeLa Cells. Mycopathologia 2018;183:691–700. <https://doi.org/10.1007/S11046-018-0259-4/FIGURES/5>.
- [43] Anderson BL, Mendez-Figueroa H, Dahlke JD, *et al.* Pregnancy-induced changes in immune protection of the genital tract: defining normal. Am J Obstet Gynecol 2013;208:321.e1-321.e9. <https://doi.org/10.1016/J.AJOG.2013.01.014>.
- [44] Nagashima M, Yamagishi Y, Mikamo H. Antifungal susceptibilities of *Candida* species isolated from the patients with vaginal candidiasis. J. Infect. Chemother. 2016;22:124–6. <https://doi.org/10.1016/j.jiac.2015.08.008>.
- [45] Morales DK, Hogan DA. *Candida albicans* Interactions with Bacteria in the Context of Human Health and Disease. PLoS Pathog 2010;6:1–4. <https://doi.org/10.1371/JOURNAL.PPAT.1000886>.
- [46] Parolin C, Marangoni A, Laghi L, *et al.* Isolation of Vaginal Lactobacilli and Characterization of Anti-*Candida* Activity. PLoS One 2015;10:e0131220. <https://doi.org/10.1371/JOURNAL.PONE.0131220>.
- [47] Araújo D, Mil-Homens D, Henriques M, *et al.* Anti-EFG1 2'-OMethylRNA oligomer inhibits *Candida albicans* filamentation and attenuates the candidiasis in *Galleria mellonella*. Mol Ther Nucleic Acids 2022;27:517–23. <https://doi.org/10.1016/j.omtn.2021.12.018>.
- [48] Barry AL, Brown SD. Fluconazole disk diffusion procedure for determining susceptibility of *Candida* species. J Clin Microbiol 1996;34:2154–7. <https://doi.org/10.1128/jcm.34.9.2154-2157.1996>.
- [49] Silva S, Henriques M, Martins A, *et al.* Biofilms of non-*Candida albicans* *Candida* species: quantification, structure and matrix composition. Med Mycol 2009;47:681–9. <https://doi.org/10.3109/13693780802549594>.
- [50] Ferreira C, Gonçalves B, Boas DV, *et al.* *Candida tropicalis* biofilm and human epithelium invasion is highly influenced by environmental pH. Pathog Dis 2016;74. <https://doi.org/10.1093/FEMSPD/FTW101>.
- [51] Alves CT, Wei X-Q, Silva O, *et al.* *Candida albicans* promotes invasion and colonisation of *Candida glabrata* in a reconstituted human vaginal epithelium. J. Infect. 2014;69:396–407. <https://doi.org/10.1016/j.jinf.2014.06.002>.
- [52] Pereira TC, Barros PP de, de Oliveira Fugisaki LR, *et al.* Recent Advances in the Use of *Galleria mellonella* Model to Study Immune Responses against Human Pathogens. J Fungi (Basel) 2018;4. <https://doi.org/10.3390/JOF4040128>.
- [53] Kozics K, Bučková M, Puškárová A, *et al.* The effect of ten essential oils on several cutaneous drug-resistant microorganisms and their cyto/genotoxic and antioxidant properties. Molecules 2019;24:pii: E4570. <https://doi.org/10.3390/molecules24244570>.
- [54] Soares IH, Loreto S, Rossato L, *et al.* *In vitro* activity of essential oils extracted from condiments against fluconazole-resistant and -sensitive *Candida glabrata*. J Mycol Med 2015;25:213–7. <https://doi.org/10.1016/j.mycmed.2015.06.003>.
- [55] Omran SM, Esmailzadeh S. Comparison of anti-*Candida* activity of thyme, pennyroyal, and lemon essential oils versus antifungal drugs against *Candida* species. Jundishapur J Microbiol 2009;2:53–60.
- [56] Brun P, Bernabè G, Filippini R, *et al.* *In Vitro* Antimicrobial Activities of Commercially Available Tea Tree (*Melaleuca alternifolia*) Essential Oils. Curr Microbiol 2019;76:108–16. <https://doi.org/10.1007/s00284-018-1594-x>.

- [57] Mondello F, De Bernardis F, Girolamo A, *et al.* *In vitro* and *in vivo* activity of tea tree oil against azole-susceptible and -resistant human pathogenic yeasts. *J. Infect. Chemother.* 2003;51:1223–9. <https://doi.org/https://doi.org/10.1093/jac/dkg202>.
- [58] Jafri H, Ahmad I. *Thymus vulgaris* essential oil and thymol inhibit biofilms and interact synergistically with antifungal drugs against drug resistant strains of *Candida albicans* and *Candida tropicalis*. *J Mycol Med* 2020;30:100911. <https://doi.org/10.1016/j.mycmed.2019.100911>.
- [59] Rajkowska K, Nowicka-Krawczyk P, Kunicka-Styczynska A. Effect of clove and thyme essential oils on *Candida* biofilm formation and the oil distribution in yeast cells. *Molecules* 2019;24:pii: E1954. <https://doi.org/10.3390/molecules24101954>.
- [60] Hammer KA, Carson CF, Riley T V. Antifungal activity of the components of *Melaleuca alternifolia* (tea tree) oil. *J Appl Microbiol* 2003;95:853–60. <https://doi.org/10.1046/J.1365-2672.2003.02059.X>.
- [61] Cox SD, Mann CM, Markham JL. Interactions between components of the essential oil of *Melaleuca alternifolia*. *J Appl Microbiol* 2001;91:492–7. <https://doi.org/10.1046/j.1365-2672.2001.01406.x>.
- [62] Carson CF, Riley T V. Antimicrobial activity of the major components of the essential oil of *Melaleuca alternifolia*. *Journal of Applied Bacteriology* 1995;78:264–9. <https://doi.org/10.1111/j.1365-2672.1995.tb05025.x>.
- [63] Roxo M, Zuzarte M, Gonçalves MJ, *et al.* Antifungal and anti-inflammatory potential of the endangered aromatic plant *Thymus albicans*. *Sci Rep* 2020;10:18859. <https://doi.org/10.1038/S41598-020-75244-W>.
- [64] Tyagi AK, Malik A. Liquid and vapour-phase antifungal activities of selected essential oils against *Candida albicans*: Microscopic observations and chemical characterization of *Cymbopogon citratus*. *BMC Complement Altern Med* 2010;10:1–11. <https://doi.org/10.1186/1472-6882-10-65/TABLES/3>.
- [65] Wang Y, Liu H, Zhan F. Effects of Natural Borneol on Germ Tube Formation and Preformed Biofilm Activity in *Candida albicans*. *Nat Prod Commun* 2022;17. https://doi.org/10.1177/1934578X221129128/ASSET/IMAGES/LARGE/10.1177_1934578X221129128-FIG2.JPEG.
- [66] Zheng N, Guo R, Wang J, *et al.* Contribution of *Lactobacillus iners* to Vaginal Health and Diseases: A Systematic Review. *Front Cell Infect Microbiol* 2021;11. <https://doi.org/10.3389/FCIMB.2021.792787>.
- [67] Anh DN, Hung DN, Tien TV, *et al.* Prevalence, species distribution and antifungal susceptibility of *Candida albicans* causing vaginal discharge among symptomatic non-pregnant women of reproductive age at a tertiary care hospital, Vietnam. *BMC Infect Dis* 2021;21:1–10. <https://doi.org/10.1186/S12879-021-06192-7/TABLES/2>.
- [68] Pedro NA, Fontebasso G, Pinto SN, *et al.* Acetate modulates the inhibitory effect of *Lactobacillus gasseri* against the pathogenic yeasts *Candida albicans* and *Candida glabrata*. *Microbial Cell* 2023;10:88. <https://doi.org/10.15698/MIC2023.04.795>.
- [69] Amabebe E, Anumba DOC. The Vaginal Microenvironment: The Physiologic Role of Lactobacilli. *Front Med (Lausanne)* 2018;5. <https://doi.org/10.3389/FMED.2018.00181>.
- [70] Zangl I, Pap IJ, Aspöck C, Schüller C. The role of *Lactobacillus* species in the control of *Candida* via biotrophic interactions. *Microbial Cell* 2020;7:1. <https://doi.org/10.15698/MIC2020.01.702>.
- [71] Tachedjian G, Aldunate M, Bradshaw CS, *et al.* The role of lactic acid production by probiotic *Lactobacillus* species in vaginal health. *Res Microbiol* 2017;168:782–92. <https://doi.org/10.1016/J.RESMIC.2017.04.001>.
- [72] Borges S, Silva J, Teixeira P. The role of lactobacilli and probiotics in maintaining vaginal health. *Arch Gynecol Obstet* 2014;289:479–89. <https://doi.org/10.1007/S00404-013-3064-9>.
- [73] Matsubara VH, Bandara HMHN, Mayer MPA, *et al.* Probiotics as Antifungals in Mucosal Candidiasis. *Clin Infect Dis* 2016;62:1143–53. <https://doi.org/10.1093/CID/CIW038>.
- [74] Reid G, Younes JA, Van Der Mei HC, *et al.* Microbiota restoration: natural and supplemented recovery of human microbial communities. *Nat Rev Microbiol* 2011;9:27–38. <https://doi.org/10.1038/NRMICRO2473>.

- [75] Dos Santos CI, França YR, Campos CDL, *et al.* Antifungal and Antivirulence Activity of Vaginal *Lactobacillus* Spp. Products against *Candida* Vaginal Isolates. *Pathogens* 2019, Vol 8, Page 150 2019;8:150. <https://doi.org/10.3390/PATHOGENS8030150>.
- [76] Xu J, Chatterjee M, Baguley TD, *et al.* Inhibitor of the Tyrosine Phosphatase STEP Reverses Cognitive Deficits in a Mouse Model of Alzheimer's Disease. *PLoS Biol* 2014;12:e1001923. <https://doi.org/10.1371/JOURNAL.PBIO.1001923>.
- [77] Silva S, Henriques M, Oliveira R, *et al.* Characterization of *Candida parapsilosis* infection of an *in vitro* reconstituted human oral epithelium. *Eur J Oral Sci* 2009;117:669–75. <https://doi.org/10.1111/J.1600-0722.2009.00677.X>.

CHAPTER IV

EFFECT OF OREGANO ESSENTIAL OIL IN *CANDIDA* INFECTION

Vulvovaginal candidiasis (VVC), one of the most prevalent vaginal infectious diseases, faces increasing challenges attributed to drug-resistant *Candida* strains. Considering the therapeutic limitations, essential oils (EOs) are emerging as promising alternatives due to their high antimicrobial activity. Among these, oregano essential oil (OEO) stands out for its proven efficacy against *Candida* species resistant to conventional treatments. Nevertheless, the direct application of OEO presents some limitations, including cytotoxicity. Interestingly, vapor-phase of essential oils (VP-EOs) have more advantages than their direct application. Therefore, focusing exclusively on the vapor-phase application seems to be particularly effective. Thus, a comprehensive study of the mode of action of the vapor-phase of the OEO becomes crucial for potential clinical applications.

In addition to vapor-phase application, microencapsulation emerges as an innovative approach to address the challenges associated with the direct application of OEO. This method aims to overcome the problems associated with OEO, providing protection, and facilitating a controlled and prolonged release of the OEO. Microencapsulation serves as a strategic solution, addressing concerns about cytotoxicity and enhancing the overall therapeutic potential of OEO.

These advances mark a significant step in the search for effective and safe treatments for VVC, offering a comprehensive approach to harnessing the benefits of EOs while minimizing the associated disadvantages.

CHAPTER IV.1

EFFECT OF VAPOR-PHASE OREGANO ESSENTIAL OIL ON RESISTANT *CANDIDA* SPECIES BIOFILMS: MECHANISMS OF ACTION

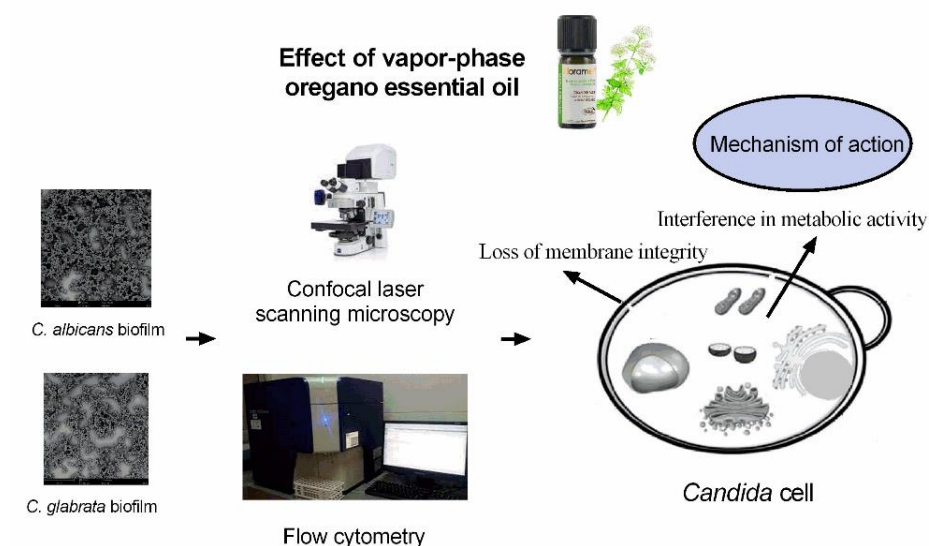


Figure 29. Subchapter IV.1 workflow.

Oregano essential oil has gained attention for its remarkable efficacy against *Candida* species. This suggests that OEO may offer a valuable solution for the treatment of VVC. This subchapter aims to evaluate the effect of vapor-phase of oregano essential oil (VP-OEO) on biofilms of antifungal-resistant vaginal isolates of *Candida* species (*Candida albicans* and *Candida glabrata*) and determine its mode of action. Furthermore, a reconstituted vaginal epithelium was used to mimic vaginal conditions and evaluate the effect of VP-OEO on *Candida* species infection.

This subchapter is based on the following publication: Fernandes L, Costa R, Silva S, Henriques M, Costa-de-Oliveira S, Rodrigues ME. Effect of Vapor-Phase Oregano Essential Oil on Resistant *Candida* Species Biofilms: Mechanisms of Action. Microbiol Spectr 2023;11. <https://doi.org/10.1128/SPECTRUM.05124-22>.

And presented as poster: Fernandes L, Costa-de-Oliveira S, Costa R, Silva S, Henriques M, Rodrigues ME. Effect of vapor-phase essential oil on resistant *Candida* species: Mechanisms of action. FEMS Conference on Microbiology 2022, Belgrade, Serbia.

And part of this subchapter was presented as poster: Fernandes L, Blázquez E, Tejada A., Ribeiro, A, Silva S, Mira N, Cussó L, Costa-de-Oliveira S, Rodrigues ME, Henriques M. Oregano Essential Oil: An Effective and Non-Toxic Approach for prevent or treat Resistant *Candida* Species. Congress of Microbiology and Biotechnology 2023 (FEMs grant), 2023, Covilhã, Portugal.

1. Introduction

Antimicrobial resistance is increasing over time, threatening the effective prevention and treatment of an ever-increasing range of infectious diseases [1]. One of the most common and frequent fungal infections is candidiasis, ranging from superficial mucosal infections (oral cavity, vagina, penis, gastrointestinal tract, or other parts) to systemic and potentially fatal diseases (disseminated candidiasis) [2]. Indeed, vulvovaginal candidiasis (VVC) affects millions of women of reproductive age every year, causing physical discomfort, pain, and mental distress and representing considerable direct and indirect costs [3,4]. VVC is caused by *Candida* species, namely, *Candida albicans* and *Candida glabrata*, both phenotypically and genetically different [5]. Although *C. albicans* is more frequently associated with candidiasis, the incidence of infections caused by *C. glabrata* is increasing, showing overall greater resistance to common antifungal agents, especially azoles [6–8]. These species are associated with biofilm formation, one of the main virulence factors associated with increased resistance to antifungal agents [6,7]. Even though several years ago, antifungal agents exerted a potent effect, these agents are currently no longer effective [9]. Thus, discovering new and effective alternative therapies or improving/reinventing already used therapies is fundamental.

From the past until today, plants and their derivatives have been employed in traditional medicine, alternative medicine, or phytotherapeutic applications for the prevention and treatment of several diseases [10]. In this sense, essential oils (EOs) have been suggested as potential sources of new therapeutic products due to their antileishmanial, antiviral, anti-inflammatory, antioxidant, hepatoprotective, antitumor, and antimicrobial activities [11,12]. Furthermore, EOs have advantages such as fewer side effects, less toxicity, and better biodegradability than available antimicrobial agents [13]. These natural products are biosynthesized by glandular trichomes and other secretory structures in plants, being liquids that are particularly rich in volatile molecules such as monoterpenes and sesquiterpenes with their oxygenated derivatives (phenols, oxides, esters, ketones, and aldehydes), phenylpropanoids (alcohols, aldehydes, phenols, and short-chain aliphatic hydrocarbon), and nitrogenous or sulfured components [14,15]. The inhibitory activities of EOs against many fungal pathogens have been widely reported [16], including EOs from the *Lamiaceae* family such as *Origanum* species and *Thymus* species, which have been described as being particularly effective against various microorganisms, particularly *Candida* species [17–21]. In fact, oregano EO (OEO) was able to inhibit, *in vitro*, the germination and development of the filamentous form of *C. albicans* [8,17,22]. Moreover, Hacıoglu *et al.* [23] also showed previously that OEO inhibits the three phases of *Candida* biofilms, i.e.,

their adhesion, formation, and mature state. Those authors also showed the ability to reduce biofilm formation by up to 50 % when biofilms were formed on surfaces previously coated with OEO [23].

The literature provides information about the impact of the direct application of EOs on *Candida* species [10,11,16], but few data are available on the antifungal effect of the vapor-phase of essential oils (VP-EOs) [24], mainly on *Candida* species biofilms. Despite this, studies have shown that VP-OEO showed greater antimicrobial activity than its liquid phase [25,26].

The objective of this subchapter was to evaluate the abilities of the vapor-phase of oregano essential oil (VP-OEO) to inhibit biofilm formation and destroy mature biofilms of antifungal-resistant vaginal isolates of *Candida* species. Furthermore, the mode of action of VP-OEO against two antifungal-resistant vaginal isolates of *Candida* species (*C. albicans* and *C. glabrata*) was evaluated.

2. Materials and Methods

2.1 Oregano essential oil

This study evaluated the antifungal activity of oregano (*Origanum compactum*) EO (Florame, France) with 100 % purity. OEO samples were stored at room temperature under dark conditions.

Chromatographic analysis (gas chromatography [GC]) of the OEO was performed by Florame (Saint-Rémy-de-Provence, France) (Annex I.5). The OEO samples were introduced into the GC system (6890 GC and 5975 mass spectrometry [MS] systems) under the following conditions: a 1- μ L injection split of 1/200 was used, an HP5 MS capillary column (30 m/ 0.25 mm) with a film thickness of 0.25 μ m was used, the column temperature was 60 °C to 250 °C, helium was used as the carrier gas at a constant flow rate of 1.0 mL min⁻¹, and the mass range was set at 40 to 450 atomic mass units (amu). The acquisition time was 100 min.

2.1.1 Oregano essential oil cytotoxicity

To assess the toxicity of VP-OEO, the *Galleria mellonella* survival model was used, and the rate of larval survival was determined. For this, 10 *G. mellonella* larvae were placed into glass petri dishes containing a glass well where the oil was placed (controlled atmosphere with a volume of 1.5×10^6 cm³), allowing its diffusion without contact with the larvae (Subchapter III.2). Larvae were stored at 37 °C under dark conditions, and larval survival was monitored over 72 h. In addition, the total number of hemocytes present in the hemolymph of the larvae after exposure to VP-OEO was evaluated at 4 h and 72 h, as described previously by Araújo *et al.* [27]. Briefly, 5 larvae sanitized with 70 % (v/ v) ethanol were

punctured in the abdomen with a sterile needle, and the hemolymph was collected into a sterile microtube and diluted 10 times in phosphate-buffered saline (PBS). Next, the hemocytes were counted with a Neubauer chamber, and the results were presented as the logarithm of the concentration (Log_{10}). The control was carried out in the same way, without the larvae being exposed to VP-OEO. All experiments were performed in triplicate in a minimum of three independent assays.

2.2 Microorganisms and culture conditions

In this study, 13 drug-resistant *Candida* isolates, *C. albicans* (n = 3), *C. glabrata* (n = 8), *C. krusei* (n = 1), and *C. guilliermondii* (n = 1), belonging to the Biofilm Research Group of the Centre o Biological Engineering, were used (Subchapter II.3). The clinical isolates were subcultured from a frozen stock (Sabouraud dextrose broth (SDB; Liofilchem) medium with 20 % (v/ v) glycerol at $-80\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$) onto Sabouraud dextrose agar (SDA; Liofilchem) plates and incubated for 24 h. Prior to testing, a preinoculum was prepared in SDB with colonies from the SDA plates for 18 h at $37\text{ }^{\circ}\text{C}$ under agitation (120 rpm). Next, the cellular suspensions were centrifuged and washed twice with PBS ($5,000 \times g$ for 10 min at $4\text{ }^{\circ}\text{C}$).

For biofilm assays, vaginal *Candida* isolates cells were cultivated in simulated vaginal fluid (SVF). SVF consisted of 58 mM NaCl (Biochem), 18 mM KOH (AppliChem), 2 mM $\text{Ca}(\text{OH})_2$ (Frilabo), 1.75 mM glycerol (Biochem), 6.7 mM urea (Frilabo), 33 mM glucose (Biochem), and 6.7 g L^{-1} yeast nitrogen base (YNB;(Difco)). In addition, natural compounds in the vaginal fluid, such as acetic acid (17 mM; pKa, 54.76) and lactic acid (22 mM; pKa, 53.85), were added to maintain the pH at 4.2 as described previously in subchapter III.1 [28,29].

2.3 Planktonic antimicrobial susceptibilities to oregano essential oil

The inhibitory effects of direct contact with OEO (DC-OEO) and VP-OEO on the growth of 13 drug-resistant *Candida* isolates were first evaluated by the agar disk diffusion method, as described in the previous chapters and by Tran *et al.* [30] and Vihanova *et al.* [31], with some modifications. Briefly, the agar surface was inoculated using a swab dipped in a cell suspension adjusted to 1×10^8 cells mL^{-1} . After the inoculum was dried, a sterile filter paper disk (6 mm) (Liofilchem) was impregnated with 25 μL of OEO and directly and indirectly (for vapor-phase action) applied, that is, placed on top of the plates on the cell suspension (direct application) and placed on the cover of the SDA plate, without touching the cell suspension, and incubated inverted (indirect application). Plates with disks without OEO were also included as controls. All plates were then sealed with parafilm and incubated at $37\text{ }^{\circ}\text{C}$, and the diameters

of the zones around the disks were measured within 24 h. All experiments were performed in triplicate. The experiments were carried out in glass petri dishes due to the high corrosive power of 100 % EOs, according to Chapter II.

2.4 Effect of the vapor-phase of oregano essential oils on biofilms

The antifungal effects of the VP-OEOs on both biofilm formation and mature biofilms (24 h old) of two fluconazole-resistant *Candida* isolates (*C. glabrata* Cg8 and *C. albicans* Ca2) were evaluated. The *Candida* isolate biofilms were developed as described previously in Chapter II and III and by Stepanović *et al.* [32]. After the pre-inoculum was resuspended and washed twice with PBS, the initial cell concentration was adjusted to 1×10^8 cells mL⁻¹ in SVF. Next, the cellular suspension was transferred to glass wells inside glass petri plates (1 mL/ well) (controlled atmosphere with a volume of 1.5×10^6 cm³) and incubated aerobically for 24 h (120 rpm at 37 °C). The effect of the VP-OEO was evaluated from two different perspectives: (i) prophylactic treatment, where the therapeutic agent (25 µL of OEOs [100 %]) was placed on a sterile blank disk, which was positioned near the wells (the set was kept inside a glass plate), and added at time zero, and (ii) infection treatment, where the therapeutic agent was added to preformed biofilms (24 h).

After 24 h of treatment, the wells were washed with a saline solution, and the resulting biofilm-cell suspensions were then serially diluted in PBS, plated onto SDA plates, and incubated aerobically for 24 h at 37 °C. Next, the number of grown colonies was counted. The results were expressed as Log CFU per milliliter. The experiment was performed in triplicate with three independent assays.

2.4.1 Determination of the mechanism of action

2.4.1.1 Biofilm time-dependent killing assay

After the above-described assays, it was decided to perform this part of the study only with *C. glabrata* Cg8 and *C. albicans* Ca2 biofilms. The effect of VP-OEO on these species was evaluated by a time-dependent killing assay. This assay was performed according to methods described previously by Sumiyoshi *et al.* [33], with some modifications. *Candida* biofilms were formed as described above. Inhibition of both biofilm formation and preformed biofilms over time after exposure to VP-OEO (0, 2, 4, 8, 12, 14, 17, 18, and 24 h) was measured. For this, after antifungal treatment and subsequent washing with a saline solution, the suspensions were serially diluted in PBS, plated onto SDA plates, and incubated

aerobically (24 h at 37 °C). Afterward, the number of grown colonies was counted, and the results were expressed as Log reductions in the CFU per milliliter. Each analysis was performed in triplicate.

2.4.1.2 Flow cytometry

According to the biofilm time-dependent killing assay, preformed and mature biofilms of *C. glabrata* Cg8 and *C. albicans* Ca2 after 10, 12, 14, and 18 h of exposure to VP-OEO were analyzed by flow cytometry. Biofilms were formed as described above, and samples of 1 mL were collected after VP-OEO exposure. *Candida* cells killed by treatment with 70 % ethanol for 20 min and *Candida* cells without treatment were used as controls. The cells were centrifuged at 4 °C for 3 min at $10,000 \times g$, the pellets were resuspended and washed twice with PBS, the suspension concentration was adjusted to 1×10^6 cells mL⁻¹, and the cells were counted in a Neubauer chamber, as described previously by Pina-Vaz *et al.* [34]. Two fluorescent probes were used, propidium iodide (PI) and FUN-1 {2-chloro-4-[2,3-dihydro-3-methyl-(benzo-1,3-thiazol-2-yl)-methylidene]phenylquinolinium iodide}. To evaluate cell membrane integrity, cell suspensions were incubated for 30 min in the presence of PI (1.0 µg mL⁻¹) (Sigma-Aldrich, Munich, Germany) in the dark at room temperature. To assess the metabolic activity, the cellular suspensions were centrifuged, and the pellets were resuspended in sterilized H₂O supplemented with 2 % glucose and incubated in the presence of 0.5 µM FUN-1 (Invitrogen Molecular Probes, OR, USA) for 30 min with protection from light. For each isolate, nonstained cells (autofluorescence) and the fluorescence of nontreated and dead controls were also evaluated. The samples were analyzed with a FACSCalibur cytometer (BD Biosciences, Sydney, Australia) equipped with 3 photomultipliers (PMTs), standard filters, and a 15-mW, 488-nm argon laser using CellQuest Pro software (version 4.0.2). The cell scattergram (forward scatter [FS] and side scatter [SS]) and the intensity of fluorescence at fluorescent channel FL1 (green fluorescence, 530 nm), FL2 (yellow-green fluorescence, 575 nm), and FL3 (red fluorescence, 630 nm) were logged using a logarithmic scale.

PI results are expressed as a percentage of cells showing high fluorescence in FL3, and FUN-1 results are expressed as the staining index (SI), defined as the ratio between the mean fluorescence of the treated cell suspensions (VP-OEO) and the value corresponding to the control cells (no exposure to VP-OEO), in FL2 [34]. Each assay was performed in triplicate.

2.4.1.3 Confocal laser scanning microscopy analysis

In order to evaluate the cell morphology and viability of *C. glabrata* Cg8 and *C. albicans* Ca2 biofilms (biofilm formation and preformed biofilms) after 12 h of exposure to VP-OEO, the Live/ Dead

BacLight bacterial viability kit (Molecular Probes, Leiden, The Netherlands) was employed. Briefly, biofilms were formed on glass coupons (inserted into the glass wells, as described above) after 12 h of exposure to VP-OEO, for both biofilm formation and preformed biofilms, and the coupons were washed with 0.85 % NaCl and then stained for 15 min in the dark with a mixture of SYTO-9 (Invitrogen Molecular Probes, OR, USA) ($3 \mu\text{L mL}^{-1}$) and PI ($3 \mu\text{L mL}^{-1}$). Samples were observed using an Olympus (Tokyo, Japan) BX61 model FluoView 1000 confocal scanning laser microscope. The combination of optical filters consisted of a laser excitation line at 488 nm, emission filters BA 505 to 540 nm (green fluorescence) with an excitation line at 559 nm, and emission filters BA 575 to 675 nm (red fluorescence). Images were acquired and analyzed with the FV10-Ver4.1.1.5 program (Olympus). The assay was repeated two independent times with two technical replicates.

