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RAFAEL ALVES DA SILVA

Combinação de óleos essenciais entre si e com clotrimazol na inibição e erradicação de biofilmes formados por espécies de *Candida*.

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RAFAEL ALVES DA SILVA

Combinação de óleos essenciais entre si e com clotrimazol na inibição e erradicação de biofilmes formados por espécies de *Candida*.

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"O conhecer surge como resposta a uma pergunta. A origem do conhecimento está nas perguntas, ou no ato mesmo de perguntar"
Paulo Freire

"Importante não é ver o que ninguém nunca viu, mas sim, pensar o que ninguém nunca pensou sobre algo que todo mundo vê."
Arthur Schopenhauer

RESUMO

Introdução: O crescente uso de produtos derivados de compostos naturais tem sido uma alternativa ao tratamento de infecções mucocutâneas, como as causadas por *Candida* spp.A atividade anti-*Candida* da associação de óleos essenciais (OE) e azóis não tem sido analisada extensivamente. **Objetivo:** Avaliar ação antifúngica e antibiofilme dos OE de *Cupressus. sempervirens, Citrus limon*, *Litsea cubeba* e *Melaleuca alternifolia* isolados e em combinação entre si e com o antifúngico clotrimazol sobre *Candida albicans* (ATCC 90028 e SV01), *C. glabrata* (ATCC 2001 e SV 02), *C. krusei* (ATCC 6258 e SV 03) e *C. parapsilosis* (ATCC 22019 e SV 04) e a toxicidade aguda no modelo *in vivo Caenorhabditis elegans*. **Métodos:** A atividade antifúngica das associações sinérgicas OE-OE e OE-clotrimazol foi aferida por microdiluição em caldo pela técnica de *checkerboard* e, posteriormente, nos biofilmes através da determinação da viabilidade celular utilizando a técnica colorimétrica com MTT-menadiona. A toxicidade para o *C. elegans* foi avaliada após 24 horas de exposição as substâncias. **Resultados:** A concentração inibitória mínima variou de 500 µg/mL a > 4000 µg/mL, e os OE isolados de *C. sempervirens* e *L. cubeba* obtiveram a menor CIM (500 µg/mL) para *C.krusei*; e o clotrimazol variou de 0,015 µg/mL-0,5 µg/mL. As concentrações capazes inibir a formação do biofilme e erradicar os biofilmes já formados variaram entre 1000 µg/mL a > 4000 µg/mL. Não foi possível determinar a dose letal (DL_{50}) para *C. sempervirens* e clotrimazol, enquanto *C. limon*, *L. cubeba* e *M. alternifolia* foi de 2000 µg/mL. Para as combinações, houve sobrevivência de 85% das larvas expostas a *M. alternifolia*-clotrimazol e *M. alternifolia*-*L. cubeba*, *C. sempervirens*-clotrimazol, e *C. sempervirens*-*C. limon*. **Conclusão**: Nosso trabalho demonstrou que menores concentrações pelo sinergismo inibiram o crescimento de *Candida* spp. na forma planctônica e em biofilmes, entretanto houve combinações efetivas e tóxicas ao *C. elegans*.

Palavras-chave: Óleos voláteis. *Candida*. Testes de Sensibilidade Microbiana. Sinergismo deSubstâncias Bioativas. *Caenorhabditis elegans*. Biofilme.