2.5 Effect of oregano essential oil on *Candida* species infection of a reconstituted human vaginal epithelium

To mimic human vaginal conditions, a commercially reconstituted human vaginal epithelium (RHVE) (0.5 cm²; SkinEthic Laboratories) was infected with *Candida* species (*C. glabrata* Cg8 and *C. albicans* Ca2), and the effect of VP-OEO on vaginal candidiasis attenuation was evaluated. For this, epithelia were infected with a cell suspension of *C. glabrata* and *C. albicans* (initial concentration adjusted to 1×10^8 cells mL⁻¹ in SVF). Epithelia devoid of *Candida* cells but with SVF exposed and unexposed to VP-OEO were used as controls. All epithelia were incubated for 24 h (120 rpm at 37 °C). After incubation, the tissues were washed once with PBS to remove nonadherent *Candida* cells. Epithelia were then bisected, with one half being used for histological analysis (2.5.1) and the other half being used for molecular studies (2.5.2) [35].

2.5.1 Microscopic observation

RHVE tissue for microscopic analysis was fixed in 2 % (v/v) paraformaldehyde, stored at room temperature, and embedded in paraffin wax using standard histological techniques. RHVE sections (20 μm) were cut, placed onto HistoBond+-coated microscope slides, and dewaxed by processing through xylene, followed by immersion in ethanol and then in water. The prepared sections were then stained using the periodic acid-Schiff method for keratinocyte and *Candida* cell staining.

After histological processing, samples were analyzed in bright field using an Olympus BX51 epifluorescence microscope coupled with a DP72 digital camera (Olympus Portugal SA, Portugal). All tissue images were acquired using Olympus Cell-B software.

2.5.2 Quantification of *Candida* cells

DNA present in the tissues was extracted using a DNA extraction kit (DNeasy blood and tissue kit; Qiagen). *Candida* cells were quantified using real-time PCR by employing a CFX96 real-time PCR system (Bio-Rad, Berkeley, CA, USA). Each reaction mixture consisted of 10 µL of a working concentration of SsoFast EvaGreen supermix, 0.2 µL of each primer (Table 9) and 4 µL of DNA, in a final reaction mixture volume of 20 µL.

Table 9. Primers for real time-PCR assay for quantification of *Candida albicans* and *Candida glabrata* cells

Target	Primer	Sequence (5' -> 3')
<i>Candida albicans</i>	Forward	GAGCGTCGTTTCTCCCTCAAACCGCTGG
	Reverse	GGTGGACGTTACCGCCGCAAGCAATGTT
<i>Candida glabrata</i>	Forward	ATTTGCATGCGCTTGCCACGAATCC
	Reverse	ACGTCTGATCCAATCAATGGCTGGTGA

Negative controls were performed using a reaction mixture with nuclease-free water replacing the DNA template. PCR cycling conditions consisted of an initial denaturation step at 94 °C for 3 min followed by 40 cycles of denaturation at 95 °C for 10 s and primer annealing at 55 °C for 30 s. For each *Candida* isolate, calibration curves (CT [threshold cycle] versus Log cells) were constructed using the same PCR protocol as the one described above (Subchapter III.2), from serial dilutions of *Candida* cell concentrations, as described previously by Alves *et al.* [35].

2.5.3 Lactate dehydrogenase assay

The release of lactate dehydrogenase (LDH) from the RHVE into the culture medium was used as a measure of epithelial cell damage using the CytoTox-ONE homogeneous membrane integrity assay kit (Promega). The LDH released during infection and VP-OEO treatment for the two *Candida* strains was expressed as LDH activity relative to the values for untreated *C. albicans* Ca2 and *C. glabrata* Cg8. In addition, the effect of VP-OEO on tissue, expressed as the LDH activity relative to that in tissue with SVF (control), was also evaluated. LDH activity was analyzed in a spectrophotometer (FLUOstar Optima; BMG Labtech, Ortenberg, Germany) at a 560-nm excitation wavelength and a 590-nm emission wavelength. All experiments were performed in triplicate.

2.6 Statistical analysis

The results were statistically analyzed using the Prism software package (version 8.01; GraphPad Software). One-way analysis of variance (ANOVA) was performed, and means were compared by applying Tukey's multiple-comparison test. The statistical analyses performed were considered significant when the p value was < 0.05 .

3. Results and Discussion

The high incidence of resistant *Candida* species and the associated negative consequences and therapeutic limitations make it crucial to develop effective, inexpensive, and nontoxic alternative treatments. In this sense, OEO has demonstrated good fungicidal and bactericidal activities against different pathogens [36–38]. Recent studies also showed that the volatile part of the EO (VP-OEO) has interesting antimicrobial activity (Subchapter II.3 and III) [39,40]. Thus, considering the highly effective anti-*Candida* activity evidenced by OEO described in the literature, it is essential to deepen the knowledge of the effect of the vapor-phase of this oil alone, a phase that is often neglected.

In the first stage of this study, the *in vivo* toxicity of VP-OEO was evaluated using the *G. mellonella* model [27]. After 72 h of exposure of larvae to VP-OEO, the results revealed that was no evidence of toxicity. In fact, until 72 h, all larvae remained alive (Figure 30.A), with no significant differences in the total numbers of hemocytes in the larval hemolymphs (Figure 30.B) exposed and not exposed to VP-OEO.

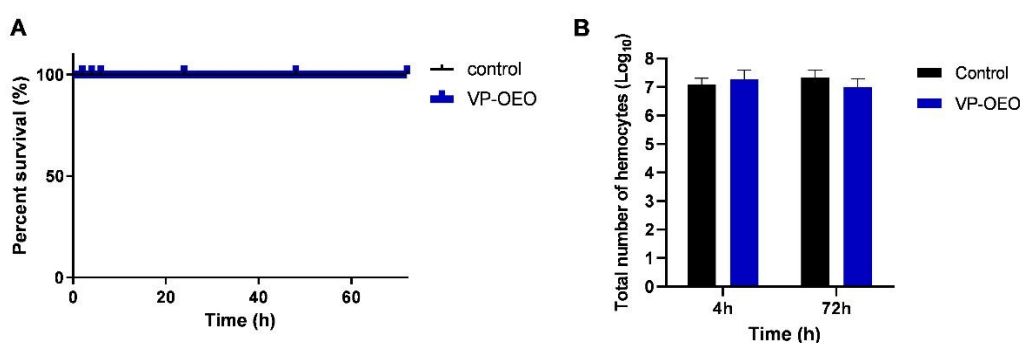


Figure 30. Toxicity of the vapor-phase of oregano essential oil (VP-OEO) measured in a *Galleria mellonella* model. **(A)** Survival curves of *G. mellonella* larvae exposed to VP-OEO and the respective controls (without exposure to VP-OEO). **(B)** Total numbers of hemocytes counted in *G. mellonella* larvae after 4 and 72 h of exposure and those unexposed to VP-OEO.

After verifying the nontoxicity of VP-OEO, the antifungal activity of VP-OEO against antifungal-resistant vaginal isolates of several *Candida* species was evaluated, and the effect of VP-OEO was compared with that of direct contact with OEO (DC-OEO). For this, an initial screening of the OEO against antifungal-resistant vaginal isolates of *C. albicans*, *C. glabrata*, *C. guilliermondii*, and *C. krusei* was performed using the disk diffusion method (DC and VP) (Table 10).

Table 10. Anti-*Candida* activity of oregano essential oil on drug-resistant isolates evaluated through the disk-diffusion method by contact direct (DC-OEO) and vapor-phase (VP-OEO)

Species	Isolate	Oregano essential oil Inhibition zone (mm)	
		Direct contact (DC-OEO)	Vapor-phase (VP-OEO)
<i>C. glabrata</i>	Cg1	52.7 ± 2.5	54.7 ± 2.1
	Cg2	51.0 ± 5.6	57.3 ± 2.5
	Cg3	38.0 ± 2.8	31.3 ± 2.3
	Cg4	68.0 ± 3.5	56.0 ± 5.3***
	Cg5	51.7 ± 2.9	50.0 ± 3.0
	Cg6	57.7 ± 4.0	44.0 ± 3.6***
	Cg7	43.0 ± 2.5	36.0 ± 3.6
	Cg8	38.3 ± 1.2	36.3 ± 2.3
<i>C. albicans</i>	Ca1	53.7 ± 3.2	30.3 ± 2.9****
	Ca2	46.3 ± 4.2	35.3 ± 3.8*
	Ca3	53.3 ± 6.1	27.5 ± 2.1****
<i>C. krusei</i>	Ck1	50.8 ± 2.2	43.7 ± 3.2*
<i>C. guilliermondii</i>	Cgi1	62.0 ± 2.7	77.3 ± 3.1***

Asterisks indicate statistical significance between DC-OEO and VP-OEO (* $p < 0.1$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$)

Both DC-OEO and VP-OEO were able to inhibit the growth of all tested isolates but with different impacts (inhibition zones of 27 to 77 mm), and different results were obtained depending on the *Candida* species. The effects of DC-OEO and VP-OEO were similar in almost all of the *C. glabrata* isolates except the *C. glabrata* Cg4 and *C. glabrata* Cg6. In these two strains, VP-OEO had a smaller effect than DC-OEO ($p < 0.001$). In contrast, significantly reduced effects on *C. albicans* and *C. krusei* were obtained with VP-OEO ($p < 0.1$ and $p < 0.0001$, respectively), and the opposite was observed for *C. guilliermondii* ($p < 0.001$). In fact, *C. guilliermondii* proved to be more susceptible to both DC-OEO and VP-OEO. The different antifungal activities of DC-OEO and VP-OEO may result from the characteristics of the EOs. Indeed, in the liquid phase (DC-OEO), the activity of the EOs depends on two factors, the diffusibility and solubility of the EOs in the medium, while the effect of VP-OEO depends only on the volatility of the EO [26]. For the study of the mechanism of action of EOs or volatile substances, volatility and hydrophilicity or

lipophilicity must be factors to be considered. Indeed, the lipophilic character allows interaction with lipid structures of the fungal cell membrane, allowing easy penetration and modifying cell membrane properties such as permeability and fluidity. Consequently, fungal cells suffer structural damage that can result in cell death [39]. However, the lipophilic molecules in the aqueous medium associate to form micelles, suppressing the fixation of EOs on the microorganism, while in the VP-EO, it allows free fixation [40]. The level of lipophilicity is related to the polar functional groups in its constitution [41].

According to these data, it can be said that the results obtained during this work are closely related to the composition of the OEO (Table 11). This EO contained the following major components: carvacrol (36.97 %, v/ v), thymol (21.44 %, v/ v), p-cymene (15.27 %, v/ v), and γ -terpinene (13.35 %, v/ v). However, several factors must be considered to predict the volatility of compounds, such as molar mass, vapor pressure, molecular structure (the existence of functional groups), Log K_{ow} (octanol/water partition coefficient), and polarity.

Table 11. Compounds identified (%) in oregano essential oil, and their characteristics, such as vapor pressure (mm Hg), water solubility (mg L⁻¹) and Log K_{ow}

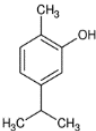
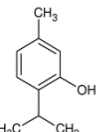
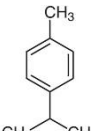
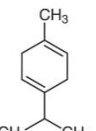
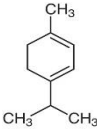
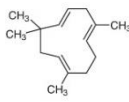
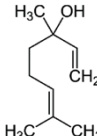
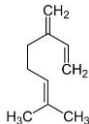
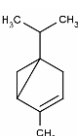
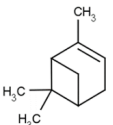
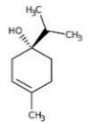
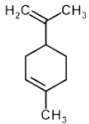
Compound, molecular formula and molar mass	In oregano oil (%)	Molecular structure	Vapor pressure (mm Hg) (at 25 °C)	Solubility in water (mg L ⁻¹)	Log K_{ow}
Carvacrol C ₁₀ H ₁₄ O (150.21 g mol ⁻¹)	36.97		0.029	1250 (at 25 °C) High solubility	3.49
Thymol C ₁₀ H ₁₄ O (150.22 g mol ⁻¹)	21.44		0.038	900 (at 25 °C) Moderately soluble	3.30
p-Cymene C ₁₀ H ₁₄ (134.21 g mol ⁻¹)	15.27		1.50	23.4 (at 25 °C) Moderately soluble	4.10
γ-terpinene C ₁₀ H ₁₆ (136.23 g mol ⁻¹)	13.35		1.1	8.68 (at 23.5 °C) Low solubility	4.36

Table 11. Compounds identified (%) in oregano essential oil, and their characteristics, such as vapor pressure (mm Hg), water solubility (mg L⁻¹) and Log K_{ow} (continuation)

Compound, molecular formula and molar mass	In oregano oil (%)	Molecular structure	Vapor pressure (mm Hg) (at 25 °C)	Solubility in water (mg L ⁻¹)	Log K _{ow}
α-terpinene C ₁₀ H ₁₆ (136.23 g mol ⁻¹)	2.04		1.66	5.915 (at 25 °C) Low solubility	4.53
α-caryophyllene C ₁₅ H ₂₄ (204.36 g mol ⁻¹)	1.97		0.008	0.014 (at 25 °C) Low solubility	6.59
Linalool C ₁₀ H ₁₈ O (154.25 g mol ⁻¹)	1.55		0.16	1590 (at 25 °C) High solubility	2.97
Myrcene C ₁₀ H ₁₆ (136.23 g mol ⁻¹)	1.39		2.29	5.6 (at 25 °C) Low solubility	4.17
α-thujene C ₁₀ H ₁₆ (136.24 g mol ⁻¹)	0.89		4.77	2.91 (at 25 °C) Low solubility	4.02
α-pinene C ₁₀ H ₁₆ (136.24 g mol ⁻¹)	0.66		4.75	2.49 (at 25 °C) Low solubility	4.83
Terpinen-4-ol C ₁₀ H ₁₈ O (154.25 g mol ⁻¹)	0.63		0.048	386.6 (at 25 °C) Moderately soluble	3.26
Limonene C ₁₀ H ₁₆ (136.24 g mol ⁻¹)	0.55		1.54	4.58 (at 25 °C) Low solubility	4.38

p-Cymene, *γ*-terpinene, *α*-terpinene, myrcene, *α*-thujene, *α*-pinene, and limonene are the most volatile components (vapor pressure of >1). As monoterpenes (such as *γ*-terpinene, *α*-terpinene, myrcene, *α*-thujene, *α*-pinene, and limonene) do not have functional groups, these compounds have the highest vapor pressure and therefore are the most volatile and also the most lipophilic (higher Log K_{ow}).

Indeed, Muñoz *et al.* [42] demonstrated previously that limonene acts on yeast genetic material, leading to damage to the cell wall and intracellular structures, including nuclear alterations, and induces dramatic structural changes in organelles, accompanied by cell wall disruption. Of the monoterpene compounds, the cyclic monoterpenes α -thujene and α -pinene are the most volatile (4.75 to 4.77 mm Hg of vapor pressure), although they are present at a lower percentage of the total oil composition. Nóbrega *et al.* [43] showed previously that α -pinene showed significant antifungal activity against *Candida* isolates and fungicidal action and proved to be effective in inhibiting fungal structures such as pseudohyphae and promoting a marked decrease in blastoconidia. Linalool and terpinen-4-ol are also monoterpenes but with alcohol function, so these compounds are more hydrophilic.

In turn, p-cymene, carvacrol, and thymol are aromatic compounds with a benzene ring; however, p-cymene, as it is not a phenol, unlike carvacrol and thymol, which have the hydroxyl group attached directly to the benzene ring, becomes less volatile and hydrophilic. Several studies have shown that carvacrol (high solubility) and thymol (moderate solubility) exerted interesting anti-*Candida in vitro* activity as determined by broth microdilution (DC), even against fluconazole-resistant *Candida tropicalis* and *C. glabrata* strains [44–46]. Indeed, high concentrations of carvacrol may be directly related to germ tube inhibition and are also often considered mainly responsible for modifying membrane permeability [22,47–49]. However, studies that compared the antimicrobial effects of EOs and purified components, such as carvacrol, in equal concentrations showed that EOs were more effective than the purified compounds [50]. Thus, the synergism between carvacrol and other components at lower concentrations may be more effective than purified carvacrol [22]. In fact, carvacrol together with thymol can facilitate the entry of EO compounds through the fatty acid chains that make up the lipid bilayers of the membrane and, consequently, modify the permeability and fluidity of cell membranes [51].

Deep fungal infections, caused mainly by *Candida* species, are associated with biofilm formation that leads to the development of resistance and, consequently, become extremely difficult to treat with conventional antifungal agents [52–54]. Therefore, the effects of the VP-OEO on *Candida* biofilm formation and preformed biofilms of two antifungal-resistant strains were evaluated. In this sense, vaginal isolates of *C. albicans* and *C. glabrata* were selected: *C. albicans* Ca2 presents extremely high-level resistance to fluconazole (Chapter II and III), and *C. glabrata* Cg8 presents resistance to fluconazole and ketoconazole, a less common azole. Thus, the results obtained from this assay showed that VP-OEO induced the total inhibition of these two species under the two conditions tested, that is, biofilm formation and preformed biofilms (Figure 31). Exposure to VP-OEO for 24 h led to reductions of approximately 6 Log CFU mL⁻¹ and 10 Log CFU mL⁻¹ ($p < 0.0001$) for *C. albicans* Ca2 and *C. glabrata* Cg8, respectively. Thus,

these results showed the great effectiveness of VP-OEO against both the establishment of a biofilm and mature biofilms.

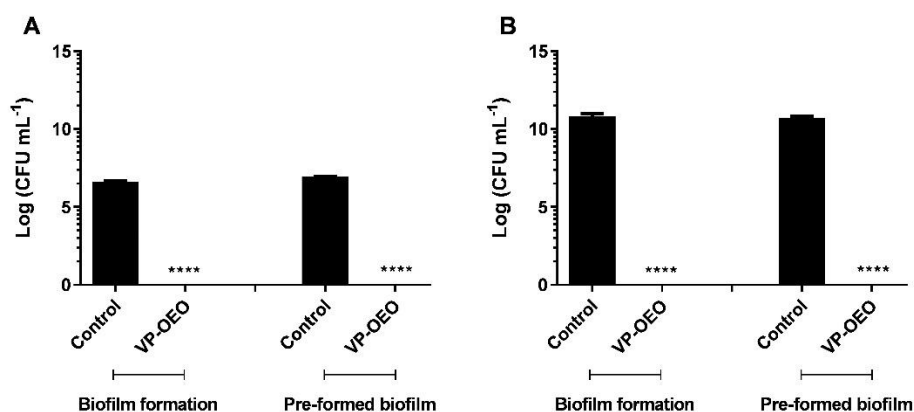


Figure 31. Effect of the vapor-phase of oregano essential oil (VP-OEO) on biofilm formation and preformed biofilms of the antifungal-resistant *Candida* isolates (A) *Candida albicans* Ca2 and (B) *Candida glabrata* Cg8. * indicate a statistically significant reduction in biofilm cell cultivability in comparison with the respective controls (*, $P < 0.1$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

These results are supported by previous study (Subchapter II.3) where the antifungal activity of tea tree VP-EO (VP-TTEO) was evaluated: VP-TTEO induced a significant inhibitory effect on biofilm formation (2 to 3.5 Log reductions) and caused a significant reduction in mature biofilms (3 to 5 Log reductions) (subchapter II.3). In fact, it has been shown that due to their diffusibility and mode of contact, EOs can be effective in controlling biofilm cultures compared to planktonic cells [26]. Furthermore, Karpanen *et al.* [55] noted previously that thymol showed increased activity against *Staphylococcus epidermidis* cells growing in biofilms compared to planktonic cells; thus, those authors suggested that thymol, being a phenolic compound, has hydrophilic and hydrophobic properties that may increase the diffusion of this compound in a biofilm and allow its access to fungal cells where it alters the permeability of the plasma membranes. In this study, although thymol has a low vapor pressure value (0.038 mm Hg), it is one of the main constituents of OEO, so it can be suggested that the observed effect of VP-OEO on the *C. albicans* Ca2 and *C. glabrata* Cg8 biofilms is due, in part, to this compound.

The mechanisms of action of EOs in their liquid phase have been described in the literature by several authors [39,47]. However, the antimicrobial activity mediated by the VP-EO is often neglected, underestimated, or ignored, so there are few studies focusing on the mechanisms of action of the VP-EO alone. A recognized and important fact is that the mechanism of action of the EOs can diverge according

to the way in which the EOs come into contact with microorganisms; therefore, it is crucial to try to understand the mode of action involved in the effect of VP-EO. For this, assessments of cell damage and metabolic activity with VP-OEO were performed by flow cytometry and confocal laser scanning microscopy (CLSM) analyses. Flow cytometry has been used to determine the quantitative and qualitative properties of cells isolated from biofilms. Thus, the effect of VP-OEO was based on the detection of increased membrane permeability of *Candida* cells to propidium iodide (PI) (percentage of dead cells) and the measurement of metabolic activity using the dye 2-chloro-4-[2,3-dihydro-3-methyl-(benzo-1,3-thiazol-2-yl)-methylidene]phenylquinolinium iodide (FUN-1) (staining index [SI]), from the moment when VP-OEO started to exert an effect on cell viability according to a time-dependent killing assay (Figure 32 and 33).

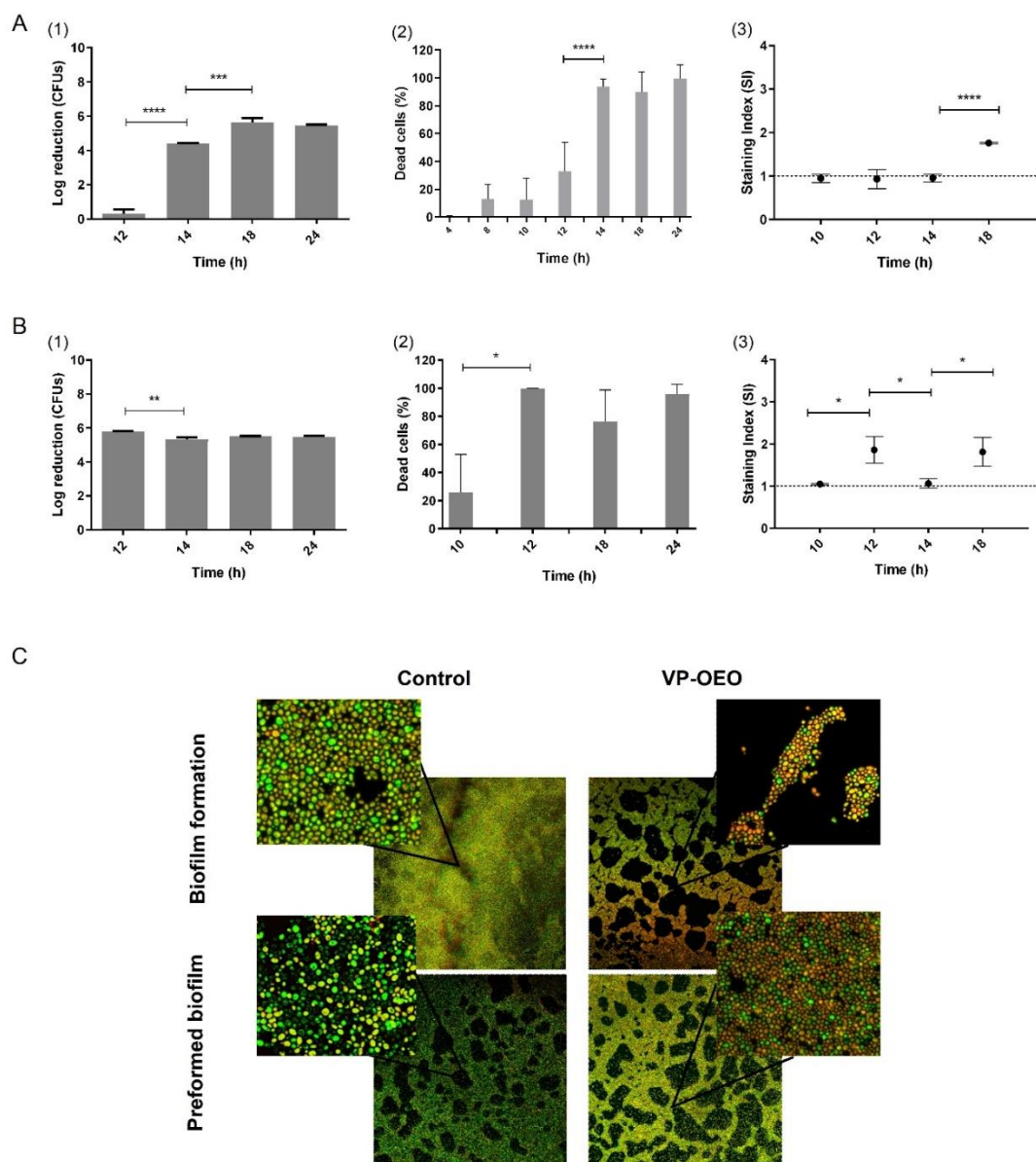


Figure 32. Effects of the vapor-phase of oregano essential oil (VP-OEO) on (A) biofilm formation and (B) preformed biofilms of *Candida albicans* Ca2. Evaluated by a (1) time-killing assay, (2) cell viability, and (3) metabolic activity (an SI of <1 indicates metabolic activity). (C) Confocal laser scanning microscopy images of *C. albicans* Ca2 biofilms (biofilm formation and preformed biofilms) after 12 h of exposure to VP-OEO. CLSM images show the staining patterns for live cells (SYTO-9 [green]) and dead cells (propidium iodide [red]). * indicate a statistically significant reduction in biofilm cell cultivability compared to that at the previous time point (* $p < 0.1$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).

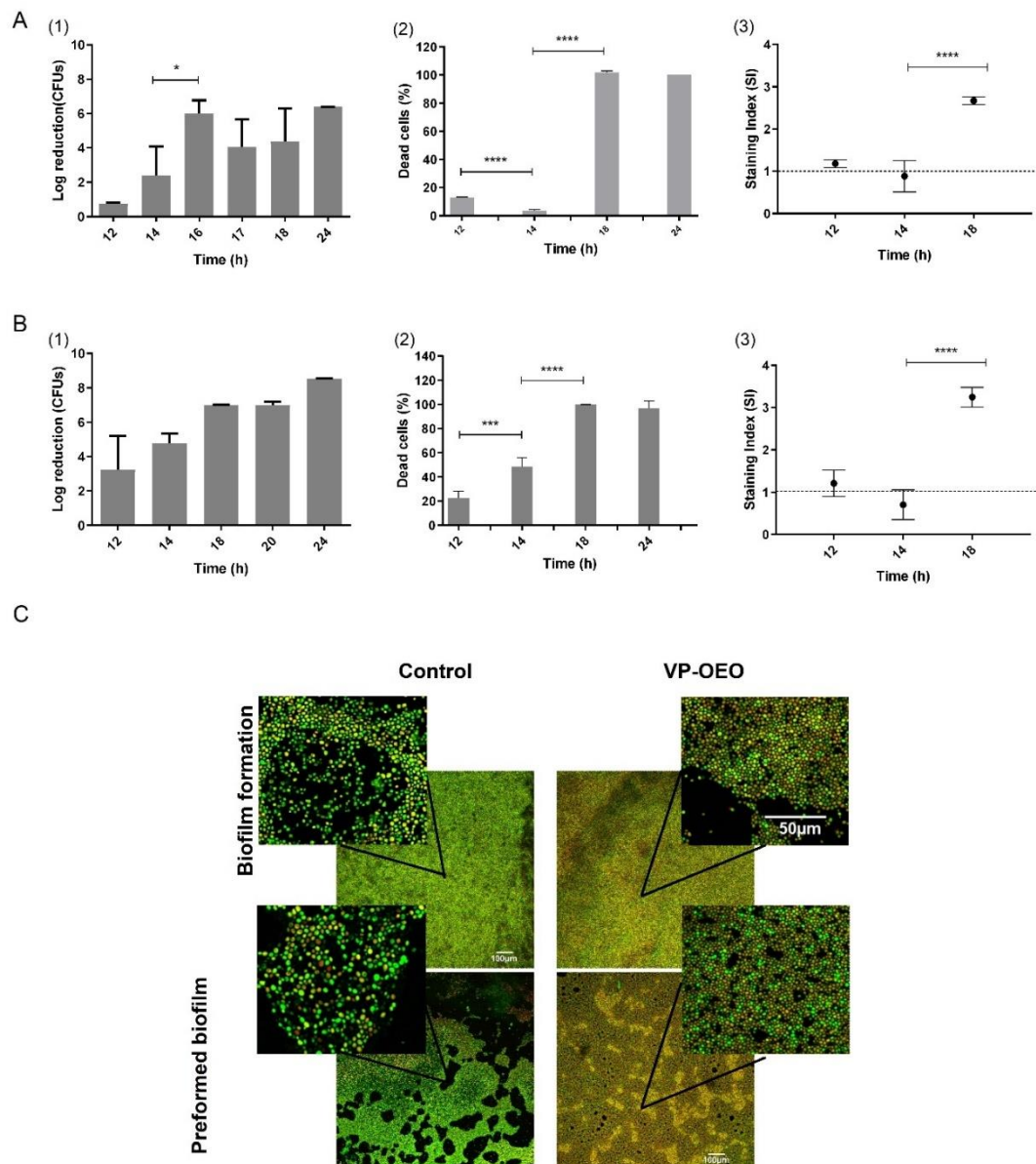


Figure 33. Effects of the vapor-phase of oregano essential oil (VP-OEO) on (A) biofilm formation and (B) preformed biofilms of *Candida glabrata* Cg8. Evaluated by a (1) time-killing assay, (2) cell viability, and (3) metabolic activity (an SI of <1 indicates metabolic activity). (C) Confocal laser scanning microscopy images of *C. glabrata* Cg8

biofilms (biofilm formation and preformed biofilms) after 12 h of exposure to VP-OEO. CLSM images show the staining patterns for live cells (SYTO-9 [green]) and dead cells (propidium iodide [red]). * indicate a statistically significant reduction in biofilm cell cultivability compared to that at the previous time point (* $p < 0.1$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).