ABSTRACT

Introduction: The increasing use of plant-derived products for therapeutic purposes has been an alternative to the treatment of mucocutaneous infections, such as those caused by *Candida* spp. The anti-*Candida* activity of the association of essential oils (EO) and azoles is not widely studied. **Aim:** To evaluate the antifungal and antibiofilm activity of EO of *C. sempervirens*, *C. limon*, *L. cubeba* and *M. alternifolia* and their combinations with each other and with clotrimazole on *Candida* spp., besides checking the acute toxicity in the *in vivo* model *Caenorhabditis elegans*. **Methods:** The antifungal activity of the synergistic associations OE-OE and OE-clotrimazole was measured by microdilution using the checkerboard technique and, later, in biofilms using MTT-menadione. Toxicity to *C. elegans* was evaluated after 24 hours of exposure. **Results:** The minimum inhibitory concentration ranged from 500 µg/mL to > 4,000 µg/mL, and the EO of *C. sempervirens* and *L. cubeba* had the lowest MIC (500 µg/mL) for *C. krusei*; and clotrimazole ranged from 0.015 - 0.5 µg/mL. Biofilm eradication and inhibition ranged from 1000 µg/mL to > 4,000 µg/mL. It was not possible to determine the lethal dose (LD_{50}) for *C. sempervirens* and clotrimazole, while for *C. limon*, *L. cubeba* and *M. alternifolia* it was 2000 µg/mL. There was 85% survival of larvae exposed to *M. alternifolia*-clotrimazole and *M. alternifolia*-*L. cubeba*, *C. sempervirens*-clotrimazole, and *C. sempervirens*-*C. limon*. **Conclusion:** Our study showed that lower concentrations by synergism could inhibit the planktonic and biofilm form of *Candida* spp., however there were effective and toxic combinations to *C. elegans*.

Keywords: Volatile oils. *Candida*. Microbial Sensitivity Tests. Synergism of Bioactive Substances. *Caenorhabditis elegans*. Biofilm.

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LISTA DE ABREVIATURAS E SIGLAS

µg/mL	Micrograma / mililitro
mg/mL	Miligrama / mililitro
SGLT2	Inibidores de cotransportador sódio-glicose 2
CIM	Concentração inibitória mínima
CFM	Concentração fungicida mínima
CL_{50}	Concentração letal capaz de eliminar 50% da população
DL_{50}	Dose letal capaz de eliminar 50% da população
CVV	Candidíase vulvovaginal
CVVR	Candidíase vulvovaginal recidiva
CFU/mL	Colony Forming Unit per milliliter
DMSO	Dimetilsufóxido
RPMI	Roswell park memorial institute
MBIC	Minimum biofilm-inhibiting concentration
MBICc	Minimum biofilm-inhibiting concentration combined
MBEC	Minimum biofilm-eradication concentration
MBECc	Minimum biofilm-eradication concentration combined
PBS	10 mM phosphate buffer, 2.7 mM potassium chloride, 137 mM sodium chloride, pH 7.4
MTT	(3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide;
FICI	Fractional inhibitory concentration index
NGM	Nematode growth medium

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APRESENTAÇÃO

Esta dissertação está estruturada no formato alternativo aprovado pelo Colegiado do Programa de Pós-graduação em Ciências da Saúde da Universidade Federal de Uberlândia, o qual permite que os resultados do estudo sejam apresentados em formato de artigo científico.

A formatação e a estruturação do texto foram feitas de acordo com as recomendações do Programa de Pós-Graduação e a Normalização de Trabalhos Acadêmicos, disponíveis em www.bibliotecas.ufu.br/treinamentos (acesso em 29/07/2022).

O trabalho está organizado, portanto, nas seguintes seções: (1) Introdução; (2) Fundamentação teórica, que aborda o referencial teórico que norteou as hipóteses do presente trabalho; (3) Objetivos, em que são expostos os propósitos do estudo; (4) Metodologia; (5) Manuscrito, com os resultados da pesquisa; (6) Considerações finais, que discorre sobre a síntese dos principais resultados do estudo.

1 INTRODUÇÃO

O gênero *Candida* inclui espécies comensais, que não oferecem riscos à saúde dos indivíduos imunocompetentes, e que habitam pele e mucosas do trato gastrointestinal, vaginal e da cavidade oral. Entretanto, infecções superficiais podem estar associadas a *Candida albicans* e, eventualmente, por *Candida* não-*albicans* (GONÇALVES *et al.*, 2016). Nesse sentido, a candidíase vulvovaginal (CVV) é uma infecção mucocutânea relevante para as mulheres, visto que 50% de mulheres saudáveis em idade reprodutiva irão desenvolver um quadro de CVV, e a sua recorrência considerável (mais de 4 casos em 12 meses), presente em até 10% dos casos (SOBEL, 2007; WILLEMS *et al.*, 2020). Anualmente, estima-se que os custos em decorrência da redução da qualidade de vida por esta causa, sobretudo, pela redução da produtividade, somam US$14,39bilhões (DENING *et al.*, 2018).