The time-dependent killing assay is used to study the activity of an antimicrobial agent and can determine the fungicidal ($>3 \text{ Log}_{10} \text{ CFU mL}^{-1}$) or fungistatic ($<3 \text{ Log}_{10} \text{ CFU mL}^{-1}$) activity of an agent over time Sumiyoshi *et al.* [33]. Thus, from this assay, it was found that after 10 to 14 h of exposure to VP-OEO, a reduction in cell viability begins. In reality, after 12 h of exposure to VP-OEO, 100 % of *C. albicans* Ca2 cells were already PI stained (Figure 32.B2) in mature biofilms, although during biofilm formation, only 50 % of cells were stained with PI (Figure 32.A2). The same effect was observed in relation to metabolic activity at 12 h, where mature biofilms showed no metabolic activity (Figure 32.B3), while during biofilm formation, cells ceased to have metabolic activity only after 18 h of exposure to VP-OEO (Figure 32.A3). Since after the absorption and accumulation of FUN-1 in the cell's cytoplasm, the probe is converted only by metabolically active yeast cells, the fluorescence intensity was significantly higher in *Candida* cells exposed to VP-OEO than in untreated cells (control), corresponding to an SI of >1 . Thus, an SI of >1 after treatment indicated a serious impairment of yeast metabolism [56].

To confirm the cell morphology and viability of *C. albicans* Ca2 and *C. glabrata* Cg8 biofilms (formation and preformed biofilms) after 12 h of contact with VP-OEO, the biofilms were observed by confocal microscopy (Figure 32.C and 33.C). Visualization by live/ dead staining showed a reduction in *C. albicans* Ca2 biofilms after exposure to VP-OEO (Figure 32.C). The control shows green, which means that the 24-h-old and 48-h-old biofilms had developed, and most of the cells were alive. However, biofilms exposed to VP-OEO presented more dead cells (red staining) and a poorly developed and sagging architecture (Figure 32.C) compared to the untreated biofilm.

Regarding the effect of VP-OEO on *C. glabrata* Cg8, it was found that VP-OEO started to exert an effect after 12 to 14 h of exposure (Figure 33.A1 and Figure 33.B1), relatively later than for *C. albicans* Ca2 (Figure 32). In fact, from the microscopy images (Figure 33.C), it seems that 12 h of exposure to VP-OEO did not interfere with the structure of the biofilms; despite this, biofilms had more dead cells (red), for both biofilm formation and mature biofilms. Indeed, there was a total loss of metabolic activity and damage to the cell membrane only after 18 h of exposure to VP-OEO, with 100 % of the cells being stained with PI, for both biofilm formation and mature biofilms, although, there was also a great difference in cell viability at 14 h between mature biofilms (50 % PI-stained cells) and biofilm formation

(only 4 % PI-stained cells), with the same trend being observed for *C. albicans* Ca2 cells (Figure 32). This trend observed in the two *Candida* species, with VP-OEO having a faster effect on mature biofilms than on the formation stage, contradicts what was expected since mature biofilms present greater resistance. Interestingly, these results are in agreement with those of previous studies in which the impact of EO (VP-TTEO) was greater on mature biofilms than on biofilm formation; Subchapter II.3 suggests that the mechanism of action of EOs on biofilms can be potentiated by characteristics acquired by mature biofilms.

Based on these findings, two of the mechanisms of action of VP-OEO are interference with the integrity of the *Candida* membrane and interruption of cell metabolism. VP-OEO seems to cause serious primary cellular damage to *Candida* cells once the passage of PI into the cells occurred in a significant way, with this aspect being indicative of the existence of membrane damage/lesions. In fact, after membrane destabilization, cells lose their proper homeostasis, which leads to an organic imbalance that blocks their metabolic functions, and consequently, cells lose functionality [9,57]. As described above, carvacrol, one of the main constituents of OEO (36.97 %), may be directly related to the modification of membrane permeability by chemical reactions with amino and hydroxylamine groups of membrane proteins [22,47–49]. Corroborating the results of this research, López *et al.* [58] previously observed that the inhibition of planktonic cells of *C. albicans* by exposure to VP-OEO can be attributed largely to carvacrol. Furthermore, Niu *et al.* [59] found that carvacrol induced reactive oxygen species (ROS) production and mitochondrial dysfunction in *C. albicans*; still, those authors suggested that carvacrol treatment led to the apoptosis of *C. albicans* cells independent of ROS production.

The reconstituted human vaginal epithelium (RHVE) model has been successfully used as a model for antifungal drug delivery and for studies of the *in vitro* mechanisms of tissue degradation and virulence gene expression after infection with *Candida* species [35]. Thus, in the last step of this research, the RHVE was used to mimic vaginal conditions and evaluate the effect of VP-OEO on the attenuation of *Candida* species infection (Figure 34).

The colonization of the RHVE by *Candida* species was evaluated after 24 h, and the results confirm the effectiveness of VP-OEO against colonization by both *Candida* species (Figure 34). The greatest effect was observed against *C. albicans* Ca2, with a reduction in numbers of around 2 Logs ($p < 0.001$) in the RHVE exposed to VP-OEO (Figure 34.A and 34.B). Instead, although the effect of VP-OEO on *C. glabrata* Cg8 was not as pronounced as that on *C. albicans* Ca2, the cell concentration in the control was also considerably lower, as shown in Figure 34.B.

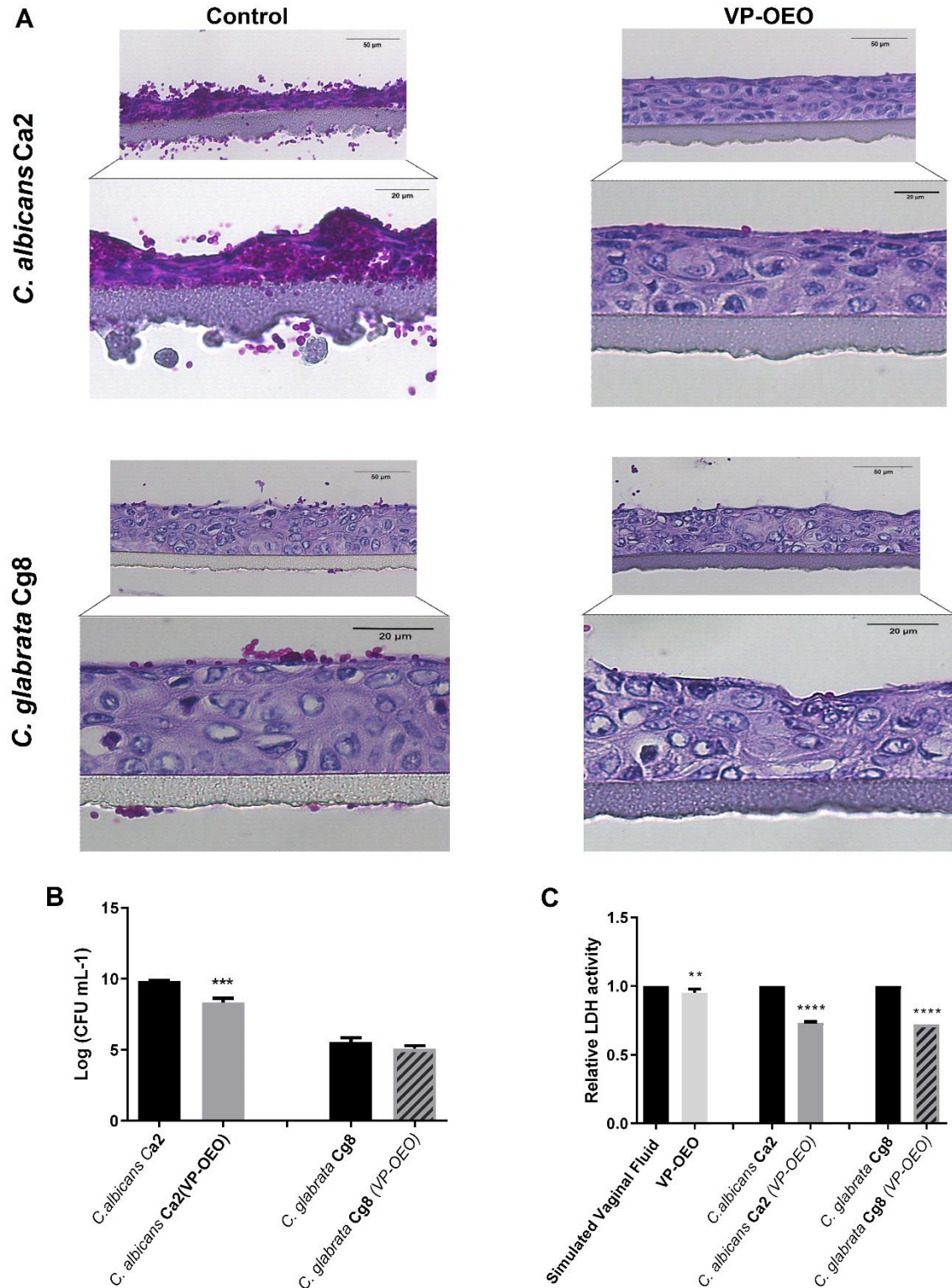


Figure 34. Effect of the vapor-phase of oregano essential oil (VP-OEO) on *Candida* species infection of the reconstituted human vaginal epithelium (RHVE). **(A)** *Candida albicans* Ca2 and *Candida glabrata* Cg8 infection of the RHVE and effect of VP-OEO after 24 h. **(B)** *C. albicans* Ca2 and *C. glabrata* Cg8 infection of the RHVE after 24 h (Log CFU per milliliter). **(C)** Relative lactate dehydrogenase (LDH) activity measured in the RHVE culture supernatant after 24 h of incubation with the *C. albicans* Ca2 and *C. glabrata* Cg8 strains compared to the untreated epithelium

(without VP-OEO) and effect of VP-OEO compared to simulated vaginal fluid. * indicate a statistically significant reduction in biofilm cell cultivability in comparison with that of the respective control (** $p < 0.001$; **** $p < 0.0001$).

LDH is a stable cytosolic enzyme that is released after cell lysis, and the amount of color formed during the LDH assay is proportional to the number of lysed cells [60]. Thus, to quantify possible damage to the RHVE induced by VP-OEO, LDH activity levels were measured after 24 h of exposure (Figure 34.C), and the results revealed no significant effects on the RHVE compared to the control SVF. These results highlight the safe application of the EO and confirm the *in vivo* cytotoxicity results (Figure 30). In addition, there was also a significant decrease ($p < 0.0001$) in the amount of LDH released by the RHVE infected by *Candida* species when treated with VP-OEO compared to the untreated RHVE infected with the same strains of *C. albicans* and *C. glabrata*. So, the RHVE model confirms the effectiveness of VP-OEO for the treatment of *Candida* vaginal infections.

4. Conclusions

The antifungal agents used for *Candida* therapy are often toxic and have low efficacy, particularly against resistant strains of *Candida* species. The replacement of these agents with natural substances is a field to be investigated to reduce the concentration of antifungal agents and increase the effectiveness of treatments. The clinical application of EOs may be discouraged due to their lipophilic character, which makes them potentially toxic when used neat or at high concentrations. However, this study shows that the application of VP-OEO is safe, does not present cytotoxicity, and is active against both *Candida* species (*C. albicans* and *C. glabrata*). In fact, the results demonstrated that VP-OEO causes damage to the cell membrane and disrupts the cell metabolism of both *Candida* species studied.

CHAPTER IV.2

DESIGN AND EVALUATION OF MICROENCAPSULATED OREGANO ESSENTIAL OIL AS AN ALTERNATIVE TREATMENT TO *CANDIDA ALBICANS* INFECTION

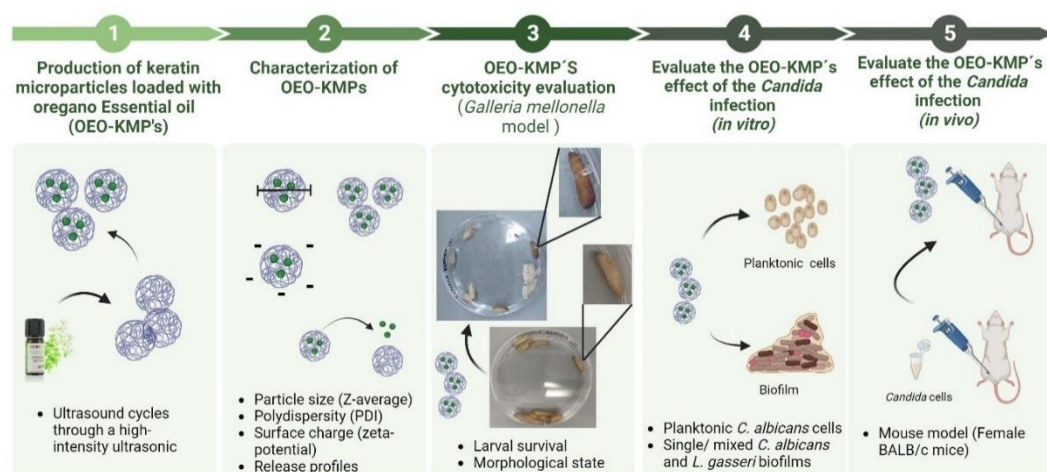


Figure 35. Subchapter IV.2 workflow (Created with BioRender.com (BT26ABXE9C)).

The application of essential oils has some limitations, and to overcome them, micro or nanoencapsulation emerged as an efficient technique. The main goal of this subchapter was to produce and characterize microparticles of keratin loaded with oregano essential oil (OEO-KMP's) and evaluate their anti-*Candida* activity through *in vitro* and *in vivo* experiments. Additionally, the study evaluated the impact of OEO-KMP's on *Lactobacillus* species, which are beneficial bacteria in the vaginal microflora.

This subchapter is based on the following manuscript: Fernandes L, Tejada A, Blázquez E, Araujo D, Ribeiro A, Silva S, Cussó L, Costa-de-Oliveira S, Rodrigues ME, Henriques M. Development and evaluation of microencapsulated oregano essential oil as an alternative treatment for *Candida albicans* infections. (In submission)

And part of this subchapter was presented as poster: Fernandes L, Blázquez E, Tejada A., Ribeiro, A, Silva S, Mira N, Cussó L, Costa-de-Oliveira S, Rodrigues ME, Henriques M. Oregano Essential Oil: An Effective and Non-Toxic Approach for prevent or treat Resistant *Candida* Species. Congress of Microbiology and Biotechnology 2023 (FEMs grant), 2023, Covilhã, Portugal.

1. Introduction

Vulvovaginal candidiasis (VVC) is a common fungal infection that affects women and can manifest at different stages of life, with peaks during pregnancy or when the immune system is weakened [61]. At the beginning of VVC infection, patients may experience itching, redness, and burning of the vulva and vaginal mucosa, leading to frequent urination, dyspareunia, dysuria, and a consequent impaired quality of life [61]. *Candida albicans* is the most common found species, accounting for approximately 20 % to 90 % of the recovered isolates [62]. One of the main virulence factors of *C. albicans* is the yeast-to-hyphal transition, which occurs after adhesion to the epithelial cells of the vulvovaginal mucosa. Subsequent invasion by hyphae activates epithelial signaling pathways leading to pro-inflammatory responses and subsequent biofilm formation [61]. The eradication of fungal biofilms requires critical medical intervention, including antifungal treatment through different routes of drug administration [63]. Presently, azole antifungals are the main choice for preventing and treating invasive fungal infections. However, these agents have several limitations and disadvantages, including drug resistance and adverse effects [64]. Its long-term use is associated with hepatotoxicity and hormone-related effects, including gynecomastia, alopecia, oligospermia, azoospermia, decreased libido, impotence and hyponatremia [64]. In this context, natural products are currently being evaluated for their antimicrobial activity in order to develop alternative therapies, with lower costs, fewer adverse reactions and thus less negative impact [65].

Previous studies have reported that essential oils (EOs) and their vapor-phase (VP-EOs) exhibit antifungal activities against *Candida* species, having biocompatibility and low cost [47,66,67] (Chapter II and III). Oregano EO has been shown to have anticandidal properties (Subchapter III.1 and IV.1). In fact, subchapter IV.1 demonstrated high antifungal activity of VP of OEO (VP-OEO), with a significant reduction in *C. albicans* and *Candida glabrata* biofilms, interfering with membrane integrity and metabolic activity. However, the EOs activity depends on several factors such as photosensitivity, high volatility, low water-miscibility and degradability at high temperature, which reduces their bioavailability. Therefore, micro or nanoencapsulation has been investigated as an effective technique to protect, control and prolong the release of EOs, improve the water-solubility and bioavailability of lipophilic compounds, and minimize the inconvenience caused by possible EOs side effects [68,69]. Among the numerous typed drug delivery systems, such as liposomes or nanoliposomes, nanoemulsions, films or nanogels, microparticles are promising due to their permeation and retention effect [70].

Proteins have drawn attention due to their potential as drug carriers and for controlled drug release. In this sense, several types of protein can be used for fabricating microcapsules, such as keratin. Keratin

is a natural protein found in different sources such as wool, human hair, nails, feathers and horns or hooves [71,72]. This compound is a promising option for building drug carriers due to its biodegradability, biocompatibility, absorbability, non-immunogenicity and reduction sensitivity [70,73]. Moreover, due to a cysteine-rich structure and the presence of several functional groups, such as amid, carboxyl or sulfhydryl, can be easily modified with biomolecules to improve its solubility, stability and can be applied in a wide range of biomedical and biotechnological applications [71]. Indeed, keratin-based drug carriers have been explored, including micro or nanoparticles, nanogels or films [72].

Thus, the aim of this part of this work was to produce and characterize keratin microparticles (KMPs) loaded with oregano essential oil (OEO-KMP's) and to evaluate the anti-*Candida* activity of OEO-KMP's using *in vitro* and *in vivo* assays. The effect of OEO-KMP's on *Lactobacillus* species, beneficial bacteria in the vaginal microflora, was also evaluated.

2. Materials and Methods

2.1 Oregano essential oil encapsulated into microparticles of keratin

2.1.1 Oregano essential oil and conditions

In this study, oregano EO (*Origanum compactum*, florame®, France) (100 % pure) was encapsulated in keratin microparticles. The OEO was stored in a desiccator at room temperature and protected from light. The chromatographic analysis is detailed in the subchapter IV.1 (Annex I.5).

In this work two synthetic fluids were used: simulated vaginal fluid (SVF) and simulated sweat fluid (SSF). The SVF was prepared according to subchapter III.1 and SSF was adapted from the international standard ISO 105-E01:2013 [74] and consisted in 6.25 g L⁻¹ of NaCl (Biochem Chemopharma, France), 0,625 g L⁻¹ of L-histidine monohydrochloride monohydrate (Sigma-Aldrich) and 2,5 g L⁻¹ disodium hydrogen orthophosphate anhydrous (Merck- Sigma-Aldrich), the pH was adjusted to 5.5 with acetic acid (Fisher chemical).

2.1.2 Keratin extraction and purification

Keratin was extracted from natural wool samples. The wool samples were washed according to the IAEA/RL/50 1978 instructions to remove contaminants and lipids. The washing process involved several steps: first, the samples were washed with distilled water and acetone, with agitation at 250 rpm for 20 min, in four alternating cycles; then, the wool samples were subjected to a bleaching process using a kit,

followed by thorough washing until all residues were removed; after this, the samples were kept at 37 °C overnight.

The protocol, adapted from Tinoco *et al.* [75], was followed for keratin extraction, and it consisted of a solution that was prepared using urea (8 M), sodium metabisulfite (0.5 M), and sodium dodecyl sulfate (SDS) (0.2 M). The volume-to-mass ratio of dry hair (in grams) was consistently held at 10:1. Afterward, the mixture underwent heating at 100 °C for 30 minutes. Subsequent steps included incubation at 37 °C with agitation at 500 rpm overnight, followed by centrifugation at 4000 rpm for 15 minutes, and the resulting supernatant was subsequently filtered. The keratin solution obtained was dialyzed for five days using a dialysis membrane with a 14 kDa cut-off (Merk-Sigma), with the distilled water being changed twice daily. After five days, the solution was centrifuged for 15 min at 4000 rpm, and, as in the previous step, the resulting supernatant was filtered. The quantification of protein concentration was performed using the method (Bio-Rad), according to the manufacturer's instructions [76]. Subsequently, the keratin was lyophilized for subsequent use.

2.1.3 Preparation of the keratin microparticles for encapsulation of oregano essential oil

The protein solutions were prepared with phosphate buffer saline (PBS 1x) pH 7.4 to a final concentration of 10 mg mL⁻¹ with 5 % (v/ v) of OEO. For the homogenization process, the samples were subjected to ultrasound cycles (total time: 6 min; cycles: 8 s and 2 s; amplitude: 40 %) by a high-intensity ultrasonic equipment (SONICS, Vibra-Cell™). The free OEO was separated from the particle's formulations, by size-exclusion chromatography using a 5 kDa of cut-off PD-10 Desalting Column (GE-Healthcare), according to manufacturer's protocol [77].

2.1.4 Determination of oregano essential oil encapsulated into microparticles of keratin encapsulation efficiency

The encapsulation efficiency (EE) of OEO within the keratin microparticles was measured using a standard calibration curve of OEO in ethyl acetate (Figure 36). This curve was obtained using the maximal peak absorbance values of different OEO concentrations, recently prepared, measured by UV-Vis spectrophotometer (275 nm) (Synergy H1; BIOTEK). Then the free OEO was extracted from the aqueous phase with ethyl acetate and analyzed by spectrophotometry. Each sample was assayed in triplicate. The EE of OEO was evaluated by the difference between the initial oil concentration in the sample and the free oil concentration after encapsulation.

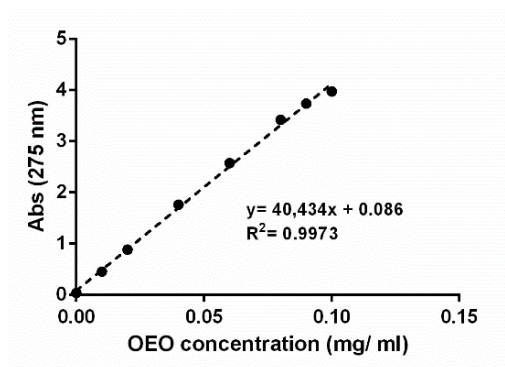


Figure 36. The standard calibration curve of oregano essential oil (OEO) in ethyl acetate.

2.1.5 Physicochemical properties of the oregano essential oil encapsulated into microparticles of keratin

The physicochemical properties (mean size diameter, polydispersity index (PDI) and surface charge (ξ -potential)) of the OEO-KMP's were measured using a Zetasizer Nano ZS (Malvern Instruments) at 25 °C. The samples were measured with a 1:10 dilution with PBS (1x) and read in triplicate. The stability of the OEO-KMP's was monitored for 5 months. The results of this assay are presented as the mean $\pm$ standard deviation.

2.1.6 Evaluate the oregano essential oil encapsulated into microparticles of keratin release, *in vitro*

To measure the amount of OEO released, a standard calibration curve in SFV was obtained using the maximal peak absorbance values of different OEO concentrations prepared with SFV (Figure 37), using a UV-Vis spectrophotometer.

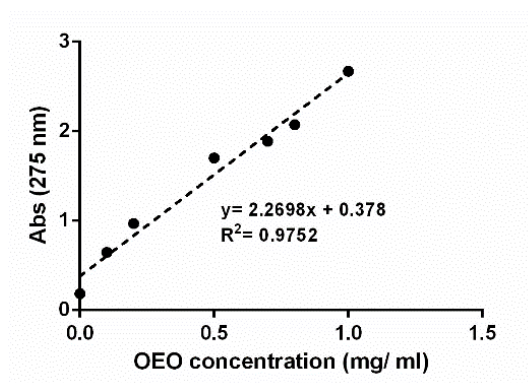


Figure 37. The standard calibration curve of oregano essential oil (OEO) in simulated fluid vaginal (SFV).

The *in vitro* release of OEOs for 72 h was accomplished using a dialysis membrane (14 kDa cut-off). For this, 2 mL of the solution OEO-KMP 's was placed inside the dialysis membrane and incubated in 15 mL of SFV, with agitation (250 rpm) at room temperature and in dark conditions. At specific time intervals, 200 μ L of the SFV solution was withdrawn in triplicate and spectrophotometrically analyzed by absorption reading at 275 nm. The amount released (%) was determined by comparing the released oil with the initial concentration of OEO encapsulated. The stability of OEO-KMPs in interaction with SFV was also evaluated, encompassing an analysis of their physicochemical properties, including average diameter, PDI and surface potential ξ , as previously described.

2.1.7 Oregano essential oil encapsulated into microparticles of keratin cytotoxicity

To assess the *in vivo* skin toxicity of OEO-KMP 's, the *Galleria mellonella* model was used. The *G. mellonella* larvae were maintained on a diet based on pollen grains at 25 °C in the dark and carefully selected at the final stage of development when the weight was approximately 250 mg, as described previously by Araújo *et al.* [27]. Then, 5 μ L of OEO-KMP 's was spread over the *G. mellonella* larvae body (n=10), two control groups were carried out under the same conditions, one with PBS (n=10) and the other with the empty keratin solution (n=10). The three set of larvae were kept at 37 °C and in dark conditions. Morphological state (movement, cocoon formation and melanization) and larvae survival were monitored over 72 h. The results were shown as the percent survival (%) and health index. The experiment was performed in two independent assays.

2.2 Evaluate the oregano essential oil encapsulated into microparticles of keratin effect on the *Candida* infection *in vitro* assays

2.2.1 Microorganisms and culture conditions

This study used *Candida albicans* SC 5413 obtained from the American Type Culture Collection (ATCC) and *Lactobacillus gasseri* ATCC 33323 (acquired from DSMZ). The *C. albicans* species were subcultured from a frozen stock (Sabouraud dextrose broth (SDB; Liofilchem) medium with 20 % (v/ v) glycerol (Biochem Chemopharma, France), at -80 ± 2 °C onto Sabouraud dextrose agar (SDA; Liofilchem) plates and incubated for 24 h at 37 °C. The *L. gasseri* was kept in Mann Rogosa and Sharpe broth (MRSB; Liofilchem) broth with 20 % (v/ v) glycerol at -80 ± 2 °C. This specie was subcultured from the frozen stock onto MRSB (1 %) and incubated for 72 h at 37 °C and 5 % CO₂ environment in saturated humidity as described in subchapter III.2.

2.2.2 Oregano essential oil encapsulated into microparticles of keratin effect on planktonic *Candida albicans* cells

The inhibitory effect of OEO-KMP's on the growth of *C. albicans* was evaluated by the agar disk diffusion method, as described previously and by Tran *et al.* [78]. Briefly, the SDA surface was inoculated using a swab dipped in a cell suspension adjusted to 1×10^8 cells mL⁻¹. After the inoculum was dried, a sterile filter paper disk (6 mm) (Liofilchem) was impregnated with 25 μ L of OEO-KMP's. Plates with disks without OEO-KMP's were also included as controls. Incubation of all plates occurred at 37 °C, and the diameters (mm) of the zones surrounding the disks were measured within 24 h.