A melhoria do espectro antifúngico aliado a diminuição da toxicidade em tecidos vitais, um efeito adverso comum à classe de antifúngicos, é um atributo desejável no delineamento deprodutos com ação nas diferentes formas de *Candida* spp. (PEDROSO *et al.*, 2019). Atualmente, o difícil manejo, erradicação e crescente resistência a ação de drogas nos biofilmesformados por espécies do gênero estão fortemente relacionados ao aumento do tempo de internação, dos custos assistenciais, e à mortalidade de pacientes (LAFLEUR; KUMAMOTO; LEWIS, 2006; HARRIOT *et al.*, 2010; SARDI *et al.*, 2013; SILVA *et al.*, 2017; RODRÍGUEZ-CERDEIRA et a, 2019; KARPIŃSKI *et al.*, 2021).

Nos últimos anos, pesquisas otimizando o potencial biológico pela combinação entre substâncias e/ou composições isoladas com promissora ação fungicida e/ou fungistática têm apresentado crescente fonte de inovação, sobretudo em aplicações de compostos derivados de plantas, como óleos essenciais (BAKKALI *et al.*, 2008; RAUT; KARUPPAYIL, 2014; CANNAS *et al.*, 2016; SWAMY; AKHTAR; SINNIAH, 2016).

A rica diversidade de quimiotipos de baixo peso molecular e a lipofilicidade intrínsecados óleos essenciais podem favorecer a penetração em estruturas de revestimento da *Candida* spp., características biológicas atrativas pela complementaridade de mecanismos de ação quando associada com substâncias antifúngicas, incluindo o clotrimazol (AHMAD; KHAN; MANZOOR, 2013; KHAN; MALIK; AHMAD, 2013;

NIKOLIC *et al.*, 2017; JAFRI *et al.*, 2020).

Azóis representam opções seguras com efetiva ação em infecções superficiais em diversos níveis de gravidade (CROWLEY; GALLAGHER, 2014; ALLEN *et al.*, 2015). Pertencente a classe, o clotrimazol é um fungistático imidazólico no tratamento da CVV e dermatófitos, com ação na membrana citoplasmática e inibindo a síntese de ergosterol, contudo, apresentatoxicidade sistêmica importante, porém adequada ação fungistática quando veiculado emformulações tópicas (CROWLEY; GALLAGHER, 2014; KASPER *et al.*, 2015; CARBONE *etal.*, 2019).

O uso preditivo de animais oferece segurança relacionada a aspectos tóxicos em estudos para o desenvolvimento de medicamentos e produtos voltados a saúde. Os animais vertebrados Zebrafish (*Danio rerio*), sobretudo ratos e camundongos (murinos) constituem modelos convencionais amplamente conhecidos na validação *in vivo* (SCORZONI *et al.*, 2016). Os modelos não invertebrados *in vivo Bombyx mori, Caenorhabditis elegans*, *Galleria mellonella, Drosophila melanogaster* são tendências atuais no estudo da patogenicidade de *Candida* spp. (KEAN *et al.*, 2020). Nesse sentido, identificar a toxicidade aguda de compostos com potencial farmacológico em *Caenohabditis elegans* assume importância na triagem de produtos efetivos para infecções mucocutâneas contra espécies de *Candida* (TAMPAKAKIS; OKOLI; MYLONAKIS, 2008; SCORZONI *et al.*, 2008; SEGAL; FRENKEL, 2018; KEAN *etal.*, 2020; ABDULLAHI *et al.*, 2021; HERNANDO-ORTIZ *et al.*, 2021).