The broth microdilution method according to Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI M27-A4), with some modifications, was used. Briefly, the initial cell concentration for *C. albicans* was adjusted for 1×10^5 and 1×10^8 Colony-Forming Units (CFU) mL⁻¹ in SFV and dispensed into 96-well round bottom microtiter plates with the OEO-KMP's solution, both in a proportion of 1:2. Positive controls (*C. albicans* suspension) and negatives controls (SFV or keratin solution) were included. Microtiter plates were incubated at 37 °C for a duration of 24 h. It was not possible to compare the turbidity of the solution between the conditions, as described in CLSI M27-A4, since OEO-KMP's is an opaque liquid. Then, each well was sub-cultured onto SDA plates and incubated for 24 h at 37 °C and the number of grown colonies (CFUs) was counted. The results were expressed as Log CFU mL⁻¹. Both experiments were performed in triplicate with three independent assays.

2.2.3 Oregano essential oil encapsulated into microparticles of keratin effect on single and mixed *Candida albicans* and *Lactobacillus gasseri* biofilms *in vitro*

The effect of the OEO-KMP's on single and mixed *C. albicans* and *L. gasseri* biofilms was evaluated. Biofilms were developed as described in the previous chapters and by Stepanović *et al.* [32]. Briefly, the *C. albicans* pre-inoculum in SDB was prepared and incubated for 18 h at 37 °C under agitation (120 rev/ min) and *L. gasseri* was pre-cultured in MRSB with 1 % (v/ v) taken from the subculture during 24 h at 37 °C in an environment of 5 % CO₂ in saturated humidity. Then, both cellular suspensions were centrifuged and washed twice with PBS (5000 g for 10 min, at 4 °C).

To study the effect in single *C. albicans* and *L. gasseri* biofilms, 1 mL of standardized suspension (1×10^7 cells mL⁻¹ prepared in SFV) of *C. albicans* and *L. gasseri* strains, separately, was transferred to 24-well plate. For mixed-species cultures, 500 μ L of *C. albicans* suspension was combined with 500 μ L of *L. gasseri* suspension, both suspensions adjusted for 2×10^7 cells mL⁻¹. The 24-well plates were incubated (24 h at 37 °C, 120 rev/ min). Then, several volumes of OEO-KMP's (5, 12.5, 25, 50, 150 and 250 μ L)

were applied, for an additional 24 h. As positive control, biofilms were formed without any contact with the OEO-KMP's while SVF was used as negative control. The number of cultivable cells in the single and dual biofilms was calculated using the CFU counting methodology. For this, the biofilms were washed with PBS to remove planktonic fraction and the suspensions with biofilm cells were serially diluted in PBS and then passed to SDA for single biofilm, SDA supplemented with 20 mg L⁻¹ of gentamycin and MRSA supplemented with 1 mg L⁻¹ of amphotericin B for dual biofilms. The plates were incubated (5 % CO₂ environment, at 37 °C for 24 h), and the number of colonies grown was counted and translated into CFU per milliliter (Log (CFU mL⁻¹)).

2.3 Evaluation of the effect of the oregano essential oil encapsulated into microparticles of keratin in a mouse model

2.3.1 Microorganism culture

Candida albicans SKCA23-ACTgLuc strain was grown on SDA chloramphenicol plates (Condalab, Spain) overnight at 37 °C prior to the experiments. The inoculum with a concentration of 1.33×10^8 CFU mL⁻¹ in PBS was made by selecting two to three colonies from the 24 h culture of *C. albicans* SKCA23-ACTgLuc in SDA and adjusting with Neubauer chamber.

2.3.2 *Candida albicans* infection and the treatment with oregano essential oil encapsulated into microparticles of keratin

Twenty-one BALB/c mice (Charles River) of 6 – 8 weeks and 19.29 ± 2 g were housed under a 12-h light-dark cycle at 23 °C and 1 °C and 50 % and 5 % humidity in the animal facility of *Hospital General Universitario Gregorio Marañón*, Madrid, Spain (ES280790000087). All animal procedures conformed to EU Directive 2010/63EU and national regulations (RD 53/2013). All animal procedures were approved by the HGUGM. Animal Experimentation Ethics Committee, the local Ethics Committees, and the Animal Protection Board of the *Comunidad Autónoma de Madrid* (PROEX 083/18). All animals were allowed access to food and water ad libitum.

Mice were divided into two groups: infected untreated (n= 10) and infected treated with OEO-KMP's (n=11). Over time, the body weight of each animal was monitored. Mice were injected with 17β-Estradiol (E2) 0.5 mg mL⁻¹ dissolved in sesame oil (Sigma-Aldrich, USA). After 72 h, 2×10^6 cells of *C. albicans* in 15 µL of PBS were inoculated into the vagina, as described by Relloso *et al.* [79]. Following a 24-hour infection period, 15 µL of OEO-KMP's containing an OEO concentration of 17.88 mg mL⁻¹ (for the treated group) or PBS (for the untreated group) were administered intravaginally. Microbiological

cultures from the vaginal lavage and vaginal tissue samples were obtained after 24 h of OEO-KMP's or PBS (control) applications, to assess the presence of *C. albicans* and *Lactobacillus* species and measure the toxicity of the treatment in the vaginal tissue, respectively.

In all the procedures, animals were awake except at final point, when mice were anesthetized with 3% sevoflurane in 100 % oxygen for vaginal lavages and sacrificed for the removal of vaginas for histological studies.

2.3.2.1 Quantification of *Candida albicans* and *Lactobacillus* species

Vaginal lavage was performed by flushing the vaginal vault with 50 µL of sterile PBS. This process was repeated four times, utilizing a 100 µL pipette tip, as described in previous studies by Salinas-Muñoz *et al.* and Relloso *et al.* [79,80]. Following the lavage, serial dilutions were performed. These dilutions were subsequently incubated onto two types of agar plates: SDA for *C. albicans* quantification and MRSA for quantifying *Lactobacillus* species. The agar plates were subsequently incubated for 24 h at 37 °C. After incubation, the CFU's were counted to determine the microbial content in the samples.

2.3.2.2 Histopathological analysis of mucosal tissues

After the animal sacrifice, vaginal tissue was initially fixed in 4 % (w/ v) formaldehyde (Kalttek, Italy) and stored at room temperature. Then, vaginal tissue was embedded in paraffin wax and sections of 20 µm were cut and placed on HistoBond+ coated microscope slides. After that, by processing in xylene, the tissues were deparaffinized and immersed in ethanol and water. Staining was carried out by the periodic acid-Schiff method. Then, the vaginal tissues were examined in a bright field using an Olympus BX51 epifluorescence microscope coupled to a DP72 digital camera (Olympus Portugal SA, Portugal) and the images were acquired using Olympus Cell-B software.

Candida-free and infected vaginal tissues subsequently treated with OEO-KMP's were examined for histopathological changes in the mucosal tissues. The evaluation involved examining various factors, including epithelial lesions, glandular cystic dilatation, inflammatory infiltrates, hemorrhage, vascular proliferation/degeneration, edema/fibrosis, necrosis, and calcifiers in the vaginal epithelium. A scoring system was employed to quantify the severity of these factors, with scores ranging from 0 (no alterations) to 4 (very severe). These scores were then summed to determine the level of vaginal irritation, categorized as minimal (1-4), mild (5-8), moderate (9-11), or severe (12-16) [81]. The criteria for evaluation were based on standards outlined in the Biological evaluation of medical devices, particularly the tests for irritation and skin sensitization [82].

2.4 Statistical analysis

All results obtained during this study were statistically analysed using the Prism software package (GraphPad Software version 8.0.1). In the microparticles characterization, one-way ANOVA (analysis of variance) and Tukey's multiple comparisons test were used to compare the different hours with the initial time. For the analysis of the OEO release rate two-way ANOVA and Dunnett's multiple comparisons tests were used to compare the different hours with the initial rate. The same test was used to compare the effect of OEO-KMP's on biofilms versus untreated biofilm. For all *in vitro* assays, three independent experiments were carried out and each analysis was performed in triplicate. In *in vivo* assay, non-parametric test and Mann-Whitney test were used, and one of the points was removed as it was considered an outlier. The experiments were carried out with a confidence level of 95 %. Statistical significance was assumed at $p < 0.05$.

3. Results and Discussion

3.1 Oregano essential oil encapsulated into microparticles of keratin characterization

The possible toxicity, strong taste, chemical instability, restricted administration routes and volatility of OEO limit its application [83]. In this sense, micro or nanotechnology is receiving particular attention to overcome these obstacles. In fact, this technology allows EOs protection from light and temperature alterations, increasing their solubility in aqueous environments, prolonging the release, and improving their bioaccessibility and bioavailability [84]. Electrospray, spray and precipitation methods have been used for the synthesis of micro and nanoparticles of keratin for drug delivery applications [85]. In the present work, the OEO-KMP's were produced by ultrasound cycles through a high intensity ultrasonic and characterized regarding OEO-KMP's morphological and physicochemical parameters, such as OEO encapsulation efficiency, particles stability (PDI, size and surface charge) and OEO release profile.

EE is a crucial characteristic for evaluating and validating micro or nanocapsules as a delivery system [86]. Indeed, a high OEO EE of 99.42 ± 0.10 % was obtained, which clearly, demonstrates the high capacity of keratin load OEO during the homogenization process by ultrasound cycles. In fact, this method is a facile and versatile synthetic tool for nanostructured materials [87]. Moreover, microcapsules formed from keratin have shown attractive properties, such as high excellent biocompatibility, surface area, biodegradability and easy functionalization, being a promising option for controlled and targeted administration of compounds [88]. Furthermore, the significant hydrogen and disulfide bonds confer to keratin some mechanical properties, such as rigidity, stability, strength and resistance to proteolytic degradation [73].

Also, the proper functioning of the nano or micro delivery systems is deeply influenced by their size, shape, dispersion and surface chemistry [89,90]. In addition, the ability of microcapsules to retain OEO can vary depending on time and storage conditions. Because of the importance of these factors in OEO delivery application, in this study the OEO-KMP 's properties were evaluated over time (Figure 38).

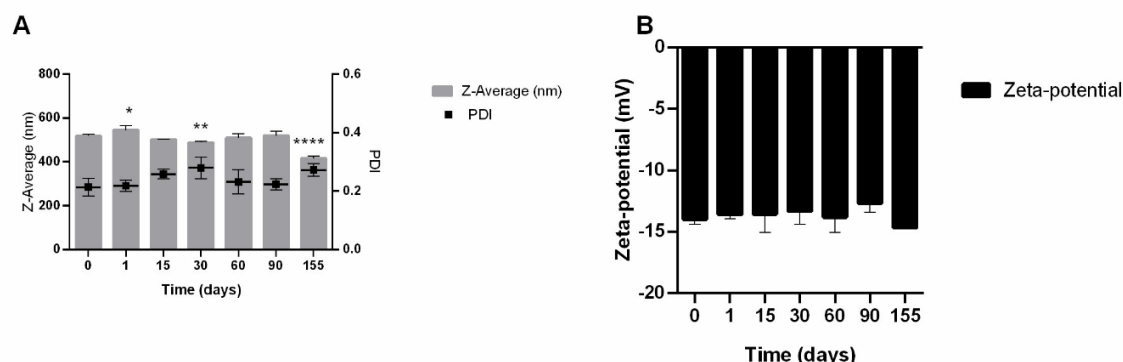


Figure 38. Characterization of keratin microparticles prepared in Phosphate-buffered saline (PBS) encapsulating oregano oil (OEO-KMP 's) for 5 months (155 days). **(A)** particle size (Z-average) and polydispersity (PDI); **(B)** surface charge (zeta-potential). *indicate statistical difference in particle size when compared to the results obtained at time 0 (initial conditions) (* $p < 0.1$, ** $p < 0.01$, **** $p < 0.0001$).

Although there was a slight statistical difference ($p < 0.01$) on days 1 and 30, and a significant difference ($p < 0.0001$) between day 1 and day 155, the OEO-KMP 's particles exhibit a mean size (Z-average) of approximately 500 nm over 90 days of storage (Figure 38.A). These differences observed might be related with some rearrangements on the protein network on OEO-KMP surface. The small dimensions of micro or nanoparticles enable them to cross diverse biological barriers, facilitating the transportation of drugs to various levels inside the microorganism [90]. In fact, as particle size decreases, specific surface area, reactivity, and the bioavailability of encapsulated drugs increase, leading to an amplified functional capacity of the bioactive agent, remarkably improving antimicrobial efficacy [90].

The PDI serves as a metric to evaluate the uniformity of particle sizes. Relatively to the OEO-KMP 's, the values of this parameter were between 0.2- 0.3, without significative difference for 155 days (Figure 38.A). A PDI value below 0.3 indicates a well-proportioned size distribution in a colloidal system, minimizing the risk of precipitation [90,91].

Zeta-potential stands as another crucial parameter. In addition to being a good indicator of the stability of particles in suspension due to the extent of electrostatic repulsion/attraction between particles but also as an indicator of particle involvement with biological systems [90]. Relatively to OEO-KMP 's, this parameter also remained stable over time (Figure 38.B), between -14 and -15 mV due to the carboxyl

groups within keratin [70]. From the standpoint of colloidal stability, a colloidal suspension highly negative is considered stable, the repulsive forces prevent agglomeration, thus contributing to the overall stability of the system [75]. Therefore, based on these three parameters, it can be concluded that the OEO-KMP's prepared are stable and uniform in neutral aqueous media (PBS).

The selection of the EO, along with the decision regarding the delivery system, impacts the achieved therapeutic [90]. A good drug delivery system should be able to encapsulate molecules and release them over time under physiological conditions [71]. The *in vitro* release profiles of OEO encapsulated in keratin-based particles, particles was studied by incubating the OEO-KMP 's in SVF and SSF for 72 h (Figure 39).

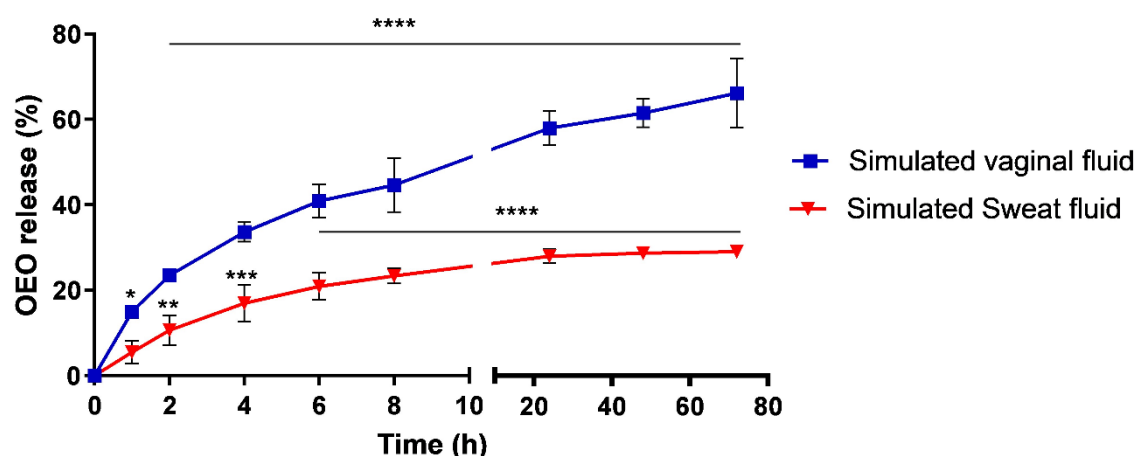


Figure 39. *In vitro* release profiles of oregano essential oil from keratin-based particles in simulated vaginal and sweat fluids, over 72 h. * indicate statistical difference compared to the results obtained at time 0 h (* $p < 0.1$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

The OEO release reached approximately 50 % and 20 % in the first 8 h of incubation in SVF and SSF, respectively (Figure 39). After 72 h of incubation, there was a maximum OEO release of 65 % in SVF and 30 % in SSF. This difference can be related to the affinity of the OEO compounds with the release medium, allowing a higher and faster rate of oil release [92,93]. The release rate of active compounds from polymeric nano/microcapsules is dependent on several factors which may be related to the properties of the substance incorporated in the microcapsules, such as solubility or desorption of the surface-bound or adsorbed substance, or the matrix properties of the nanocapsules such as the diffusion of the substance through the matrix, erosion or degradation/disintegration of the matrix, penetration of the release medium through the matrix or the combination of erosion and diffusion processes [92,94]. Moreover, it was observed that the OEO release profiles showed an increased effect in the first hours of

incubation before reaching a stable release profile. The higher initial release can be attributed to the OEOs molecules adsorbed on the particle surface and to the EO entrapped near the surface of the matrix, since the rate of dissolution of the substance near the surface is high, the amount of substance released will be also high in the first few hours [92,94].

In addition, the stability of the OEO-KMP's was evaluated after 72 h of contact with both fluids (SSF and SVF) and it was observed that the size of the OEO-KMP's showed significant differences when in contact with the SVF ($p < 0.0001$) (Table 12) compared with OEO-KMP's in PBS at time 0 (Figure 38). This may indicate that the particles when in contact with SVF undergoes structural changes, allowing the release of OEO. Moreover, the differences observed on particles sizes when incubated in both fluids can be related to the fluids' pH, since SVF has the lowest pH (4.2) compared to SSF of 5.5.

Table 12. Characterization of keratin microparticles prepared in Phosphate-buffered saline (PBS) encapsulating oregano oil (OEO-KMP's) when in contact with simulated vaginal fluid and simulated sweat fluid for 72 h. * indicate statistical difference in particle size when compared to the results obtained at time 0 (Figure 38) (**** $p < 0.0001$)

	Particle size (Z-average nm)	PDI	Zeta- potential (mV)
Phosphate-buffered saline	517.8 ± 8.09	0,213 ± 0.03	-14.00 ± 0.40
Simulated vaginal fluid	1809.00 ± 273.41****	0.347 ± 0.20	-12.55 ± 0.14
Simulated sweat fluid	484.55 ± 14.83	0.336 ± 0.02	-14.05 ± 0.50

Following the analysis of OEO-KMP's stability, it becomes essential to evaluate the cytotoxicity of this formulation. To this end, considering the final application involves contact with vaginal tissue, the response resulting from the interaction between OEO-KMP's and the surface of *G. mellonella* larva's body was examined. Similar to the prior investigation involving VP-OEO (subchapter IV.1), in this current research, there was no indication of toxicity after 72 h of larval contact with keratin solution or OEO-KMP's formulations. Indeed, until 72 h, all larvae maintained their viability (Figure 40.A), and there were no significant differences in the larval health index (Figure 40.B) following contact with PBS, keratin solution or OEO-KMP's. Hence, it can be concluded that the application of OEO-KMP's is considered safe without manifesting any surface irritation signal. Several studies have indicated a reduced occurrence of side effects with nanostructured systems, which incorporate lower drug doses without compromising therapeutic efficacy [95]. Consequently, these nanocarriers have been explored for diverse applications to address the limitations associated with conventional formulations [95].

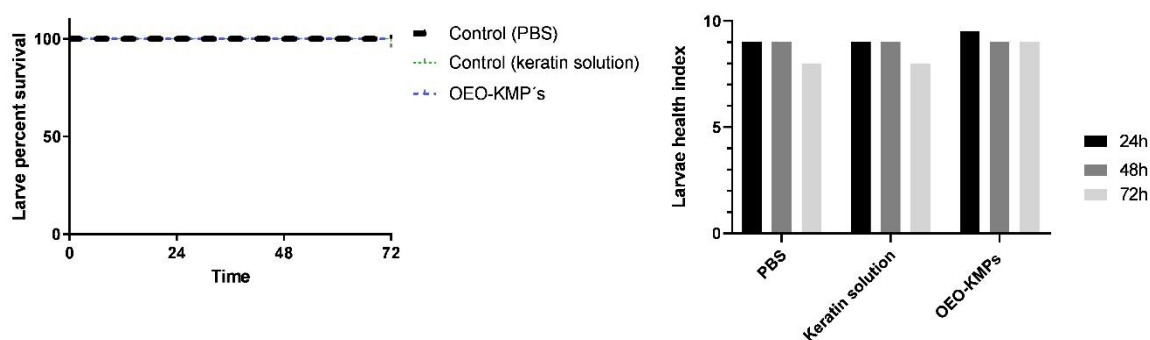


Figure 40. Keratin microparticles encapsulating oregano oil (OEO-KMP's) cytotoxicity measured in *Galleria mellonella* model. **(A)** Survival curves of *G. mellonella* larvae in contact with OEO-KMP's and respective controls (without exposure to OEO-KMP's and keratin solution) during 72 h; and **(B)** *G. mellonella* larvae health index after 24, 48 and 72 h in contact with OEO-KMP's and respective controls.

3.2 Impact of oregano essential oil encapsulated into microparticles of keratin on *Candida albicans* growth *in vitro*

The high incidence of *C. albicans* and the therapeutic limitations and associated negative consequences make it crucial to develop effective alternative to the current treatments. In this context, the previous subchapter IV.1 demonstrates that the application of VP-OEO is safe and effective against both *C. albicans* and *C. glabrata*, inducing changes in membrane integrity and metabolic activity. So, another possible strategy to apply this EO would be encapsulation in keratin microcapsules, allowing a more controlled and safer release of the OEO.

As previously mentioned, the OEO-KMP's have dimensions of approximately 500 nm and several studies corroborate that particles with dimensions around 500 nm facilitate the penetration of delivery systems into fungal cells [96]. The inhibitory activity of the OEO-KMP's on the planktonic growth of the *C. albicans* was evaluated using the disk-diffusion agar method, and the results showed a halo of 17.3 ± 0.28 mm. Furthermore, employing the broth microdilution method, it was observed that OEO-KMP's led to complete inhibition (6- 7 Log reduction) of *C. albicans* in its planktonic state. Moreover, it was evident that the keratin solution had no impact on the growth of fungal cells. These findings indicate that OEO-KMP's exert a more pronounced effect when cells grow in liquid medium. This observation reinforces the notion that the release rate of OEO-KMP's depends on the characteristics of the culture conditions.

Candida infections are often associated with biofilm formation, leading to resistance development and, consequently, the need for more potent therapies that can induce changes in the beneficial vaginal

microbiota. It is therefore essential to confirm that this alternative treatment does not cause an imbalance in the remaining microbiota. Indeed, beneficial bacteria play an important protective role in preventing gynecological diseases. In a previous work (subchapter III.2), it was shown that the vapor-phase of white thyme EO (VP-WTEO) had a significant inhibitory effect on the number of *C. albicans* cells colonizing a reconstituted human vaginal epithelium (RHVE) without changing the number of *L. gasseri* cells, making this application a safe alternative for the remaining vaginal microbiota. Despite the indication on the safety of VP-WTEO towards *L. gasseri*, it was important to evaluate if OEO, being more potent, might exert a more pronounced impact on *Lactobacillus* species. So, in this study, the OEO-KMP's concentration that inhibits/damages *C. albicans* biofilms but does not influence the permanence and viability of *Lactobacillus* species was determined. To this end, the OEO-KMP's effect in the OEO concentration range 0.23- 11.6 mg mL⁻¹ on single biofilms of *C. albicans* (Figure 41.A) and on mixed biofilms of *C. albicans* (Figure 41.B) and *L. gasseri* (Figure 41.C) was evaluated. In both types of biofilms, single or mixed, the effect of OEO-KMP's followed the same pattern, with the concentration of 0.45 mg mL⁻¹ eradicating completely (p< 0.01) the *C. albicans* biofilm. So, the results demonstrated that only 2.5 % of OEO-KMP's corresponding to 0.45 mg mL⁻¹ of OEO encapsulated allows to eradicate *C. albicans* mature biofilms and preserve the *L. gasseri*, at the same time (Figure 41). Therefore, a safe concentration for eradicating *C. albicans* biofilms has been determined at 0.45 mg mL⁻¹ of the OEO-KMP's.

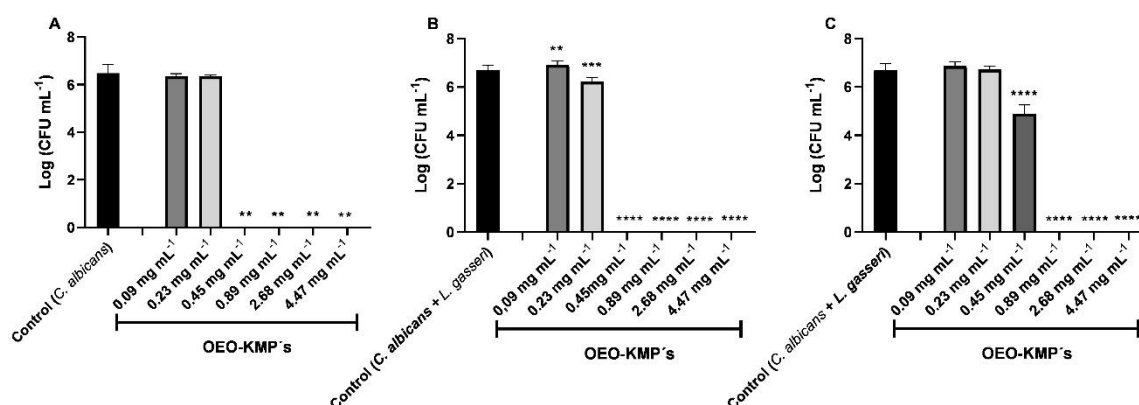


Figure 41. Effect of the keratin microparticles encapsulating oregano oil (OEO-KMP's) on single and mixed infection of *Candida albicans* with *Lactobacillus gasseri*. (A) Single infection of *C. albicans*; (B) Mixed infection (*C. albicans*); (C) Mixed infection (*L. gasseri*). *indicate statistical reduction of biofilms cell cultivability in comparison with the respective control (**p< 0.01, *** p< 0.001, **** p< 0.0001).

3.3 *In vivo* effect of oregano essential oil encapsulated into microparticles of keratin on *Candida albicans* infection

A well-designed vaginal drug delivery system based on microparticles encapsulating a drug, should ensure even distribution throughout the vaginal cavity, prolonged retention at the administration site, and sustained drug release without causing adverse effects on the local vaginal epithelium [95,97]. In this sense, the mouse vaginal model is an appropriate tool to mimic human vaginal conditions as much as possible and to evaluate new therapies, as well as to verify the host's defenses against fungal infections [98,99]. So, in this work, the efficacy of OEO-KMP's *in vivo* was evaluated using an experimental VVC mouse model (Figure 42.A). For this, a mouse model with VVC was established with the support of E2, a hormone which is an effective precipitator of VVC. This hormone allowed mice colonization by *C. albicans*, since, unlike the human vaginal flora, *Candida* species is not naturally present as a colonizing organism in the vaginal flora of mice [98,100]. E2 allows the reduction of extravasation of neutrophils, effector cells that can phagocytose pathogens and mediate tissue damage, being one of the first lines of defence against invading pathogens [101]. Thus, female BALB/c mice were infected intravaginally with *C. albicans*, and the dose of E2 was reinforced, to maintain the ideal conditions for inducing infection (Figure 42.A). After 24 h of infection, the OEO-KMP's or PBS (control group) were applied intravaginally, allowing the formulation to encounter the localized infection during 24 h, as shown in Figure 42.A. After this time, vaginal lavages were performed and the amount of *C. albicans* in the vaginal canal was quantified by CFU counting. The mice were then sacrificed, and the vaginal tissues isolated and analysed under a microscopy. Over time, the body weight of each animal was also monitored, an important parameter that allows the detection of side effects of the treatment on the animal's health, which can be reflected in excessive weight gain or loss [102]. Throughout the entire procedure, infection and subsequent treatment, there were no significant changes in the body weight of the treated animals compared to the control group. In fact, with the same pattern being observed over time (Figure 42.B).

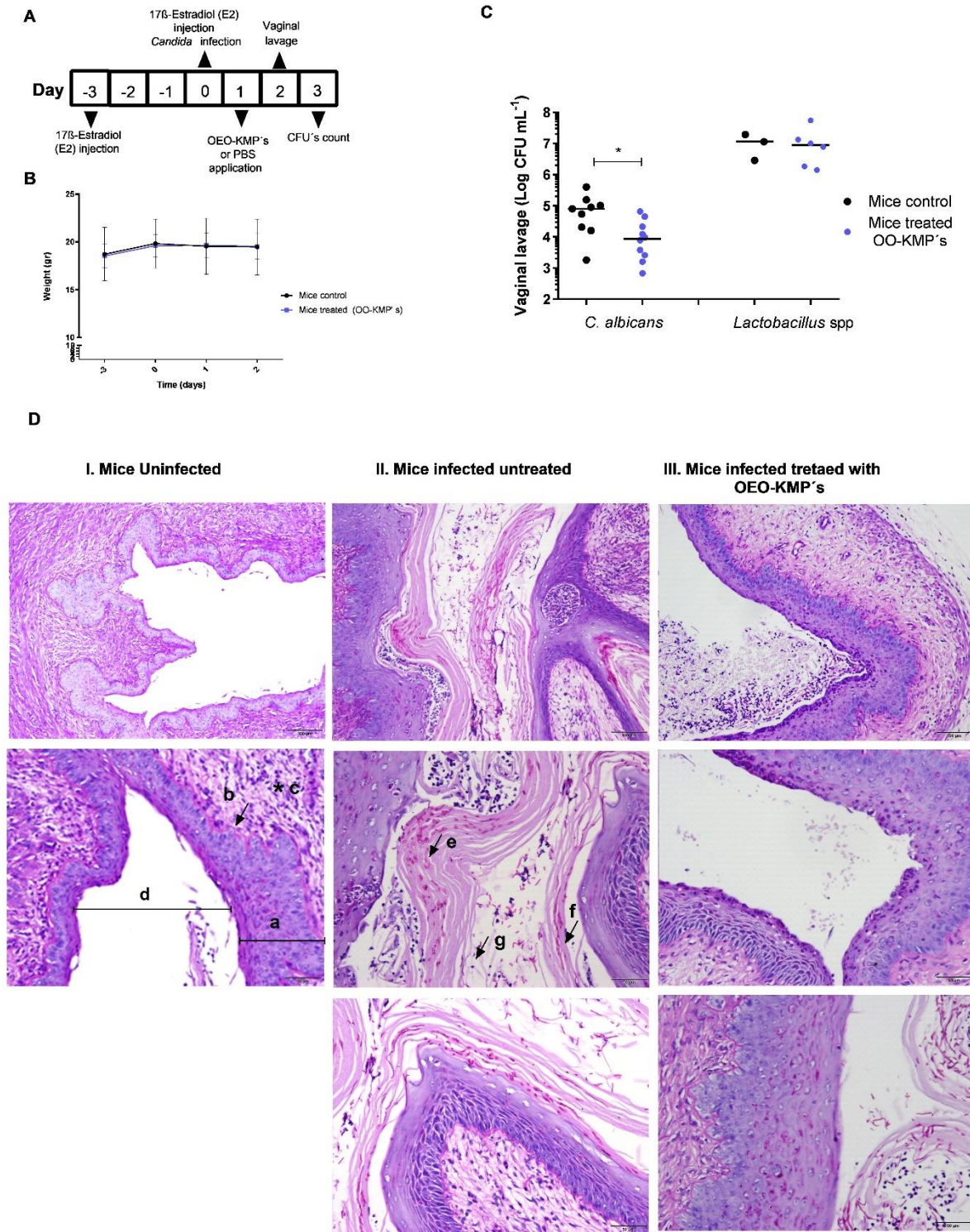


Figure 42. Effect of OEO-KMP's on mice model of vaginal candidiasis. **(A)** Timeline of *Candida albicans* infection and OEO-KMP's treatment of mice; **(B)** Mice weight according to the timeline of infection; **(C)** *C. albicans* and *Lactobacillus* cells detected in mice vaginal lavage fluid (Log CFU mL⁻¹) represented is the median with 95 % confidence interval (*p < 0.05); **(D)** Histological images of vaginal tissue stained with periodic acid-Schiff (PAS), increasing magnification along the column: **I.** Mice uninfected (negative control); **II.** Mice infected with *C. albicans*,

untreated (positive control); III. Mice infected with *C. albicans*, treated with OEO-KMP's for 24 h after infection. (a) layer of superficial squamous epithelial cells, (b) basal membrane, (c) lamina propria (d) vaginal lumen, (e) and (f) *C. albicans* cells and (g) neutrophils. The results were registered under 10x and 20x magnification. The data presented in this study are representative of at least two independent experiments.

The results of the CFUs in vaginal lavages showed a significant difference ($p < 0.1$) between the treated group of animals and the untreated animals after 24 h of OEO-KMP's application. In fact, a single intravaginal application of OEO-KMP's (0.69 mg of OEO) induced a 1 Log CFU mL⁻¹ reduction in *C. albicans* cells compared to control (untreated mice) (Figure 42.C). This result suggests that a single intravaginal application of OEO-KMP's is able to control the growth of *C. albicans* in an environmental niche.

A downward trend in *Candida* infection is evident, despite this dispersion in *in vivo* assays that can be considered usual, as it entails several factors that can influence the initial infection and subsequent treatment, mainly the hormonal cycle of the female mice. In fact, during the menstrual cycle, the female reproductive tract is highly susceptible to changes in sex hormones [101,103]. In humans, the menstrual cycle lasts approximately 28 days and consists of three main phases: (a) follicular phase, (b) ovulatory phase, and (c) luteal phase; in rodents this cycle, called the estrous cycle, lasts approximately 4- 5 days and the 3 corresponding phases are called (a) proestrus, (b) estrus and (c) metestrus [101,103]. During the different phases of the cycle, the vaginal epithelium changes, and in the luteal phase it keratinizes and hardens, resulting in epithelial thickening and desquamation during menstruation [100]. Indeed, hormonal effects may have implications for the development and treatment of VVC, the recurrence of VVC is frequently observed during pregnancy and in the late luteal phase, occurring just prior to menstruation [104]. Kalo-Klein *et al.* [105] observations revealed that *C. albicans* filamentation and consequently infection was maximal during the luteal phase. Gonçalves *et al.* [104] also reported that *C. albicans* is susceptible to hormone-induced biofilm dispersion. Therefore, controlling the estrous cycle in mice can be challenging due to their relatively rapid cycle, even with the administration of E2.

Moreover, one of the crucial aspects of this therapy is that it preserves the remaining microflora, particularly the *Lactobacillus* species, as shown in Figure 42.C. A single intravaginal application of OEO-KMP's does not seem to disrupt the population of *Lactobacillus* population present in the murine microflora, which remains at a concentration of approximately 1×10^7 CFU mL⁻¹ without significant differences compared to untreated mice. As mentioned above, this factor is imperative and it is worth noting that bacteria are the main component of the human microbiome and beneficial bacteria play a

fundamental role in maintaining fungi in a commensal state, acting as the first line of defence against fungal infection [106].

To better understand the effect induced by the treatment with OEO-KMP's in the vaginal environment, the vaginal tissues were histologically examined. Thus, vaginal tissues of healthy animals (negative control) (Figure 42.D.I).were compared with three conditions of infected mice: untreated and non-lavage (Annex III), untreated with lavage (positive control) (Figure 42.D.II). and treated animals (Figure 42.D.III). The vagina is a fibromuscular canal, in its relaxed state, the vaginal wall collapses and obliterates the lumen and the epithelium appears "pleated". This fibromuscular canal contains the following layers: mucosal layer: squamous stratified epithelium (mucosal layer) (Figure 42.D (a)); lamina propria: dense connective tissue, rich in elastic fibres, well vascularized (Figure 42.D (c)); muscular layer: smooth muscle and adventitial or serous layer: lax connective tissue. The oval structures and red filaments seen in the vaginal tissue images were identified as *C. albicans* cells (Figure 42.D (e and f)).

The histological images showed that infected tissue (without any vaginal treatment or lavage) is more degraded by the infection compared to the untreated, with the access of *C. albicans* hyphae observed in the layer of superficial squamous epithelial cells (Annex III). This suggests that the application of PBS alone after 24 h of infection may relieve the tissue infection process, leading to a reduction in the damage induced by the *Candida* species. Potential tissue damage from irritation includes hyperplasia, necrosis, changes in the stratum lucidum and corneum of the vaginal epithelium, detachment and separation of cells, vascular changes or damage to the vessel walls (arterioles and capillaries) [107–109].

Furthermore, the images revealed that the vaginal tissues infected with *C. albicans* (Figure 42.D.II), possessed a notable production of keratin by squamous cells, with identifiable mycotic forms, coupled with an intense exocytosis of the epithelium by neutrophils, sometimes (or frequently) aggregated on the surface of the epithelium with the formation of subcorneal pustules. Moreover, it is possible to observe the vaginal lumen with intense desquamation (desquamated cells and keratin remains) and with accumulation of neutrophils (Figure 42.D.II). Furthermore, the images show a decrease on vaginal tissues lesions with the treatment of mice with OEO-KMP, resulting in a decreased of the accumulation of inflammatory cells (neutrophils) and in formation of a superficial layer thinner with a clear reduction in intense desquamation (sloughed cells and keratin debris) in the vaginal lumen (Figure 42.D.III).

According to the literature, these layers are composed of bundles of keratin intermediate filaments, which form as epithelial cells differentiate and die. The most superficial keratinized layers are only loosely attached and are constantly lost (shedding) and replaced. As cells in the deeper (basal) layer divide and move towards the surface, they undergo several morphological and biochemical changes and when they

reach the surface, they undergo a process of keratinization. This process is important as it helps to support the surface layer of the vaginal epithelium, making it more resistant to mechanical damage, variations in pH, and infectious agents [107]. In this study, after *Candida* infection this mechanism is observed, so it could be a protective mechanism together with neutrophils. Despite the injection of estradiol, these defense cells appear to be present in the vaginal lumen (Figure 42.D.II). Moreover, the presence of cells of microorganisms smaller than *Candida* species is evident. These microorganisms probably correspond to bacteria naturally present in the vaginal fluid of mice, potentially including the lactobacilli, consistent with the results obtained concerning *Lactobacillus* persistence (Figure 42.C). While variations in tissue damage and the presence of *Candida* cells were observed within the same group (a phenomenon considered common in *in vivo* assays), it is notable that fewer *Candida* cells were associated with an increased presence of small structures, potentially indicative of *Lactobacillus*, as discussed earlier.

To support these conclusions, the assessment of vaginal irritation after the experimental procedure in the different groups was based on a histopathological evaluation of four parameters in the vaginal tissue: epithelial lesions, inflammatory infiltrates, vascular congestion, and edema/fibrosis (Table 13) [82].

Table 13. Histopathological evaluation of vaginal irritation after infection and 24 h of rectovaginal application of PBS (positive group) or OEO-KMP's. Score interpretation: minimal (1- 4), mild (5- 8), moderate (9- 11), and severe (12- 16)

Parameters	Healthy tissue (negative control)	Infected tissue without any vaginal treatment or lavage	Infected tissue untreated (control positive)	Infected tissue and treated with OEO-KMP's
Epithelial lesions	0	4	3	1
Inflammatory infiltrates	0	4	3	3
Vascular congestion	0	3	2	0
Edema/fibrosis	0	1	1	0
Total score	0	12	9	4

Healthy tissue exhibits stratified squamous epithelium with dense subepithelial connective tissue, with no sign of damage, obtaining a score of 0. On the other hand, the infected and non-interfered tissue scored 12 (severe tissue damage) (Annex III). In this group, there was a high degree of inflammatory infiltration, with the keratin layer appearing to be dissolved and with the presence of necrotic tissue remains and there was also vascular congestion. In the untreated infected group, although there was less tissue damage than in the previous group, a score of 9 (moderate) was obtained, primarily due to inflammatory infiltrate and remnants of necrotic tissue. The tissue from mice treated with the OEO-KMP's obtained a score of 4 indicating minimal damage, which is considered suitable for clinical trials of vaginal

products [81]. However, there is accumulation of inflammatory cells associated with *C. albicans* infection. Inflammation of the reproductive tissue is assessed by the magnitude of inflammatory infiltrates such as neutrophils, macrophages, and lymphocytes [110]. These results suggest that while a single application of OEO-KMP's can be used safely, the presence of inflammatory cells highlights the ongoing need for repeated treatment to address the inflammation associated with *C. albicans* infection. In fact, the potential expulsion of therapy, influenced by the dynamics of vaginal fluid and the self-cleaning action of the vaginal tract, can lead to incomplete administration of sufficient doses [95,97]. Consequently, multiple doses a day are often necessary to achieve the desired therapeutic effects [95,97]. Indeed, this, *in vivo*, study is considered preliminary, and one of the limitations is related to the small number of mice used, which made it impossible to explore specific theories, perform repeated treatments and investigate other related issues in greater depth.

Furthermore, it is crucial to recognize that the mouse model of vaginal candidiasis, while a valuable research tool for studying vaginal candidiasis due to its similarities to the chronic nature of the disease in women, still exhibits differences from human vaginal candidiasis in several physical aspects. These distinctions include the lack of *Candida* species as part of the vaginal microbiota, requiring exogenous estrogen to initiate fungal colonization, and differences in vaginal pH [79,111]. Consequently, it is essential to evaluate this therapy in alternative animal models, potentially including larger species, to obtain a more comprehensive understanding of their potential effects in cases of VVC in women.

4. Conclusions

This work confirmed the stability of OEO-KMP's over time and their *in vitro* and *in vivo* effectiveness against *C. albicans* infection. These findings constitute an important step towards understanding the potential of OEO-KMP's as antifungal agents. Significantly, our research demonstrated that a single application of OEO-KMP's effectively reduced *C. albicans* levels in an *in vivo* vaginal model while maintaining the *Lactobacillus* species population, providing the theoretical basis for the potential future clinical use of OEO-KMP's as antifungal agents, which may have a less harmful effect on women's health. The choice of this delivery method (KMP) aligns with the natural origin of OEO and its potential to minimize adverse effects. Therefore, one possible application of this therapy is its incorporation into a topical lotion for vagina use. It is worth noting that although the *in vivo* study in a murine model was successful, further investigation and complementary research is required to consolidate these findings. This research emphasizes the potential of OEO-KMP's as a novel, natural, and effective antifungal treatment, opening door to safer and more effective approaches to treating *C. albicans* infections in women's health.

References

- [1] Prestinaci F, Pezzotti P, Pantosti A. Antimicrobial resistance: a global multifaceted phenomenon. *Pathog Glob Health* 2015;109:309. <https://doi.org/10.1179/2047773215Y.0000000030>.
- [2] Pappas PG, Lionakis MS, Arendrup MC, *et al.* Invasive candidiasis. *Nature Reviews Disease Primers* 2018 4:1 2018;4:1–20. <https://doi.org/10.1038/nrdp.2018.26>.
- [3] Denning DW, Kneale M, Sobel JD, *et al.* Global burden of recurrent vulvovaginal candidiasis: a systematic review. *Lancet Infect Dis* 2018;18:e339–47. [https://doi.org/10.1016/S1473-3099\(18\)30103-8](https://doi.org/10.1016/S1473-3099(18)30103-8).
- [4] Sobel JD. Vulvovaginal candidosis. *The Lancet* 2007;369:1961–71. [https://doi.org/10.1016/S0140-6736\(07\)60917-9](https://doi.org/10.1016/S0140-6736(07)60917-9).
- [5] Brunke S, Hube B. Two unlike cousins: *Candida albicans* and *C. glabrata* infection strategies. *Cell Microbiol* 2013;15:701–8. <https://doi.org/10.1111/CMI.12091>.
- [6] Feyaerts AF, Mathé L, Luyten W, *et al.* Essential oils and their components are a class of antifungals with potent vapour-phase-mediated anti-*Candida* activity. *Scientific Reports* 2018 8:1 2018;8:1–10. <https://doi.org/10.1038/s41598-018-22395-6>.
- [7] Vale-Silva LA, Sanglard D. Tipping the balance both ways: drug resistance and virulence in *Candida glabrata*. *FEMS Yeast Res* 2015;15. <https://doi.org/10.1093/FEMSYP/FOV025>.
- [8] Giordani R, Regli P, Kaloustian J, *et al.* Antifungal effect of various essential oils against *Candida albicans*. Potentiation of antifungal action of amphotericin B by essential oil from *Thymus vulgaris*. *Phytother Res* 2004;18:990–5. <https://doi.org/10.1002/PTR.1594>.
- [9] Martins N, Costa-Oliveira S, Melo LDR, *et al.* Susceptibility testing of *Candida albicans* and *Candida glabrata* to *Glycyrrhiza glabra* L. *Ind Crops Prod* 2017;108:480–484. <https://doi.org/10.1016/j.indcrop.2017.07.008>.
- [10] Lang G, Buchbauer G. A review on recent research results (2008-2010) on essential oils as antimicrobials and antifungals. A review. *Flavour Fragr J* 2012;27:13–39. <https://doi.org/10.1002/ffj.2082>.
- [11] Bakkali F, Averbeck S, Averbeck D, *et al.* Biological effects of essential oils - A review. *Food Chem Toxicol* 2008;46:446–475. <https://doi.org/10.1016/j.fct.2007.09.106>.
- [12] Nostro A, Roccaro AS, Bisignano G, *et al.* Effects of oregano, carvacrol and thymol on *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms. *J Med Microbiol* 2007;56:519–23. <https://doi.org/10.1099/jmm.0.46804-0>.
- [13] Marino A, Nostro A, Mandras N, *et al.* Evaluation of antimicrobial activity of the hydrolate of *Coridothymus capitatus* (L.) Reichenb. fil. (*Lamiaceae*) alone and in combination with antimicrobial agents. *BMC Complement Med Ther* 2020;20:89. <https://doi.org/10.1186/s12906-020-2877-x>.
- [14] Koroch AR, Rodolfo Juliani H, Zygadlo JA. Bioactivity of essential oils and their components. *Flavours and Fragrances: Chemistry, Bioprocessing and Sustainability*, 2007, p. 87–115. https://doi.org/10.1007/978-3-540-49339-6_5.
- [15] Baser K, Demitri FC. Chemistry of essential oils. In *Flavours and Fragrances - chemistry, bioprocessing and sustainability*. Berlin, Germany: Springer; 2007.
- [16] Raut JS, Karuppaiyl SM. A status review on the medicinal properties of essential oils. *Ind Crops Prod* 2014;62:250–64. <https://doi.org/10.1016/J.INDCROP.2014.05.055>.
- [17] Manohar V, Ingram C, Gray J, *et al.* Antifungal activities of origanum oil against *Candida albicans*. *Mol Cell Biochem* 2001;228:111–7. <https://doi.org/10.1023/A:1013311632207>.
- [18] Salgueiro LR, Cavaleiro C, Pinto E, *et al.* Chemical composition and antifungal activity of the essential oil of *Origanum virens* on *Candida* species. *Planta Med* 2003;69:871–4. <https://doi.org/10.1055/S-2003-43203>.
- [19] Pina-Vaz C, Rodrigues AG, Pinto E, *et al.* Antifungal activity of Thymus oils and their major compounds. *J Eur Acad Dermatol Venereol* 2004;18:73–8. <https://doi.org/10.1111/j.1468-3083.2004.00886.x>.
- [20] Pinto E, Pina-Vaz C, Salgueiro L, *et al.* Antifungal activity of the essential oil of *Thymus pulegioides* on *Candida*, *Aspergillus* and dermatophyte species. *J Med Microbiol* 2006;55:1367–73. <https://doi.org/10.1099/JMM.0.46443-0>.

- [21] Pinto E, Gonçalves MJ, Hrimpeng K, *et al.* Antifungal activity of the essential oil of *Thymus villosus* subsp. lusitanicus against *Candida*, *Cryptococcus*, *Aspergillus* and dermatophyte species. Ind Crops Prod 2013;51:93–9. <https://doi.org/10.1016/J.INDCROP.2013.08.033>.
- [22] Pozzatti P, Loreto ES, Nunes Mario DA, *et al.* Activities of essential oils in the inhibition of *Candida albicans* and *Candida dubliniensis* germ tube formation. J Mycol Med 2010;20:185–9. <https://doi.org/10.1016/J.MYCMED.2010.06.003>.
- [23] Hacıoglu M, Oyardi O, Kirinti A. Oregano essential oil inhibits *Candida* spp. biofilms. Zeitschrift Fur Naturforschung - Section C Journal of Biosciences 2021;76:443–50. https://doi.org/10.1515/ZNC-2021-0002/DOWNLOADASSET/SUPPL/J_ZNC-2021-0002_SUPPL.DOCX.
- [24] Reyes-Jurado F, Navarro-Cruz AR, Ochoa-Velasco CE, *et al.* Essential oils in vapor phase as alternative antimicrobials: A review. Crit Rev Food Sci Nutr 2019;60:1641–50. <https://doi.org/10.1080/10408398.2019.1586641>.
- [25] Mandras N, Nostro A, Roana J, *et al.* Liquid and vapour-phase antifungal activities of essential oils against *Candida albicans* and non-*albicans* *Candida*. BMC Complement Altern Med 2016;16:1–7. <https://doi.org/10.1186/S12906-016-1316-5/TABLES/3>.
- [26] Tyagi AK, Malik A. Liquid and vapour-phase antifungal activities of selected essential oils against *Candida albicans*: Microscopic observations and chemical characterization of *Cymbopogon citratus*. BMC Complement Altern Med 2010;10:1–11. <https://doi.org/10.1186/1472-6882-10-65/TABLES/3>.
- [27] Araújo D, Mil-Homens D, Henriques M, *et al.* Anti-EFG1 2'-OMethylRNA oligomer inhibits *Candida albicans* filamentation and attenuates the candidiasis in *Galleria mellonella*. Mol Ther Nucleic Acids 2022;27:517–23. <https://doi.org/10.1016/j.omtn.2021.12.018>.
- [28] Sosinska GJ, Groot PWJ, Matto MJT, *et al.* Hypoxic conditions and iron restriction affect the cell-wall proteome of *Candida albicans* grown under vagina-simulative conditions. Microbiology (Reading) 2008;154:510–20. <https://doi.org/10.1099/MIC.0.2007/012617-0>.
- [29] Owen DH, Katz DF. A vaginal fluid simulant. Contraception 1999;59:91–5. [https://doi.org/10.1016/S0010-7824\(99\)00010-4](https://doi.org/10.1016/S0010-7824(99)00010-4).
- [30] Tran HNH, Graham L, Adukwu EC. *In vitro* antifungal activity of *Cinnamomum zeylanicum* bark and leaf essential oils against *Candida albicans* and *Candida auris*. Appl Microbiol Biotechnol 2020;104:8911–24. <https://doi.org/10.1007/s00253-020-10829-z>.
- [31] Vihanova K, Houdkova M, Promgool T, *et al.* *In vitro* growth-inhibitory effect of essential oils and supercritical carbon dioxide extracts from *Cinnamomum* spp. barks and fruits against food bacterial pathogens in liquid and vapor phase. J Food Saf 2021;41:e12900. <https://doi.org/10.1111/JFS.12900>.
- [32] Stepanović S, Vuković D, Dakić I, *et al.* A modified microtiter-plate test for quantification of staphylococcal biofilm formation. J Microbiol Methods 2000;40:175–9. [https://doi.org/10.1016/S0167-7012\(00\)00122-6](https://doi.org/10.1016/S0167-7012(00)00122-6).
- [33] Sumiyoshi M, Miyazaki T, Makau JN, *et al.* Novel and potent antimicrobial effects of caspofungin on drug-resistant *Candida* and bacteria. Scientific Reports | 123AD;10:17745. <https://doi.org/10.1038/s41598-020-74749-8>.
- [34] Pina-Vaz C, Sansonetty F, Rodrigues AG, *et al.* Cytometric approach for a rapid evaluation of susceptibility of *Candida* strains to antifungals. European Society of Clinical Infectious Diseases 2001;7:609–18. <https://doi.org/10.1046/j.1198-743x.2001.00307.x>.
- [35] Alves CT, Wei X-Q, Silva O, *et al.* *Candida albicans* promotes invasion and colonisation of *Candida glabrata* in a reconstituted human vaginal epithelium. J Infect 2014;69:396–407. <https://doi.org/10.1016/j.jinf.2014.06.002>.
- [36] Silva SL, Araújo FSM, Silva POA, *et al.* Evaluation of the antimicrobial effect of the *Origanum vulgare* L essential oil on strains of *Klebsiella pneumoniae*. Braz J Biol 2023;83. <https://doi.org/10.1590/1519-6984.269317>.
- [37] Zhan X, Tan Y, Lv Y, *et al.* The Antimicrobial and Antibiofilm Activity of Oregano Essential Oil against *Enterococcus faecalis* and Its Application in Chicken Breast. Foods 2022;11. <https://doi.org/10.3390/FOODS11152296>.

- [38] Zhou Y, Sun S, Bei W, *et al.* Preparation and antimicrobial activity of oregano essential oil Pickering emulsion stabilized by cellulose nanocrystals. *Int J Biol Macromol* 2018;112:7–13. <https://doi.org/10.1016/J.IJBIOMAC.2018.01.102>.
- [39] Ferreira EDS, Rosalen PL, Benso B, *et al.* The Use of Essential Oils and Their Isolated Compounds for the Treatment of Oral Candidiasis: A Literature Review. *Evid Based Complement Alternat Med* 2021;2021. <https://doi.org/10.1155/2021/1059274>.
- [40] Inouye S, Abe S, Yamaguchi H, Asakura M. Comparative study of antimicrobial and cytotoxic effects of selected essential oils by gaseous and solution contacts. *International Journal of Aromatherapy* 2003;13:33–41. [https://doi.org/10.1016/S0962-4562\(03\)00057-2](https://doi.org/10.1016/S0962-4562(03)00057-2).
- [41] Winiwarter S, Ridderström M, Ungell AL, *et al.* Use of Molecular Descriptors for Absorption, Distribution, Metabolism, and Excretion Predictions. *Comprehensive Medicinal Chemistry II* 2007;5:531–54. <https://doi.org/10.1016/B0-08-045044-X/00140-1>.
- [42] Muñoz JE, Rossi DCP, Jabes DL, *et al.* *In Vitro* and *In Vivo* Inhibitory Activity of Limonene against Different Isolates of *Candida* spp. *Journal of Fungi* 2020, Vol 6, Page 183 2020;6:183. <https://doi.org/10.3390/JOF6030183>.
- [43] Nóbrega JR, Silva D de F, Andrade Júnior FP de, *et al.* Antifungal action of α -pinene against *Candida* spp. isolated from patients with otomycosis and effects of its association with boric acid. *Nat Prod Res* 2021;35:6190–3. <https://doi.org/10.1080/14786419.2020.1837803>.
- [44] Chami N, Chami F, Bennis S, *et al.* Antifungal treatment with carvacrol and eugenol of oral candidiasis in immunosuppressed rats. *Braz J Infect Dis* 2004;8:217–26. <https://doi.org/10.1590/S1413-86702004000300005>.
- [45] Marcos-Arias C, Eraso E, Madariaga L, *et al.* *In vitro* activities of natural products against oral *Candida* isolates from denture wearers. *BMC Complement Altern Med* 2011;11. <https://doi.org/10.1186/1472-6882-11-119>.
- [46] Bogavac M, Karaman M, Janjušević L, Sudji J, *et al.* Alternative treatment of vaginal infections – *in vitro* antimicrobial and toxic effects of *Coriandrum sativum* L. and *Thymus vulgaris* L. essential oils. *J Appl Microbiol* 2015;119:697–710. <https://doi.org/10.1111/JAM.12883>.
- [47] Palmeira-de-Oliveira A, Salgueiro L, Palmeira-de-Oliveira R, *et al.* Anti-*Candida* Activity of Essential Oils. *Mini-Reviews in Medicinal Chemistry* 2009;9:1292–305. <https://doi.org/10.2174/138955709789878150>.
- [48] Salgueiro LR, Cavaleiro C, Gonçalves MJ, *et al.* Antimicrobial activity and chemical composition of the essential oil of *Lippia graveolens* from Guatemala. *Planta Med* 2003;69:80–3. <https://doi.org/10.1055/s-2003-37032>.
- [49] Shaban S, Patel M, Ahmad A. Improved efficacy of antifungal drugs in combination with monoterpene phenols against *Candida auris*. *Sci Rep* 2020;10:1162. <https://doi.org/10.1038/s41598-020-58203-3>.
- [50] Turgis M, Han J, Caillet S, *et al.* Antimicrobial activity of mustard essential oil against *Escherichia coli* O157:H7 and *Salmonella typhi*. *Food Control* 2009;20:1073–9. <https://doi.org/10.1016/J.FOODCONT.2009.02.001>.
- [51] Ahmad A, Khan A, Akhtar F, *et al.* Fungicidal activity of thymol and carvacrol by disrupting ergosterol biosynthesis and membrane integrity against *Candida*. *Eur J Clin Microbiol Infect Dis* 2011;30:41–50. <https://doi.org/10.1007/S10096-010-1050-8>.
- [52] Jabra-Rizk MA, Falkler WA, Meiller TF. Fungal Biofilms and Drug Resistance. *Emerg Infect Dis* 2004;10:14–9. <https://doi.org/10.3201/eid1001.030119>.
- [53] Silva S, Rodrigues CF, Araújo D, *et al.* *Candida* species biofilms' antifungal resistance. *Journal of Fungi* 2017. <https://doi.org/10.3390/jof3010008>.
- [54] Ramage G, Rajendran R, Sherry L, *et al.* Fungal biofilm resistance. *Int J Microbiol* 2012;2012:1–14. <https://doi.org/10.1155/2012/528521>.
- [55] Karpanen TJ, Worthington T, Hendry ER, *et al.* Antimicrobial efficacy of chlorhexidine digluconate alone and in combination with eucalyptus oil, tea tree oil and thymol against planktonic and biofilm cultures of *Staphylococcus epidermidis*. *J Antimicrob Chemother* 2008;62:1031–6. <https://doi.org/10.1093/JAC/DKN325>.

- [56] Pina-Vaz C, Costa-De-Oliveira S, Rodrigues AG, *et al.* Comparison of two probes for testing susceptibilities of pathogenic yeasts to voriconazole, itraconazole, and caspofungin by flow cytometry. *J Clin Microbiol* 2005;43:4674–9. <https://doi.org/10.1128/JCM.43.9.4674-4679.2005/ASSET/A0D64FDD-7F2C-49FE-9C7A-36C9639EDBDD/ASSETS/GRAPHIC/ZJM0090557760003.JPEG>.
- [57] Swamy MK, Akhtar MS, Sinniah UR. Antimicrobial properties of plant essential oils against human pathogens and their mode of action: An updated review. *Evid Based Complement Alternat Med* 2016. <https://doi.org/10.1155/2016/3012462>.
- [58] López P, Sánchez C, Battle R, *et al.* Vapor-Phase Activities of Cinnamon, Thyme, and Oregano Essential Oils and Key Constituents against Foodborne Microorganisms. *J Agric Food Chem* 2007;55:4348–56. <https://doi.org/10.1021/JF063295U>.
- [59] Niu C, Wang C, Yang Y, *et al.* Carvacrol Induces *Candida albicans* Apoptosis Associated With Ca²⁺/Calcineurin Pathway. *Front Cell Infect Microbiol* 2020;10:192. <https://doi.org/10.3389/FCIMB.2020.00192>.
- [60] Xu J, Chatterjee M, Baguley TD, *et al.* Inhibitor of the Tyrosine Phosphatase STEP Reverses Cognitive Deficits in a Mouse Model of Alzheimer's Disease. *PLoS Biol* 2014;12:e1001923. <https://doi.org/10.1371/JOURNAL.PBIO.1001923>.
- [61] Tan Y, Lin Q, Yao J, *et al.* *In vitro* outcomes of quercetin on *Candida albicans* planktonic and biofilm cells and *in vivo* effects on vulvovaginal candidiasis. Evidences of its mechanisms of action. *Phytomedicine* 2023;114:154800. <https://doi.org/10.1016/J.PHYMED.2023.154800>.
- [62] Fernandes MZ, Caetano CF, Gaspar C, *et al.* Uncovering the Yeast Diversity in the Female Genital Tract: An Exploration of Spatial Distribution and Antifungal Resistance. *Pathogens* 2023;12:595. <https://doi.org/10.3390/PATHOGENS12040595>.
- [63] Sharma K, Munjal M, Sharma RK, *et al.* Thymol encapsulated chitosan-Aloe vera films for antimicrobial infection. *Int J Biol Macromol* 2023;235. <https://doi.org/10.1016/J.IJBIOMAC.2023.123897>.
- [64] Benitez LL, Carver PL. Adverse Effects Associated with Long-Term Administration of Azole Antifungal Agents. *Drugs* 2019 79:8 2019;79:833–53. <https://doi.org/10.1007/S40265-019-01127-8>.
- [65] Soliman S, Alnajdy D, El-Keblawy AA, *et al.* Plants' Natural Products as Alternative Promising Anti-*Candida* Drugs. *Pharmacogn Rev* 2017;11:104. https://doi.org/10.4103/PHREV.PHREV_8_17.
- [66] Fernandes L, Costa R, Silva S, *et al.* Effect of Vapor-Phase Oregano Essential Oil on Resistant *Candida* Species Biofilms: Mechanisms of Action. *Microbiol Spectr* 2023;11. <https://doi.org/10.1128/SPECTRUM.05124-22>.
- [67] Córdoba S, Vivot W, Szusz W, *et al.* Antifungal Activity of Essential Oils Against *Candida* Species Isolated from Clinical Samples. *Mycopathologia* 2019;184:615–23. <https://doi.org/10.1007/s11046-019-00364-5>.
- [68] Granata G, Stracquadanio S, Leonardi M, *et al.* Essential oils encapsulated in polymer-based nanocapsules as potential candidates for application in food preservation. *Food Chem* 2018;269:286–92. <https://doi.org/10.1016/J.FOODCHEM.2018.06.140>.
- [69] Garcia LGS, da Rocha MG, Lima LR, *et al.* Essential oils encapsulated in chitosan microparticles against *Candida albicans* biofilms. *Int J Biol Macromol* 2021;166:621–32. <https://doi.org/10.1016/J.IJBIOMAC.2020.10.220>.
- [70] Zhi X, Liu P, Li Y, *et al.* One-step fabricated keratin nanoparticles as pH and redox-responsive drug nanocarriers. *J Biomater Sci Polym Ed* 2018;29:1920–34. <https://doi.org/10.1080/09205063.2018.1519987>.
- [71] Mansouri Shirazi N, Eslahi N, Gholipour-Kanani A. Production and Characterization of Keratin/Tragacanth Gum Nanohydrogels for Drug Delivery in Medical Textiles. *Front Mater* 2021;8:371. <https://doi.org/10.3389/FMATS.2021.720385/BIBTEX>.
- [72] Costa AF, Luis S, Noro J, *et al.* Therapeutic Textiles Functionalized with Keratin-Based Particles Encapsulating Terbinafine for the Treatment of Onychomycosis. *Int J Mol Sci* 2022;23:13999. <https://doi.org/10.3390/IJMS232213999/S1>.

- [73] Rouse JG, Van Dyke ME. A Review of Keratin-Based Biomaterials for Biomedical Applications. *Materials* 2010;3:999. <https://doi.org/10.3390/MA3020999>.
- [74] ISO 105-E01:2013(en), Textiles — Tests for colour fastness — Part E01: Colour fastness to water.
- [75] Tinoco A, Gonçalves J, Silva C, *et al.* Keratin-based particles for protection and restoration of hair properties. *Int J Cosmet Sci* 2018;40:408–19. <https://doi.org/10.1111/ICS.12483>.
- [76] DC Protein Assay Instruction Manual
- [77] PD-10 Desalting Columns
- [78] Tran HNH, Graham L, Adukwu EC. *In vitro* antifungal activity of *Cinnamomum zeylanicum* bark and leaf essential oils against *Candida albicans* and *Candida auris*. *Appl Microbiol Biotechnol* 2020;104:8911–24. <https://doi.org/10.1007/S00253-020-10829-Z>.
- [79] Relloso M, Aragonese-Fenoll L, Lasarte S, *et al.* Estradiol impairs the Th17 immune response against *Candida albicans*. *J Leukoc Biol* 2011;91:159–65. <https://doi.org/10.1189/JLB.1110645>.
- [80] Salinas-Muñoz L, Campos-Fernández R, Mercader E, *et al.* Estrogen receptor-alpha (ESR1) governs the lower female reproductive tract vulnerability to *Candida albicans*. *Front Immunol* 2018;9:1033. <https://doi.org/10.3389/FIMMU.2018.01033/BIBTEX>.
- [81] Gao Z, Fu R, Li X, *et al.* Safety Assessment of Microbicide 2P23 on the Rectal and Vaginal Microbiota and Its Antiviral Activity on HIV Infection. *Front Immunol* 2021;12. <https://doi.org/10.3389/FIMMU.2021.702172>.
- [82] ISO 10993-10:2010 - Biological evaluation of medical devices — Part 10: Tests for irritation and skin sensitization. <https://www.iso.org/standard/40884.html> (accessed November 3, 2023).
- [83] Hao Y, Kang J, Yang R, *et al.* Multidimensional exploration of essential oils generated via eight oregano cultivars: Compositions, chemodiversities, and antibacterial capacities. *Food Chem* 2022;374:131629. <https://doi.org/10.1016/J.FOODCHEM.2021.131629>.
- [84] Kapustová M, Granata G, Napoli E, *et al.* Nanoencapsulated Essential Oils with Enhanced Antifungal Activity for Potential Application on Agri-Food, Material and Environmental Fields. *Antibiotics (Basel)* 2021;10:1–12. <https://doi.org/10.3390/ANTIBIOTICS10010031>.
- [85] Perotto G, Sandri G, Pignatelli C, *et al.* Water-based synthesis of keratin micro- and nanoparticles with tunable mucoadhesive properties for drug delivery. *J Mater Chem B* 2019;7:4385–92. <https://doi.org/10.1039/C9TB00443B>.
- [86] Nasser M, Golmohammadzadeh S, Arouiee H, *et al.* Antifungal activity of *Zataria multiflora* essential oil-loaded solid lipid nanoparticles *in-vitro* condition. *Iran J Basic Med Sci* 2016;19:1231. <https://doi.org/10.22038/ijbms.2016.7824>.
- [87] Bang JH, Suslick KS. Applications of Ultrasound to the Synthesis of Nanostructured Materials. *Advanced Materials* 2010;22:1039–59. <https://doi.org/10.1002/ADMA.200904093>.
- [88] Ferroni C, Varchi G. Keratin-Based Nanoparticles as Drug Delivery Carriers. *Applied Sciences* 2021, Vol 11, Page 9417 2021;11:9417. <https://doi.org/10.3390/APP11209417>.
- [89] Albanese A, Tang PS, Chan WCW. The effect of nanoparticle size, shape, and surface chemistry on biological systems. *Annu Rev Biomed Eng* 2012;14:1–16. <https://doi.org/10.1146/ANNUREV-BIOENG-071811-150124>.
- [90] Dupuis V, Cerbu C, Witkowski L, *et al.* Nanodelivery of essential oils as efficient tools against antimicrobial resistance: a review of the type and physical-chemical properties of the delivery systems and applications. *Drug Deliv* 2022;29:1007–24. <https://doi.org/10.1080/10717544.2022.2056663>.
- [91] Calvo P, Remuñán-López C, Vila-Jato JL, *et al.* Development of positively charged colloidal drug carriers: Chitosan-coated polyester nanocapsules and submicron-emulsions. *Colloid Polym Sci* 1997;275:46–53. <https://doi.org/10.1007/S003960050050/METRICS>.
- [92] Ghaheh FS, Khoddami A, Alihosseini F, *et al.* Antioxidant cosmetotextiles: Cotton coating with nanoparticles containing vitamin E. *Process Biochemistry* 2017;59:46–51. <https://doi.org/10.1016/J.PROCBIO.2017.04.020>.

- [93] Hosseinzadeh S, Jafarikukhdan A, Hosseini A, *et al.* The Application of Medicinal Plants in Traditional and Modern Medicine: A Review of *Thymus vulgaris*. *Int J Clin Med* 2015;6:635–42. <https://doi.org/10.4236/IJCM.2015.69084>.
- [94] Elgegren M, Kim S, Cordova D, *et al.* Ultrasound-Assisted Encapsulation of Sacha Inchi (*Plukenetia volubilis* Linneo.) Oil in Alginate-Chitosan Nanoparticles. *Polymers* 2019, Vol 11, Page 1245 2019;11:1245. <https://doi.org/10.3390/POLYM11081245>.
- [95] Johal HS, Garg T, Rath G, *et al.* Advanced topical drug delivery system for the management of vaginal candidiasis. *Drug Deliv* 2016;23:550–63. <https://doi.org/10.3109/10717544.2014.928760>.
- [96] Thanh VM, Bui LM, Bach LG, *et al.* *Origanum majorana* L. Essential Oil-Associated Polymeric Nano Dendrimer for Antifungal Activity against *Phytophthora infestans*. *Materials* 2019;12. <https://doi.org/10.3390/MA12091446>.
- [97] Wong TW, Dhanawat M, Rathbone MJ. Vaginal drug delivery: strategies and concerns in polymeric nanoparticle development. *Expert Opin Drug Deliv* 2014;11:1419–34. <https://doi.org/10.1517/17425247.2014.924499>.
- [98] Qu S, Chen L, Tian H, *et al.* Effect of perillaldehyde on prophylaxis and treatment of vaginal candidiasis in a murine model. *Front Microbiol* 2019;10:430887. <https://doi.org/10.3389/FMICB.2019.01466/BIBTEX>.
- [99] Wang W, Burg N, Vootukuri S, *et al.* Increased Smad2/3 phosphorylation in circulating leukocytes and platelet-leukocyte aggregates in a mouse model of aortic valve stenosis: Evidence of systemic activation of platelet-derived TGF- β 1 and correlation with cardiac dysfunction. *Blood Cells Mol Dis* 2016;58:1–5. <https://doi.org/10.1016/J.BCMD.2016.01.007>.
- [100] Peters BM, Yano J, Noverr MC, *et al.* *Candida Vaginitis*: When Opportunism Knocks, the Host Responds. *PLoS Pathog* 2014;10:e1003965. <https://doi.org/10.1371/JOURNAL.PPAT.1003965>.
- [101] Latorre MC, Gómez-Oro C, Olivera-Valle I, *et al.* Vaginal neutrophil infiltration is contingent on ovarian cycle phase and independent of pathogen infection. *Front Immunol* 2022;13:1031941. <https://doi.org/10.3389/FIMMU.2022.1031941/BIBTEX>.
- [102] Zapata RC, Osborn O. Susceptibility of male wild type mouse strains to antipsychotic-induced weight gain. *Physiol Behav* 2020;220:112859. <https://doi.org/10.1016/J.PHYSBEH.2020.112859>.
- [103] Caligioni CS. Assessing Reproductive Status/Stages in Mice. *Current Protocols in Neuroscience / Editorial Board, Jacqueline N Crawley .et al* 2009;APPENDIX:Appendix. <https://doi.org/10.1002/0471142301.NSA04IS48>.
- [104] Gonçalves B, Azevedo NM, Henriques M, *et al.* Hormones modulate *Candida* vaginal isolates biofilm formation and decrease their susceptibility to azoles and hydrogen peroxide. *Med Mycol* 2020;58:341–50. <https://doi.org/10.1093/MMY/MYZ070>.
- [105] Kalo-Klein A, Witkin SS. *Candida albicans*: Cellular immune system interactions during different stages of the menstrual cycle. *Am J Obstet Gynecol* 1989;161:1132–6. [https://doi.org/10.1016/0002-9378\(89\)90649-2](https://doi.org/10.1016/0002-9378(89)90649-2).
- [106] Chow EWL, Pang LM, Wang Y. Impact of the host microbiota on fungal infections: New possibilities for intervention? *Adv Drug Deliv Rev* 2023;198:114896. <https://doi.org/10.1016/J.ADDR.2023.114896>.
- [107] The Female Reproductive System | Junqueira's Basic Histology: Text and Atlas, 15e | AccessMedicine | McGraw Hill Medical <https://accessmedicine.mhmedical.com/content.aspx?bookid=2430§ionid=190287163> (accessed November 6, 2023).
- [108] Fernando TS, Farina González J. Manual de técnicas en histología y anatomía patológica 2002.
- [109] Li T, Liu Z, Zhang X, *et al.* Local probiotic *Lactobacillus crispatus* and *Lactobacillus delbrueckii* exhibit strong antifungal effects against vulvovaginal candidiasis in a rat model. *Front Microbiol* 2019;10:449407. <https://doi.org/10.3389/FMICB.2019.01033/BIBTEX>.
- [110] Bhandari P, Rishi P, Prabha V. Positive effect of probiotic *Lactobacillus plantarum* in reversing LPS-induced infertility in a mouse model. *J Med Microbiol* 2016;65:345–50. <https://doi.org/10.1099/JMM.0.000230/CITE/REFWORKS>.

- [111] Wu X, Zhang S, Li H, *et al.* Biofilm Formation of *Candida albicans* Facilitates Fungal Infiltration and Persister Cell Formation in Vaginal Candidiasis. *Front Microbiol* 2020;11:531872. <https://doi.org/10.3389/FMICB.2020.01117/BIBTEX>.

CHAPTER V

GENERAL DISCUSSION, FINAL REMARKS AND FUTURE WORK

In this thesis, the exploration of essential oils (EO), particularly the vapor-phase, as a natural therapy for *Candida* infections, has revealed promising results. This study initially confirmed the efficacy of several EOs against *Candida auris* (Subchapter I.1 and I.2), followed by the evaluation of the effectiveness of these EOs against drug-resistant *Candida* vaginal isolates, targeting the treatment or prevention of Vulvovaginal Candidiasis (VVC) (Subchapter I.3). Throughout this process, the methodology had to be adapted and optimized (Subchapter II.1), and the impact of this therapy on the vaginal microflora was also verified (Subchapter II.2). Lastly, the EO that demonstrated the most potent antifungal effect against *Candida* species, oregano oil, was selected and the mode of action of its vapor-phase was determined (Subchapter III.1). Furthermore, another possible application of oregano EO for VVC, namely microencapsulation, was explored and culminated in the evaluation of its impact on *Candida* infections in *in vivo* experiments (Subchapter III.2).

Thus, this thesis proposes a potential, inexpensive, and nontoxic alternative treatment for preventing and treating infections caused by *Candida* species, focusing on VVC, based on a natural product. These new approaches have several advantages for women, including lower costs and easy access, since the vapor-phase presents an easier method of application avoiding contact with the skin, and its microencapsulation provides a controlled release over time. Consequently, this natural therapy reduces the negative impact on women's health. Despite this, the conclusion of this thesis has opened up a vast field with numerous possibilities for further exploration and optimization.

General Discussion and Final Remarks

Nowadays, there has been a notable increase in the occurrence of human fungal infections, particularly caused by *Candida* species. Conventional antifungal drugs, including azoles, polyenes, and echinocandins, have their limitations, such as notable side effects and sometimes inadequate fungicidal efficacy. The virulence factors of *Candida* species, being responsible for increased tolerance resistance and pathogenicity, are relevant therapeutic targets of these agents. Nonetheless, a significant limitation of many commercially available antifungals is their inability to effectively inhibit these factors, exhibiting a fungistatic effect. Which, in turn, contributes to the emergence of drug-resistant strains over time, making conventional antifungals increasingly less effective. Consequently, there is a growing demand for the discovery of novel, more effective, and less toxic therapeutic agents.

The search for substituting antifungal agents with natural substances is a promising area of research that can potentially lead to a reduction in the application of conventional antifungal agents, which has been excessive in recent years. Data collected during this thesis highlight the antifungal potential of Essential oils (EOs) as complementary strategies or alternative treatments to commonly used commercial antifungals. Extensive research into the antifungal effect of EOs has been reported, both for their direct use as biological agents and as a basis for developing novel drugs with promising therapeutic applications. On the other hand, the direct application of these natural compounds may present an important disadvantage in terms of toxicity and, consequently, many studies reported in the literature use very low concentrations of EOs, not exploiting their maximum potential. Therefore, this thesis presents two approaches to the EOs application. The first involves their vapor-phase, which avoids skin contact and potential irritability or burning sensations, facilitating its application (for instance, on women's underwear). The second method is the EOs encapsulation in microcapsules, providing protection from light and temperature alterations, increasing solubility in aqueous environments, controlled release over time, and improving bioaccessibility and bioavailability.

The initial Chapter of this thesis focuses on the evaluation of the impact of EOs, as a natural and alternative therapy, on *Candida* species strains that exhibit resistance to conventional antifungal treatments. Among the species exhibiting drug resistance, *Candida auris* has recently emerged as a significant global health threat. In fact, *C. auris* is acknowledged as a notorious nosocomial pathogen associated with high mortality rates, resulting from its high transmissibility, incorrect utilization of antifungal drugs, phenotypic plasticity, hard identification, and easy adaptation to stressful conditions. Several factors contribute to the increased virulence of *C. auris*, including its multidrug resistance, capacity for biofilm development, and the ability to evade the innate immune system's response. So, a

deeper understanding is imperative for the efficient treatment, prevention, and control of *C. auris* infections. Thus, the first step was to analyze the effect and treatment capability of four EOs (tea tree, niaouli, white thyme and cajeput) against planktonic and biofilm growth of *C. auris*. The results of this work showed that these EOs were able to inhibit the *C. auris* planktonic growth, with an MIC₅₀ between 0.78 and 1.56 % and halos of 20- 21 mm for white thyme and tea tree EOs and 13- 14 mm for cajeput and niaouli. In addition, these EOs were also able to completely inhibit biofilm formation. Moreover, white thyme and cajeput completely eradicate pre-formed biofilms, while tea tree and niaouli significantly reduce it. The findings from this part of the work emphasize the remarkable effectiveness of these four EOs in inhibiting planktonic growth and entirely preventing the formation of *C. auris* biofilms. Collectively, these results indicate that the application of these four EOs, with a particular emphasis on white thyme and cajeput, presents a highly efficient alternative for both preventing and treating infections originating from *C. auris* biofilms. So, this part of the work demonstrates that EOs are a possible alternative therapy for this important pathogen.

The effectiveness of EOs can vary considerably, even when dealing with species within the same genus, according to their chemical profile. Determining the exact antifungal properties of EOs is often challenging and may be attributed to the EOs' primary bioactive compounds or may result from a synergistic interaction between the primary and secondary components. So, in this thesis, the antifungal activity of several EOs from *Thymus* species, namely *Thymus vulgaris*, *Thymus zygis*, *Thymus satureioides* and *Thymus mastichina* against planktonic cells and biofilms of *C. auris* was assessed and compared. Importantly, it was noticed a completely different range of action between the EOs from the same genus. While *T. vulgaris* showed the highest antifungal effect with a halo of 59.75 mm, *T. mastichina* on the other side presented a halo of 13.13 mm, showing low activity. In addition, *T. vulgaris* also showed significant action in the vapor-phase mode. The chemical composition determination of *Thymus* EOs demonstrated that these EOs have different chemical profiles, including the number of phenolic compounds, which justify the different antifungal actions. So, from this research, it was observed that the EOs efficiency differs substantially between the four species of *Thymus*, with EOs from *T. vulgaris* and *T. zygis* being considered a great contribution to the treatment of *C. auris* infections. As a result, the properties of each species should not be generalized to comprise the entire genus, and it becomes essential to specify the particular species and chemotype for accurate evaluation. Therefore, the selection of natural therapies should always take into account EOs composition.

From this perspective, the application of natural therapies, specifically EOs, emerges as a promising choice for the prevention and treatment of Vulvovaginal candidiasis (VVC), the focus of this thesis. VVC has been considered a public health problem characterized by a high incidence rate, and significant implications for women's quality of life and health, representing huge treatment challenges. The increasing demand for intensive antifungal therapies has contributed to the emergence of resistant *Candida* strains to conventional antifungal agents. It should be noted that some antifungal-resistant isolates used in this study were obtained from women, some of whom had clinically significant conditions, such as pregnancy and immunosuppression. In these cases, preventing and quickly treating VVC is extremely important. So, there is an urgent need for effective therapeutic solutions to deal with the challenge of antifungal resistance and with the control of highly resistant biofilms. Therefore, a section of this thesis was dedicated to evaluating the impact of EOs, previously tested on *C. auris*, on drug-resistant *Candida* vaginal isolates. The results revealed high antifungal activity of four EOs (tea tree, cajeput, niaouli and white thyme) against drug-resistant vaginal isolates, including some extremely high resistance to common antifungals, such as *C. albicans*, *C. glabrata*, *C. guilliermondii* and *C. krusei*. In addition, the vapor-phase of EO (VP-EO) with the highest antifungal activity (tea tree) showed a significant inhibitory effect on the biofilm formation (maximum of 4 Logs of reduction) of all tested isolates and was able to provoke an expressive reduction on mature *C. albicans* biofilms (3 Logs of reduction). Additionally, the vapor-phase of tea tree EO (VP-TTEO) showed a significant inhibitory effect on the biofilm formation of all tested isolates and was able to cause an expressive reduction in mature *C. albicans* biofilms. Thus, this part of the thesis suggests that VP-EOs are an effective solution for preventing biofilm-related VVC in at-risk women, including women using intrauterine devices (IUDs) or with suppressed immune systems, or pregnant women. In this part of the thesis, tea tree, cajeput, and niaouli essential oils demonstrated significant antifungal activity, while VP-TTEO exhibited notable preventive effects against biofilm-related VVC caused by antifungal-resistant strains.

Overall, this first Chapter demonstrates that EOs are a possible therapeutic alternative and a future perspective for the hard fight against *C. auris* and drug-resistant *Candida* vaginal isolates. Importantly, it was also suggested that VP-EO is a promising solution to prevent biofilm-related VVC caused by antifungal-resistant strains. However, the selection of EOs as natural therapy should always consider the EOs composition, as observed in the work regarding the effect of Thymus EOs, where the efficacy against *C. auris* differed significantly between different EOs.

The traditional dosage forms for VVC treatment include topical creams, oral formulations, and ointments, with a growing preference and consequent focus of this thesis for drug administration via

the vaginal route. In this sense and despite the favorable outcomes achieved from EOs against clinical vaginal isolates of drug-resistant *Candida* strains, there was a need to optimize the conditions to closely replicate vaginal environment. This was essential to obtain a more comprehensive understanding of the antifungal properties of EOs and their potential effect on the VVC treatment. From this point of view, the choice of culture medium is a crucial step in the development of the process, with a strong impact on both cell growth and the evaluation of the effect of the therapy. So, the most appropriate culture medium, similar to vaginal conditions, was identified and optimized. Synthetic vaginal fluid (SVF) was chosen and compared with the more commonly used culture medium, Sabouraud Dextrose Broth (SDB), in order to validate an *in vitro* model. The results showed that *Candida* isolates maintained the same biofilm formation capacity and morphology in both media (SVF and SDB). This demonstrates that SVF does not interfere with their growth, morphology or virulence factors, including the biofilm formation. Furthermore, under the same conditions, the ability of vapor-phase of oregano and white thyme essential oils (VP-EOs) was also evaluated, to inhibit biofilm formation and to destroy mature biofilms of vaginal isolates. The results confirmed that EOs effect agrees with results acquired, previously, with SDB. This means that the effect of VP-EOs is not affected by the SVF medium, and that this fluid allows the dissolution of the volatile and bioactive compounds. Therefore, it can be deduced that the SVF medium offers a reliable and predictive tool for simulating vaginal environments. It effectively allows the evaluation of the behavior of VP-EOs designed for vaginal administration, confirming a potential effective application of VP-EOs as a prophylactic or therapeutic treatment for biofilm-related VVC.

Another crucial factor that must be considered in the development of new therapies for VVC is their potential impact on other naturally colonizing species, known as the microbiome, that colonize the vaginal mucosa. Of particular concern are those species, such as *Lactobacillus* species, that are believed to play a beneficial role in maintaining the vaginal environment's homeostasis. From this perspective, the impact of the vapor-phase of white thyme essential oil (VP-WTEO) on the vaginal species *Lactobacillus gasseri* in co-colonization with *C. albicans* of a reconstituted human vaginal epithelium (RHVE) model was evaluated. The effect of VP-WTEO on *C. albicans* biofilms (formation and mature biofilms) was initially confirmed, these results revealed that the VP-WTEO had a significant inhibitory effect on *C. albicans* biofilm formation and was able to induce a significant reduction in their mature biofilms. Furthermore, it was observed a significant reduction in the number of *C. albicans* cells colonizing the RHVE without any alteration in the number of cells of *L. gasseri* in the presence of the VP-WTEO. Additionally, tissue damage was assessed, and no significant differences were observed

between the treated tissue and the negative control, which confirmed the results of the cytotoxicity test conducted in the *Galleria mellonella* model. So, this research confirms the efficacy of VP-WTEO in controlling the *C. albicans* proliferation without causing significant disruptions to other vital species in the vaginal microflora, particularly *Lactobacillus*. This preservation of the lactic flora allows the protective role of these microorganisms in the vaginal mucosa.

The results of this third Chapter demonstrate that the SVF medium offers a reliable and predictive tool for mimicking these environments and this model can predict and confirm the potential effect of VP-EOs on vaginal infections induced by *Candida* species, being a safe alternative for the remaining vaginal microbiota, respecting the possible survival of *Lactobacillus* species.

Throughout this thesis, oregano essential oil (OEO) stood out for its remarkable antifungal efficacy against drug-resistant *Candida* species. However, their clinical application may be discouraged by their lipophilic nature, which can lead to potential toxicity when used in pure form or at high concentrations. So, in the fourth Chapter, two methods for safely applying OEO in the context of VVC are presented: one through the vapor-phase, and the other through microencapsulation. Additionally, a crucial aspect of this Chapter involves elucidating the mode of action of the vapor-phase effect. To this end, the effect of the vapor-phase of oregano EO (VP-OEO) on biofilms of antifungal-resistant vaginal clinical isolates of *Candida* species (*Candida albicans* and *Candida glabrata*) and the necessary time of exposure to VP-OEO was first evaluated and then its mode of action was determined. Furthermore, an RHVE was used to mimic vaginal conditions and evaluate the effect of VP-OEO on *Candida* species infection. The results revealed high VP-OEO antifungal activity, with a significant reduction of 4 log CFU in *Candida* biofilms. Importantly, the results show that the mechanisms of action of VP-OEO are related to membrane integrity and metabolic activity. In addition, the epithelium model confirms the effectiveness of VP-OEO.

The second part of this final Chapter involves the development and evaluation of microencapsulated OEO (OEO-KMP's) as an additional alternative approach for the application of OEO in the treatment of *Candida* infections, as mentioned above. After production and characterization regarding the morphological and physicochemical parameters of the OEO-KMP's, their effect against *C. albicans* (both in planktonic state and biofilm) was evaluated *in vitro*. Subsequently, the study moved on to *in vivo* models involving female BALB/C mice. These mice were intravaginally infected and then treated with OEO-KMP's which were analyzed regarding *C. albicans* and *Lactobacillus* species growth. The results showed that OEO-KMP's remained stable over time and exhibited high encapsulation efficiency and a controlled rate of OEO release was observed. The OEO-KMP's size was suitable for

penetration inside fungal cells, leading to a total inhibition of the *C. albicans* planktonic growth. Furthermore, the application, *in vitro*, of only 2.5 % OEO-KMP 's eradicates mature *C. albicans* biofilms while preserving the *Lactobacillus* species. In *in vivo* studies, a single intravaginal application of OEO-KMP 's induced a reduction of *C. albicans* growth (0.6 Log CFU mL⁻¹). Moreover, one of the most important factors is that this therapy keeps intact the remaining microflora concerning the *Lactobacillus* species.

In conclusion, these studies highlight the promising efficacy of OEO as an alternative for VVC treatment. Both approaches, VP-OEO and OEO-KMP, showed effective antifungal activity against drug-resistant strains while preserving vaginal health. These therapeutic options not only combat antifungal resistance but also potentially propose a safer option for women's health due to their natural characteristics, presented several advantages, such as lower costs, easy access, an easier mode of application, avoidance of skin contact, and, therefore, fewer negative impacts on women's health.

Future Work

In this thesis, the exploration of EOs as a natural therapy for *Candida* infections has demonstrated promising results, indicating its potential practical application in preventing and treating these infections. However, the search for alternative therapies in this sense involves a diverse range of natural options that can and should be further investigated. Therefore, in future research, whether continuing along this thematic line or exploring similar natural compounds, several aspects should be considered, including:

- While oregano oil stands out, followed by white thyme and tea tree oil, along with cajeput and niaouli, for their effectiveness against resistant *Candida* strains, it is important to highlight that EOs represent a vast world of possibilities. As natural alternatives, their exploration should extend beyond *Candida* treatment to include other fungal and even bacterial infections.
- *C. auris* is one of the most recent and alarming species, and in-depth studies of phenotypic characterization, pathogenicity, and resistant behavior are crucial to finding efficient treatments and precise procedures to prevent and control its infections. Furthermore, studies to analyze the mechanism of action of EOs in liquids and the vapor-phase in *C. auris* cells will be important.
- *Candida* organisms have the ability to form multi-species biofilms either of *Candida-Candida* species as well as of *Candida*-bacteria species. Thus, future studies to determine the efficacy of VP-EOs on multi-species biofilms will be also useful.
- In this thesis, the evaluation of the impact of the vapor-phase of white thyme on *Lactobacillus gasseri* was observed, which is considered a very specific study. In future research, it will be crucial to extend these studies to examine other essential oils and other strains of *Lactobacillus* species. This will allow for a more comprehensive exploration of potential interactions between different *Candida* and *Lactobacillus* species and the consequent impact of this therapy.
- Flow cytometry and fluorescence *in situ* hybridization (FISH) are techniques that had the potential to significantly improve this research. So, a more extensive utilization of these techniques would be advantageous. For instance, flow cytometry can be used to quantify the efflux of *Candida* species, as the positive regulation of multidrug efflux pumps is an important

mechanism of resistance in these species. It can also be used to determine the dormancy of *Candida* cells after therapy or to investigate the expression of surface markers and phagocytosis. The FISH technique can be used to better differentiate between *Candida* cells and *Lactobacillus* cells.

- The *in vivo* study carried out in this thesis, using the animal model of mice was preliminary, so further study of this technique exploring several factors, such as the use of larger animals, analyzing the hormonal cycle of the animals (which can interfere with the infection and subsequent treatment) would be very advantageous. These additional studies would offer complementary data that could contribute to a more comprehensive understanding of the mode of action of the oils.
- An important phase of this research would involve assessing the impact of essential oils on *Candida* infection through a clinical study. A potential collaborator for this phase could be Dr. Raquel Costa, who has the knowledge and resources required to conduct these trials, supporting the evaluation of treatment effects in women with vulvovaginal candidiasis.

Annex I

I.1 Chemical and physical analysis of tea tree essential oil (Lot 903025) report provided by the company florame® (Saint-Rémy-de-Provence, France).

I.1.1 Identification:

- **Name (International Nomenclature of Cosmetic Ingredients (INCI)/CTFA):** *Melaleuca alternifolia* (Tea tree) leaf oil
- **Latin name:** *Melaleuca alternifolia*
- **N° CAS:** 85085-48-9
- **N° EINECS (European Inventory of Existing Commercial Chemical Substances):** 285-377-1

I.1.2 General characteristics:

- **Botanical family:** Myrtaceae
- **Product acquisition:** Steam distillation of leaves and terminal branches
- **Main molecules :** Terpinen-4-ol, γ -terpinene, α -terpinene
- **Shelf life:** Contains no preservatives, can be stored in its original state away from light, air and heat according to the best before end (BBE).

I.1.3 Lot characteristics:

- **Origin:** South Africa
- **Quality:** Organic (certified by Ecocert SAS F32600)
- **Texture / Color / Odor:** Clear fluid liquid / colorless / characteristic
- **Refractive index (20°C):** 1.4775
- **Density (20°C):** 0.893
- **Rotary power (20°C):** 9.9°

I.1.4 Lot information:

- **Distillation date:** 12/2018
- **BBE:** 12/2023

I.1.5 Chromatographic analysis conditions:

- **Device:** GC with dual FID detection
- **Carrier gas:** Hydrogen
- **Polar column:** Elite Vax (100 % polyethylene glycol) 60 m / 0.25 mm / 0.25 μ m
- **Nonpolar Column:** Elite 5 (5 % diphenyl 95 % dimethylpolysiloxane) 60 m/0.25 mm/0.25 μ m

Analysis date: 03/09/2019

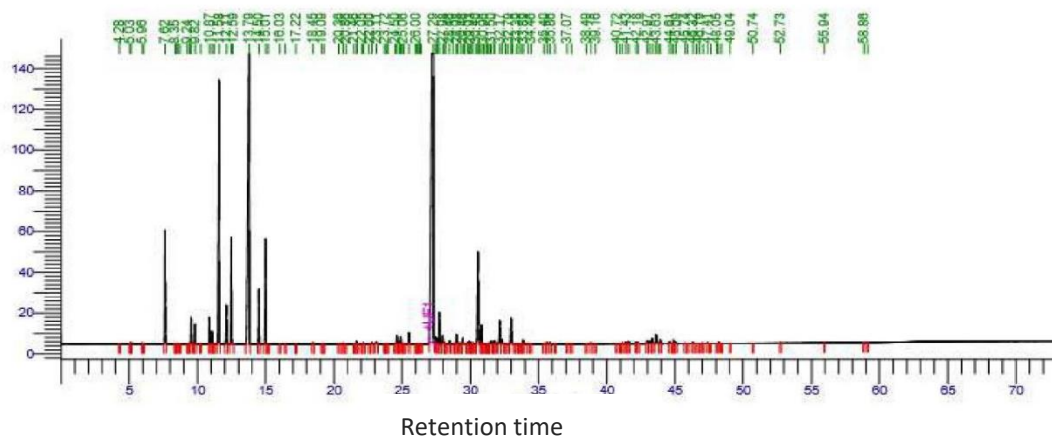


Figure 43. Chromatographic profile (polar column) obtained for the tea tree essential oil (Lot 903025) .

Table 14. Polar column analysis of tea tree Oil (Lot 903025)

Order peaks	Molecules name	Retention time (min)	Area (uV*sec)	Area (%)
6	ALPHA THUYENE+ALPHA PINENE	7.621	174708.63	3.23
13	BETA PINENE	9.537	35106.71	0.65
14	MYRCENE	9.816	25279.06	0.47
16	ALPHA PHELLANDRENE	10.874	38765.73	0.72
17		11.063	18977.32	0.35
19	ALPHA TERPINENE	11.580	554375.94	10.24
20	LIMONENE	12.108	56530.77	1.04
21	1.8 cineole + beta phellandren	12.496	199528.39	3.68
23	GAMMA TERPINENE	13.790	1171977.05	21.64
24	PARA CYMENE	14.505	78064.17	1.44
25	TERPINOLENE	15.011	182273.45	3.37
37		21.650	4934.52	0.09
43		23.110	4023.26	0.07
48	ALPHA GURGUJENE	24.626	13106.23	0.24
49		24.798	2908.32	0.05
50		24.914	12694.02	0.23
53	LINALOL	25.510	19292.58	0.36
59	TERPINENE-4-OL	27.292	2304102.68	42.54
60		27.445	9472.94	0.17
61		27.578	8815.55	0.16
62	TRANS BETA CARYOPHYLLENE	27.720	47390.94	0.87
63		27.982	18393.72	0.34
66		28.456	5128.79	0.09
68	AROMADENDRENE	28.978	16387.62	0.30
69		29.064	3199.68	0.06
71	ALLOAROMADENDRENE	29.464	10698.07	0.20
73		29.818	2822.20	0.05
74		29.932	4384.49	0.08
78	ALPHA TERPINEOL	30.567	175826.61	3.25
79		30.660	7470.76	0.14
80	LEDENE	30.812	34932.93	0.64
85		31.498	4692.54	0.09
86		31.687	4870.87	0.09
87		31.799	8641.04	0.16
88		32.167	40120.63	0.74
89	B SELINENE	32.322	7412.44	0.14
92	DELTA CADINENE	33.007	46889.96	0.87
97		33.886	6256.89	0.12
101		35.628	2582.23	0.05
113		41.623	3409.67	0.06
116		42.971	5437.12	0.10
117		43.126	4489.82	0.08
118		43.345	8506.54	0.16
119	GLOBULOL	43.629	15393.76	0.28
121	VIRIDIFLOROL	43.928	7175.49	0.13
122		44.614	3101.44	0.06
124		44.903	5668.28	0.10
		1288.910	5416221.91	100.00

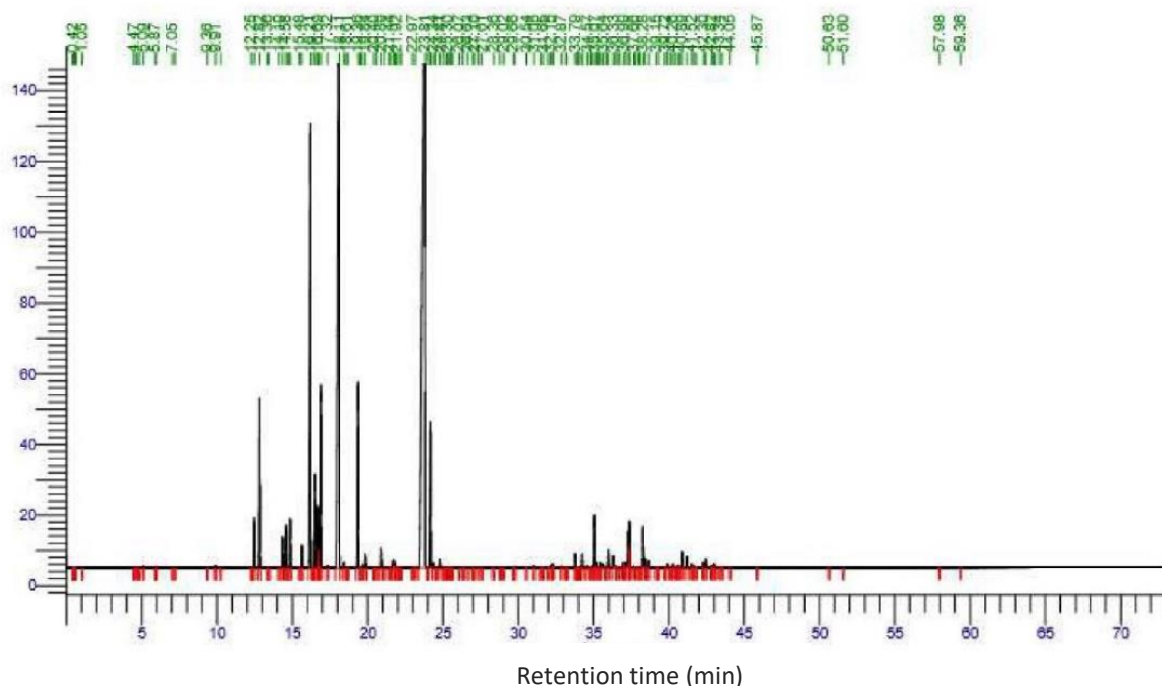


Figure 44. Chromatographic profile (non-polar column) obtained for the tea tree essential oil (Lot 903025).

Table 15. Non-polar column analysis of tea tree essential oil (Lot 903025)

Order peaks	Molecules name	Retention time (min)	Area (uV*sec)	Area (%)
18	ALPHA THUYENE	12.457	38299.14	0.71
19	ALPHA PINENE	12.819	135751.17	2.52
23	SABINENE	14.344	25385.25	0.47
24	BETA PINENE	14.578	35441.41	0.66
26	MYRCENE	14.856	39017.45	0.72
28	ALPHA PHELLANDRENE	15.621	19700.14	0.37
29	ALPHA TERPINENE	16.206	548966.31	10.19
31	PARA CYMENE	16.493	80344.96	1.49
32	LIMONENE	16.688	58398.53	1.08
33	BETA PHELLANDRENE	16.763	36761.26	0.68
34	1,8-CINEOLE	16.904	163213.77	3.03
35		17.324	2588.68	0.05
36	GAMMA TERPINENE	18.108	1151919.23	21.38
37		18.424	5192.96	0.10
40	TERPINOLENE	19.365	186181.18	3.46
42		19.657	2961.01	0.05
43		19.837	12369.57	0.23
46	LINALOL	20.891	19478.89	0.36
50		21.741	13795.86	0.26
51		21.829	3605.85	0.07
57	TERPINEN-4-OL	23.810	2273456.35	42.20
59	ALPHA TERPINEOL	24.176	174851.30	3.25
60	ALPHA COPAENE	24.343	5309.95	0.10
62		24.813	7490.93	0.14
87		32.286	3013.81	0.06
92	ALPHA GURJUNENE	33.788	13362.52	0.25
96	TRANS BETA CARYOPHYLLENE	34.249	13469.54	0.25
100	AROMADENDRENE	35.069	53710.14	1.00
101		35.192	4590.32	0.09
103		35.451	4960.53	0.09
104		35.645	4202.51	0.08
106	ALLOAROMADENDRENE	35.970	17044.86	0.32
107		36.333	12709.77	0.24
111		36.990	4829.62	0.09
112		37.085	8570.32	0.16
113	LEDENE	37.302	44215.27	0.82
114	BICYCLOGERMACRENE	37.388	45525.47	0.85
119	DELTA CADINENE	38.276	42044.94	0.78
120		38.422	8865.37	0.16
121		38.681	6841.61	0.13
125		39.902	4160.48	0.08
127		40.259	3170.96	0.06
131	GLOBULOL	40.887	15687.58	0.29
132	VIRIDIFLOROL	41.218	13036.92	0.24
133		41.525	2967.01	0.06
136		42.296	5763.16	0.11
137		42.444	8740.30	0.16
139		42.977	4836.16	0.09
		1351.683	5386800.33	100.00

I.2 Chemical and physical analysis of cajeput essential oil (Lot 801736) report provided by the company florame® (Saint-Rémy-de-Provence, France).

I.2.1 Identification:

- **Name (INCI/CTFA):** *Melaleuca leucadendra* Cajeput oil
- **Latin name:** *Melaleuca cajaputi*
- **N° CAS :** 85480-37-1
- **N° EINECS:** 287-316-4

I.2.2 General characteristics:

- **Botanical family:** Myrtaceae
- **Product acquisition:** Steam distillation of organic cajeput leaves
- **Main molecules:** 1,8-cineole, α -terpineol, Terpinolene
- **Allergens:** Limonene, linalool, geraniol
- **Shelf life:** Contains no preservatives, can be stored in its original state away from light, air and heat according to the BBE. (5 years from the date of distillation)

I.2.3 Lot characteristics:

- **Origin:** South Africa
- **Quality:** Organic (certified by Ecocert SAS F32600)
- **Texture / Color / Odor:** Clear liquid/ pale yellow/ nice fresh
- **Refractive index (20°C):** 1.4676
- **Density (20°C):** 0,914
- **Rotary power (20°C):** -1.2°

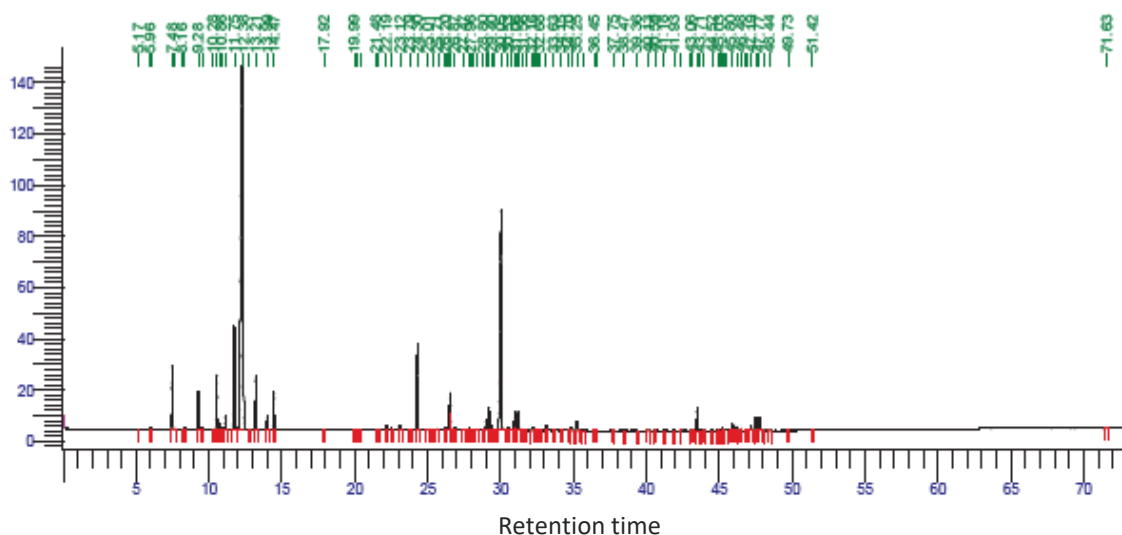
I.2.4 Lot information:

- **Distillation date:** 09/2017
- **BBE:** 09/2022

I.2.5 Chromatographic analysis conditions:

- **Device:** GC with dual FID detection
- **Carrier gas:** Hydrogen
- **Polar column:** Elite Vax (100 % polyethylene glycol) 60 m / 0.25 mm / 0.25 μ m
- **Nonpolar Column:** Elite 5 (5 % diphenyl 95 % dimethylpolysiloxane) 60 m/0.25 mm/0.25 μ m

Analysis date: 12/02/2019



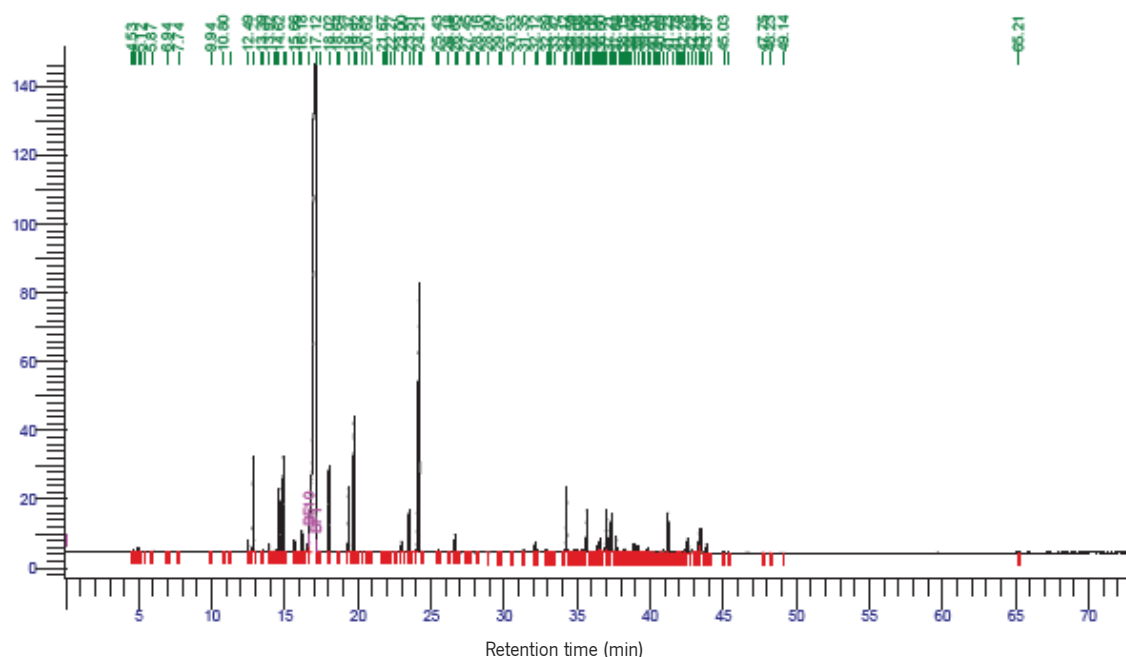


Figure 46. Chromatographic profile (non-polar column) obtained for the cajeput essential oil (Lot 801736).

Table 17. Polar noncolumn analysis of cajeput essential oil (Lot 801736)

Order peaks	Molecules name	Retention time (min)	Area (uV*sec)	Area (%)
14	ALPHA THUYENE	12.487	9033.82	0.20
15	ALPHA PINENE	12.846	76898.78	1.71
18	BENZALDEHYDE	13.924	7044.73	0.16
21	SABINENE	14.618	55361.99	1.23
22	MYRCENE	14.900	81407.53	1.81
24	ALPHA PHELLANDRENE	15.657	11717.37	0.26
26	ALPHA TERPINENE	16.180	24474.28	0.54
27	PARA CYMENE	16.597	19413.07	0.43
28	CINEOLE	17.124	2916315.41	64.83
30	GAMMA TERPINENE	18.022	76858.90	1.71
33	TERPINOLENE	19.368	58775.31	1.31
34	LINALOL	19.737	147078.71	3.27
44	BORNEOL	22.999	11096.17	0.25
45	TERPINEN-4-OL	23.513	41001.50	0.91
47	ALPHA TERPINEOL	24.214	503307.28	11.19
52		26.649	16114.28	0.36
62		31.347	2917.49	0.06
63	ACETATE DE GERANYLE	32.122	11159.22	0.25
65	ALPHA COPAENE	32.887	2836.07	0.06
70		34.280	66888.64	1.49
72		34.853	2684.59	0.06
75		35.350	4473.88	0.10
76		35.564	3046.56	0.07
77	ALPHA CARYOPHYLLENE	35.673	41300.17	0.92
83		36.430	7138.92	0.16
84		36.511	9610.44	0.21
85	D GERMACRENE	36.602	14717.22	0.33
87		36.917	4758.68	0.11
88	BETA SELINENE	37.010	57450.39	1.28
89		37.207	4083.18	0.09
90	ALPHA SELINENE	37.336	40054.39	0.89
93		37.678	17929.24	0.40
97		38.258	3407.42	0.08
102		38.902	7708.87	0.17
103		39.180	8670.84	0.15
107		39.828	3944.15	0.09
115	OXYDE DE CARYOPHYLLENE	41.231	39900.20	0.89
123	10 EPI GAMMA EUDESMOL	42.345	4291.27	0.10
124	ALPHA EUDESMOL	42.580	24934.28	0.55
127		43.369	25386.05	0.56
128	BETA EUDESMOL	43.458	25799.75	0.57
130		43.869	9213.11	0.20
		1269.604	4498182.15	100.00

I.3 Chemical and physical analysis of niaouli essential oil (Lot 100378) report provided by the company florame® (Saint-Rémy-de-Provence, France).

I.3.1 Identification:

- **Name (INCI/CTFA):** *Melaleuca viridiflora* leaf oil
- **Latin name:** *Melaleuca quinquenervia*
- **N° CAS:** 132940-73-9
- **N° EINECS:** 310-217-5

I.3.2 General characteristics:

- **Botanical family:** Myrtaceae
- **Product acquisition:** Steam distillation of leaves
- **Main molecules:** 1,8-cineole, Viridiflorol
- **Allergens:** Limonene, linalool, Citronellol
- **Shelf life:** Contains no preservatives, can be kept as is for 5 years away from light, air and heat.

I.3.4 Lot characteristics:

- **Origin:** Madagascar
- **Quality:** Organic (certified by Ecocert SAS F32600)
- **Texture / Color / Odor:** Clear fluid liquid / colorless / characteristic
- **Refractive index (20°C):** 1,464
- **Density (20°C):** 0,910

I.3.5 Lot information:

- **Distillation date:** 09/2017
- **BBE:** 09/2022

I.3.6 Chromatographic analysis conditions:

- **Device:** GC 6890 MS 5975
- **Mass range:** 40-450 uma
- **Column:** HP5ms/ 30 m/ 0.25 mm/ 0.25 µm
- **Injection divided:** 1 EL - ratio 1/200
- **Carrier gas:** Helium / Flow rate: 1 mL/min
- **Oven:** 2°/min, 60-250°C for 5 min
- **Acquisition time:** 100 min

Analysis date: 14/03/11

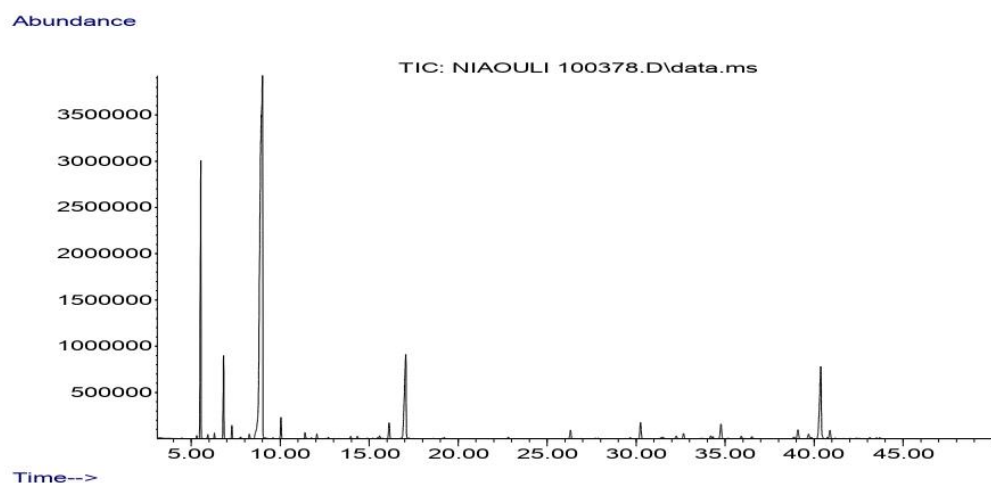


Figure 47. Chromatographic profile obtained for the niaouli essential oil (Lot 100378).

Table 18. Column analysis of niaouli essential oil (Lot 100378)

Identification	Area (%)
α -Thuyène	0,11
α -Pinène	12,00
Camphène	0,18
Benzaldéhyde	0,22
β -Pinène	3,46
β -Myrcène	0,56
α -Phellandrène	0,11
α -Terpinène	0,26
p-Cymène	57,14
Limonène*	
1,8-Cinéole	
trans- β -Ocimène	0,04
γ -Terpinène	1,04
Terpinolène	0,34
Benzoate de méthyle	0,05
Linalol*	0,24
Alcool fenchylique	0,07
Isopulegol	0,13
Bornéol	0,06
Terpinèn-4-ol	1,01
α -Terpinéol	8,55
Citronellol*	0,07
Patchoulène	0,06
β -Caryophyllène	1,22
α -Caryophyllène	0,20
Alloaromadendrène	0,38
β -Sélinène	0,20
Lédène	1,19
γ -Cadinène	0,18
δ -Cadinène	0,16

I.4 Chemical and physical analysis of white thyme essential oil (Lot 800180) report provided by the company florame® (Saint-Rémy-de-Provence, France).

I.4.1 Identification:

- **Name (INCI/CTFA):** *Thymus satureioides* (White thyme) oil
- **Latin name:** *Thymus satureioides*

I.4.2 General characteristics:

- **Botanical family:** Lamiaceae
- **Product acquisition:** Steam distillation of flowered tops
- **Main molecules:** Borneol, thymol, carvacrol
- **Allergens:** Limonene, linalool
- **Shelf life:** Contains no preservatives, can be stored in its original state away from light, air and heat according to the BBE. (5 years from the date of distillation)

I.4.3 Lot characteristics:

- **Origin:** Morocco
- **Quality:** Organic (certified by Ecocert SAS F32600)
- **Texture / Color / Odor:** Clear liquid/light yellow/characteristic
- **Refractive index (20°C):** 1,4843
- **Density (20°C):** 0,938
- **Rotary power (20°C):** -6.0°

I.4.4 Lot information:

- **Distillation date:** 06/2016
- **BBE:** 06/2022

I.4.5 Chromatographic analysis conditions:

- **Device:** GC with dual FID detection
- **Carrier gas:** Hydrogen
- **Polar column:** Elite Vax (100 % polyethylene glycol) 60 m / 0.25 mm / 0.25 µm
- **Nonpolar Column:** Elite 5 (5 % diphenyl 95 % dimethylpolysiloxane) 60 m/0.25 mm/0.25 µm

Analysis date: 19/01/2018

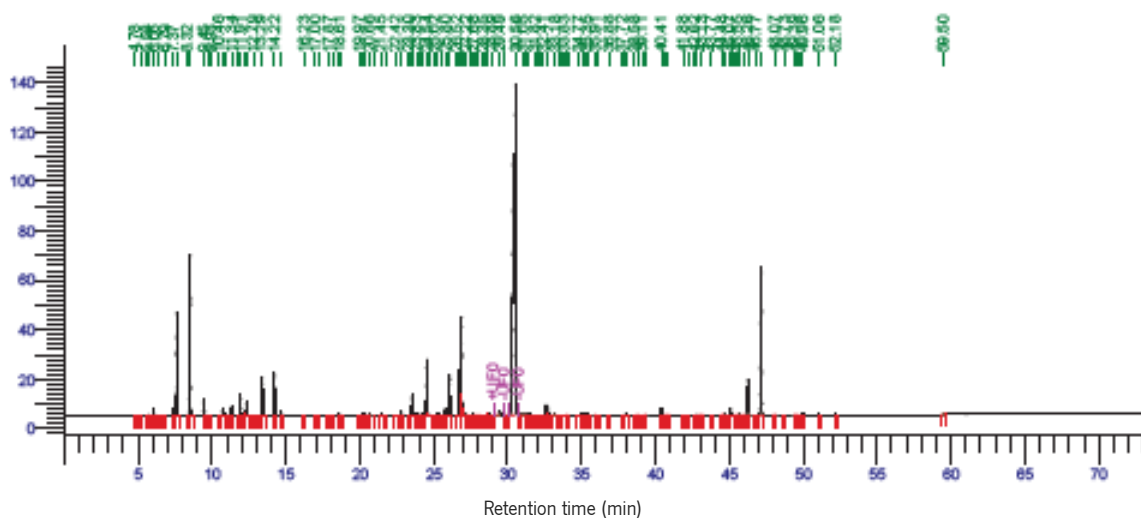


Figure 48. Chromatographic profile (polar column) obtained for the white thyme essential oil (Lot 800180).

Table 19. Polar column analysis of white thyme essential oil (Lot 800180)

Order peaks	Molecules name	Retention time (min)	Area (uV*sec)	Area (%)
6	ETHANOL	6.054	7094.70	0.24
9	TRICYCLEN	7.369	9965.88	0.34
10	ALPHA PINENE + alpha thuyene	7.616	128746.13	4.41
12	CAMPHE	8.516	210892.53	7.22
13	BETA PINENE	9.448	23931.73	0.82
18	MYRCENE	10.726	10753.14	0.37
19		10.911	2647.77	0.09
20	ALPHA TERPINENE	11.338	14926.23	0.51
22	LIMONENE	11.914	31283.70	1.07
23		12.240	5574.19	0.19
24	1.8 CINEOLE	12.336	22850.53	0.78
28	GAMMA TERPINENE	13.406	55362.02	1.89
29	PARA CYMENE	14.219	62950.12	2.15
30	TERPINOLENE	14.682	8260.80	0.28
40		20.259	3274.99	0.11
44		21.448	2705.77	0.09
47		22.801	6685.40	0.23
50	CAMPHE	23.541	37470.10	1.28
53		24.265	2864.65	0.10
54	LINALOL	24.537	86627.94	2.97
57		25.258	3590.73	0.12
59		25.798	12035.88	0.41
60	TERPINEN-4-OL	26.075	72461.93	2.48
62	ACETATE BORNYLE	26.736	84606.05	2.90
63	BETA CARYOPHYLLENE	26.884	192970.47	6.61
64		26.982	10848.40	0.37
67		27.684	5296.29	0.18

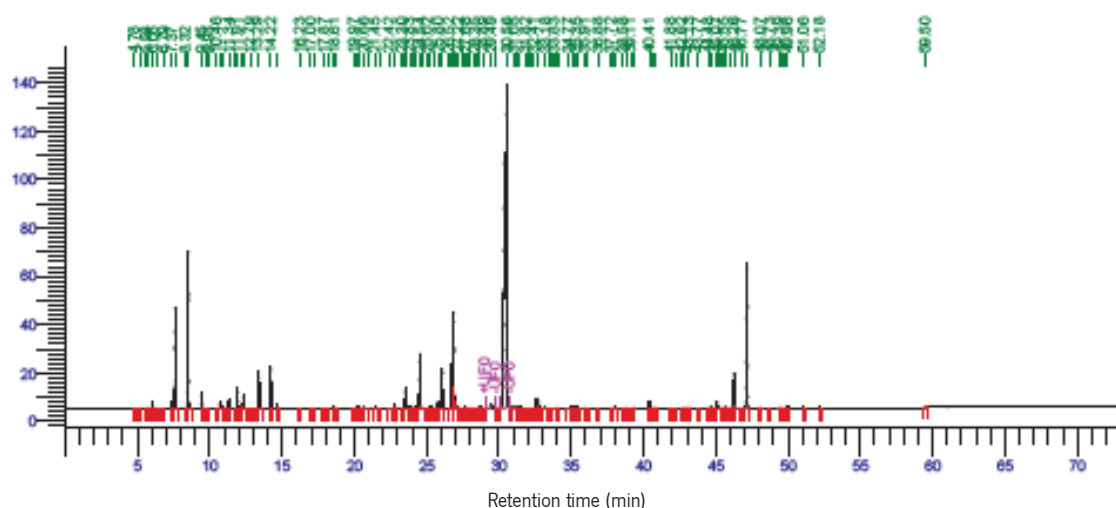


Figure 49. Chromatographic profile (non-polar column) obtained for the white thyme essential oil (Lot 800180).

Table 20. Non-polar column analysis of white thyme essential oil (Lot 800180)

Order peaks	Molecules name	Retention time (min)	Area (uV*sec)	Area (%)
6	ETHANOL	6.054	7094.70	0.24
9	TRICYCLEN	7.369	9965.88	0.34
10	ALPHA PINENE + alpha thuyene	7.616	126746.13	4.41
12	CAMPHENE	8.516	210892.53	7.22
13	BETA PINENE	9.448	23931.73	0.82
18	MYRCENE	10.726	10753.14	0.37
19		10.911	2647.77	0.09
20	ALPHA TERPINENE	11.338	14926.23	0.51
22	LIMONENE	11.914	31283.70	1.07
23		12.240	5574.19	0.19
24	1.8 CINEOLE	12.336	22850.53	0.78
26	GAMMA TERPINENE	13.406	55362.02	1.89
29	PARA CYMENE	14.219	62950.12	2.15
30	TERPINOLENE	14.682	8260.80	0.28
40		20.259	3274.99	0.11
44		21.448	2705.77	0.09
47		22.801	6685.40	0.23
50	CAMPHE	23.541	37470.10	1.28
53		24.265	2864.65	0.10
54	LINALOL	24.537	86627.94	2.97
57		25.258	3590.73	0.12
59		25.798	12035.88	0.41
60	TERPINEN-4-OL	26.075	72461.93	2.48
62	ACETATE BORNYLE	26.736	84606.05	2.90
63	BETA CARYOPHYLLENE	26.884	192970.47	6.61
64		26.982	10848.40	0.37
67		27.684	5298.29	0.18
72		28.622	3471.21	0.12
73		28.673	4397.72	0.15
75		29.487	12665.27	0.43
77	ALPHA TERPINEOL + borneol	30.557	1377321.51	47.14
78		31.047	5737.33	0.20
79		31.216	7760.70	0.27
80		31.452	3305.62	0.11
85	DELTA CADINENE	32.602	16630.66	0.57
86	GAMMA CADINENE	32.717	14615.49	0.50
87		33.183	2886.21	0.10
95		35.180	3397.70	0.12
97		35.455	4032.47	0.14
103		38.027	2552.99	0.09
108	OXYDE DE CARYOPHYLLENE	40.409	11249.26	0.39
121		45.065	11614.11	0.40
127	THYMOL METHYL ETHER+THYMOL	46.264	66214.83	2.27
129	CARVACROL	47.155	251009.52	8.59
135		49.960	2748.62	0.09
138		59.499	3254.96	0.11
		1179.615	2921546.24	100.00

I.5 Chemical and physical analysis of moroccan oregano essential oil (Lot 10468) report provided by the company florame® (Saint-Rémy-de-Provence, France).

I.5.1 Identification:

- **Name (International Nomenclature of Cosmetic Ingredients (INCI)/CTFA):** *Origanum vulgare*/ Oregano oil
- **Latin name:** *Origanum compactum*
- **N° CAS:** 84012-24-8

I.5.2 General characteristics:

- **Botanical family:** Lamiaceae
- **Product acquisition:** Entire plant (steam distillation)
- **Main molecules:** Isothymol, Thymol, g-Terpinene, p-Cymene, Pulegone

I.5.3 Lot characteristics:

- **Origin:** Morocco
- **Texture / Color / Odor:** Clear, yellow liquid with characteristic odour
- **Refractive index (20°C):** 1,488
- **Density (20°C):** 0,925

I.5.4 Chromatographic analysis conditions:

- **Device:** GC 6890 MS 5975
- **Carrier gas:** Hélium, / Flow rate: 1 mL/min
- **Column:** HP5ms 30 m / 0.25 mm / 0.25 µm
- **Mass range:** 40-450 uma
- **Injection divided:** 1 µL - ratio 1/200
- **Oven:** 60-250°C
- **Acquisition time:** 100 min
- **Note:** Verification of the analytical system: Tune OK, Vacuum OK, Absence of leaks OK.
Equipment and metrology under internal quality assurance

Analysis date: 02/03/09

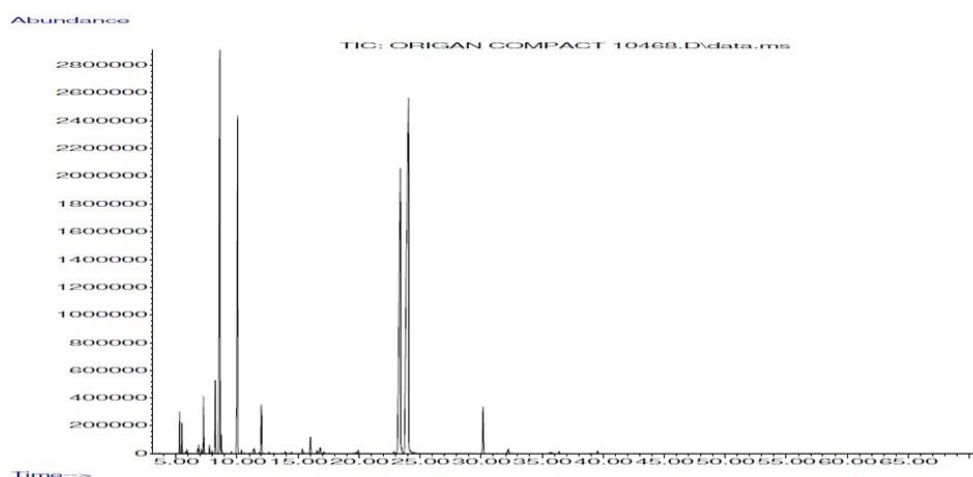


Figure 50. Chromatographic profile obtained for the oregano essential oil (Lot 10468).

Table 21. Column analysis of oregano essential oil (Lot 10468)

Retention time (min)	Identification	Area (%)
5,301	α -Thuyène	0,89
5,496	α -Pinène	0,66
5,920	Camphène	0,10
6,858	1-Octène-3-ol	0,35
7,092	3-Octanone	0,12
7,263	Myrcène	1,39
7,749	α -Phellandrène	0,23
7,963	3-Carène	0,07
8,220	α -Terpinène	2,04
8,592	<i>p</i> -Cymène	15,27
8,715	Limonène*	0,55
9,544	<i>trans</i> - β -Ocimène	0,05
10,053	γ -Terpinène	13,35
10,382	4-Thujanol <i>trans</i>	0,11
11,420	Dehydroparacymène	0,28
11,844	4-Thujanol <i>cis</i>	0,04
12,006	Linalol*	1,55
13,973	1,3,8- <i>p</i> -Menthatriène	0,07
15,368	Bornéol	0,23
16,020	Terpinène-4-ol	0,63
16,825	α -Terpinéol	0,30
17,130	<i>trans</i> -Dihydrocarvone	0,08
19,773	Pulégone	0,06
19,939	Thymol méthyl éther	0,13
23,382	Thymol	21,44
24,049	Carvacrol (Isothymol)	36,97
30,168	β -Caryophyllène	1,97
35,654	β -Bisabolène	0,06
35,797	D Germacrène	0,04
36,401	δ -Cadinène	0,09
39,554	Oxyde de caryophyllène	0,12

*Allergènes

Annex II

II.1 Complete chromatography analysis of the composition of essential oils from *Thymus vulgaris*, *Thymus zygis*, *Thymus satureioides* and *Thymus mastichina***Table 22.** Complete chromatography analysis of the composition of essential oils from *Thymus vulgaris*, *Thymus zygis*, *Thymus satureioides* and *Thymus mastichina*, here shown in percentage (%)

RI ^a	RI ^b	Compound	Percent (%) in oil samples			
			<i>T. satureioides</i>	<i>T. mastichina</i>	<i>T. vulgaris</i>	<i>T. zygis</i>
921	1030	Tricyclene	0.5		t	T
929	1030	α-Pinene	3.0	0.2	0.2	0.2
943	1075	Camphene	5.9	0.3	0.3	0.4
959	1442	Oct-1-en-3-ol	0.2	-	0.3	0.4
962	1255	Octan-3-one	-	-	-	0.2
967	1382	Octan-3-ol	-	-	-	0.1
970	1118	β-Pinene	0.8	0.1	t	0.2
964	1124	Sabinene	0.1	t	t	T
980	1162	Myrcene	0.6	0.3	0.2	0.1
997	1171	α-Phellandrene	0.1	t	t	T
1010	1187	α-Terpinene	0.0	t	t	T
1011	1275	p-Cymene	3.8	0.8	2.2	4.3
1020	1215	1,8-cineole	2.7	8.5	10.0	7.2
1020	1204	Limonene	0.8	0.3	t	T
1046	1249	γ-Terpinene	0.8	0.2	0.4	0.9
1050	1459	E-Sabinene hydrate	0.1	0.6	0.4	-
1055	1438	Z-Linalool oxide (THF)	0.1	0.4	0.1	-
1070	1466	E-Llinalool oxide (THF)	-	-	0.2	-
1071	1439	Cymenene	0.1	-	-	0.1
1076	1288	Terpinolene	0.2	0.4	t	0.1
1082	1543	Linalool	4.5	31.9	7.2	6.6
1106	1555	cis-p-Menth-2-en-1-ol	-	0.2	-	-
1118	1515	Camphor	2.5	1.6	0.7	1.8
1119	1647	E-Pinocarveol	-	0.4	-	0.9
1125	1673	E-Verbenol	-	0.2	-	-
1144	1664	Isoborneol	0.1	3.3	-	-
1145	1695	Borneol	29.3	5.8	3.4	4.9
1158	1597	Terpinen-4-ol	2.3	2.3	1.7	3.9
1165	1622	Myrtenal	-	0.5	-	-
1169	1692	α-Terpineol	15.9	10.0	0.5	1.2
1176	1786	Myrtenol	-	0.2	-	-
1175	n.d	trans-Dihydrocarvone	-	-	-	0.3
1195	1830	trans-Carveol	0.1	0.2	-	-
1206	n.d	Bornyl formiate	0.6	0.3	-	-
1214	1679	Neral	-	0.3	-	-
1223	1601	Carvacrylmethyl oxide	0.9		0.2	0.1
1233	1842	Geraniol	-	0.6	-	-
1240	1555	Linalyl acetate	T	3.8	t	T
1264	1574	Bornyl acetate	1.9	0.8	t	T
1263	2183	Thymol	1.7	4.4	63.1	26.5

1277	2205	Carvacrol	7.0	0.5	5.5	22.7
1301	1683	Myrtenyl acetate	-	1.2	-	-
1328	1688	α -Terpenyl acetate	-	4.5	-	0.1
1342	1722	Neryl acetate	-	0.2	-	-
1342	1455	α -Cubebene	T	-	-	-
1359	1755	Geranyl acetate	-	0.5	-	-
1369	1487	α -Copaene	0.2	-	-	-
1376	1517	β -Bourbonene	0.1	0.2	-	-
1397	n.d.	Isocaryophyllene	T	-	-	-
1401	1526	α -Gurjunene	0.1	-	-	0.1
1409	1590	<i>E</i> -Caryophyllene	5.3	1.9	1.1	2.3
1427	1600	Aromadendrene	0.1	t	0.1	1.0
1442	1662	α -Humulene	0.2	t	t	T
1447	1636	<i>allo</i> -Aromadendrene	0.2	0.4	-	0.3
1466	1699	Germacrene D	0.1	0.2	-	-
1470	1715	β -Selinene	T	-	-	-
1479	1726	Bicyclogermacrene	0.1	0.7	-	-
1486	1724	α -Muuroleone	0.1	0.1	-	-
1488	1720	Z- α – Bisabolene	-	-	-	0.8
1498	1751	γ -Cadinene	0.3	0.3	-	-
1502	1825	Z-Calamenene	0.1	0.4	-	0.1
1508	1751	δ -Cadinene	0.3	-	0.1	0.2
1557	1975	Caryophyllene oxide	0.2	1.0	0.3	-
1553	2113	Spathulenol	T	1.5	-	1.0
1562	2063	Globulol	-	-	-	1.7
1607	2153	γ -Eudesmol	-	0.3	-	-
1607	2277	Caryophylla-2(12),6(13)-dien-5- α -ol	0.1	-	-	-
1615	2172	τ -Muurolol	T	-	-	-
1615	2153	τ -Cadinol	-	0.3	-	-
1622	2215	β -Eudesmol	-	0.8	-	0.2
Monoterpene hydrocarbons			16.7	2.6	3.3	6.3
Oxygen containing hydrocarbons			69.7	83.2	93	76.2
Sesquiterpene hydrocarbons			7.2	4.2	1.3	4.8
Oxygen containing sesquiterpenes			0.3	3.9	0.3	1.9
Other compounds			0.2	0	0.3	0.7
Total identified			94.1	93.9	98.2	89.9

Compounds listed in order of their elution on the SPB-1column.

^aRetention index on the SPB-1 column relative to C8 - C24 *n*-alkanes.

^bRetention indices on the SupelcoWax-10 column relative to C8 - C24 *n*-alkanes.

t= traces ($\leq 0.05\%$).

Annex III

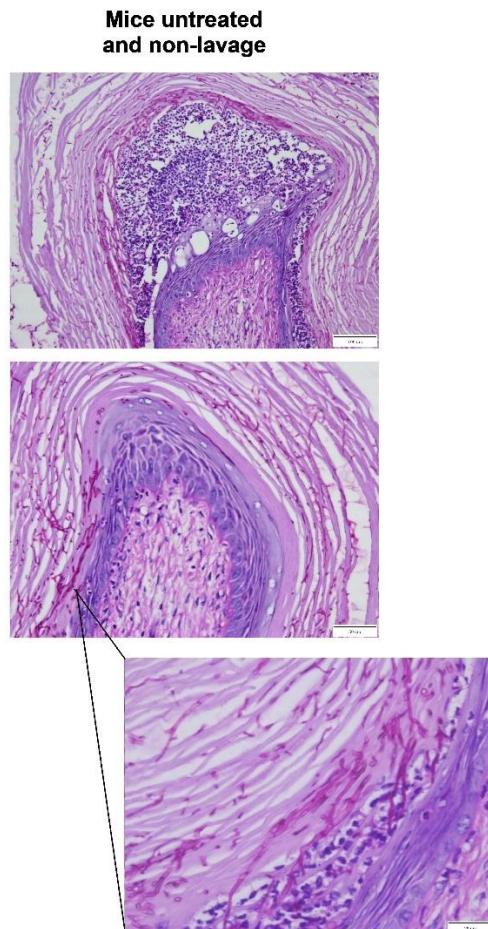
III.1 Histological images of vaginal tissue of mice infected with *Candida albicans* without any vaginal treatment

Figure 51. Histological images of vaginal tissue of mice infected with *C. albicans* without any vaginal treatment or lavage, stained with periodic acid-Schiff (PAS), increasing magnification along the column. The results were registered under 10x and 20x magnification. The data presented in this study are representative of at least two independent experiments.

Annex IV

IV.1 Confirmation of publication and licensing rights of BioRender (figure 1)



49 Spadina Ave. Suite 200
Toronto ON M5V 2J1 Canada
www.biorender.com

Confirmation of Publication and Licensing Rights

January 1st, 2024
Science Suite Inc.

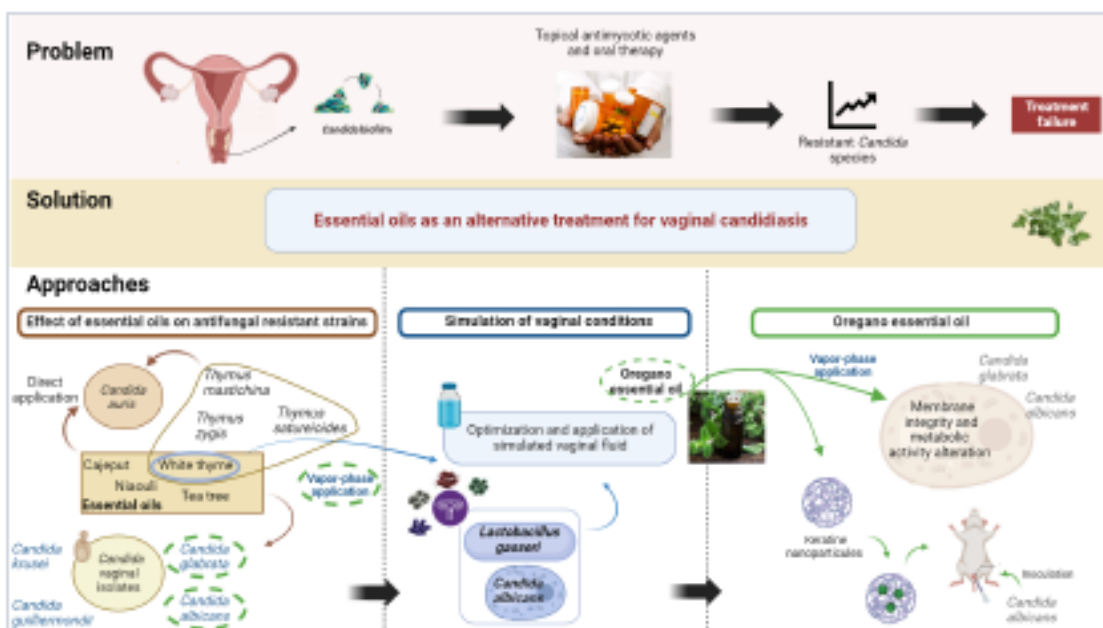
Subscription: Individual
Agreement number: KW26ABVIPV
Journal name: Thesis

To whom this may concern,

This document is to confirm that Teresa Barbosa has been granted a license to use the BioRender content, including icons, templates and other original artwork, appearing in the attached completed graphic pursuant to BioRender's [Academic License Terms](#). This license permits BioRender content to be sublicensed for use in journal publications.

All rights and ownership of BioRender content are reserved by BioRender. All completed graphics must be accompanied by the following citation: "Created with BioRender.com".

BioRender content included in the completed graphic is not licensed for any commercial uses beyond publication in a journal. For any commercial use of this figure, users may, if allowed, recreate it in BioRender under an Industry BioRender Plan.



For any questions regarding this document, or other questions about publishing with BioRender refer to our [BioRender Publication Guide](#), or contact BioRender Support at support@biorender.com.

IV.2 Confirmation of publication and licensing rights of BioRender (figure 35)



49 Spadina Ave. Suite 200
Toronto ON M5V 2J1 Canada
www.biorender.com

Confirmation of Publication and Licensing Rights

January 1st, 2024

Science Suite Inc.

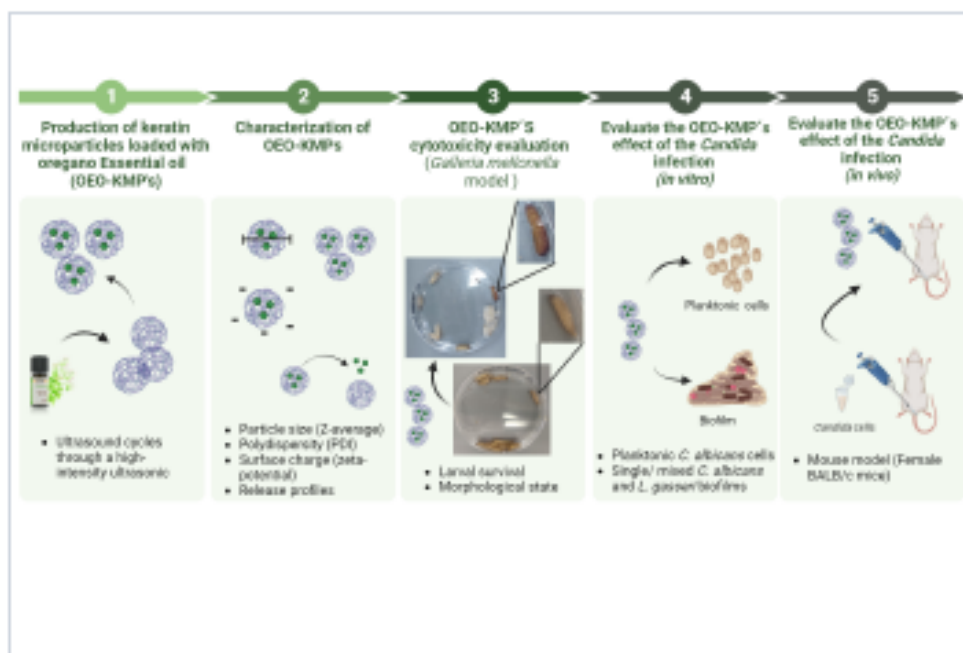
Subscription: Individual
Agreement number: BT26ABXE9C
Journal name: Thesis

To whom this may concern,

This document is to confirm that Teresa Barbosa has been granted a license to use the BioRender content, including icons, templates and other original artwork, appearing in the attached completed graphic pursuant to BioRender's [Academic License Terms](#). This license permits BioRender content to be sublicensed for use in journal publications.

All rights and ownership of BioRender content are reserved by BioRender. All completed graphics must be accompanied by the following citation: "Created with BioRender.com".

BioRender content included in the completed graphic is not licensed for any commercial uses beyond publication in a journal. For any commercial use of this figure, users may, if allowed, recreate it in BioRender under an Industry BioRender Plan.



For any questions regarding this document, or other questions about publishing with BioRender refer to our [BioRender Publication Guide](#), or contact BioRender Support at support@biorender.com.