2 FUNDAMENTAÇÃO TEÓRICA

2.1 Gênero *Candida*

O gênero *Candida* faz parte da diversidade de espécies comensais da microbiota humana, podendo ser encontrado na mucosa da cavidade oral, vaginal, na uretra, no intestino ena superfície corporal (DIGNANI; SOLOMKIN; ANAISSIE, 2003; CIUREA *et al.*, 2020). A redução da capacidade imune ou algum tipo de injúria no tecido mucocutâneo pode modificar a relação com o hospedeiro, revelando a patogenicidade de algumas espécies do gênero e, assim, característica oportunista em infecções fúngicas (SPANAKIS; APERIS; MYLONAKIS, 2006;SOBEL, 2007; PAPAS *et al.*, 2015; CIUREA *et al.*, 2020).

O evidente aumento dos casos de infecções fúngicas, bem como a gravidade das mesmas, observada a partir da década de 1980, com prevalência de *Candida albicans* como principal agente etiológico (CIUREA *et al.*, 2020). Por outro lado, a crescente incidência de *Candida* não-*albicans*, como *C. parapsilosis*, *C. orthopsilosis, C. krusei*, *C. glabrata*, *C. tropicalis*, *C. lusitaniae*, *C. dublinienesis*, *C. guilliermondii,* e recentemente *C. auris* (KEAN *et al.*, 2020), representam um novo contexto epidemiológico do gênero no Brasil e no mundo (SPANAKIS; APERIS; MYLONAKIS, 2006; DOI *et al.*, 2016; MATTA; SOUZA; COLOMBO, 2017).

2.1.1 Infecções causadas por espécies de *Candida*

Dentre as aproximadamente 200 espécies que compõem o gênero *Candida*, apenas 20 espécies apresentam relevância clínica como agente causador de infecções mucocu tâneas ou invasivas em humanos (MATTA; SOUZA; COLOMBO, 2017).

Candida albicans representa a espécie mais conhecida do gênero por apresentar, dentre outros aspectos, patogenicidade e virulência característicos, exemplificados pelo dimorfismo, produção de enzimas hidrolíticas, como proteinases e fosolipases, além da capacidade de aderir a tecidos e mucosa (COLOMBO et al., 2017). Entretando, nas ultimas décadas têm-se notado o aumento de casos em que espécies não-*albicans*, como *C. glabrata*, *C. krusei*, *C. parapsilosis*, *C. tropicalis*, e recentemente *C. auris*, como agentes etiológicos de infecção fúngica em diferentes sítios anatómicos e em diversas po pulações pelo mundo, em relação aos casos originados por *C. albicans* (PAPPAS *et al*., 2018).

As infecções ocasionadas por *Candida* spp., ou candidíase, ou ainda, candidose, são

condicionadas a múltiplos fatores, que podem influenciar a gravidade das manifestações sistêmicas. Condições inerentes ao hospedeiro, como o sítio anatômico e alterações sistêmicas ou locais do sistema imune, podem oportunizar o estabelecimento e avanço de *Candida* spp. no processo infeccioso (PAPAS *et al*., 2015; CIUREA *et al.*, 2020).

Condições comuns na internação hospitalar podem favorecer a imunossupressão e imunocomprometimento do paciente, incluindo o tempo prolongado de internação, procedimentos cirúrgicos, o uso de dispositivos invasivos, uso prologado ou repetitivo de antimicrobianos com largo espectro de ação, e além de nutrição parenteral, podem favorecer a translocação da *Candida* spp., comensal na pele e mucosas, para a forma oportunista no sangue, trato urinário, pulmonar ou cavidade intra-abdominal (COLOMBO *et al.*, 2013; DOI *et al.*, 2016; CIUREA *et al.*, 2020).

Neste sentido, a menor sensibilidade e a resistência intrínseca ao fluconazol, como visto em *C. glabrata* e *Candida krusei,* têm sido um dos fatores relacionados ao aumento de casos de candidiase invasiva dentre as espécies *C.* não-*albicans* em diferentes países (COLOMBO *et al*., 2017). Um estudo sobre infecção de corrente sanguínea conduzido na região de Nice, na França, identificou que, dos 304 isolados de 209 pacientes, *C. albicans* representou 44% do total, enquanto *C. glabrata* e *C. parapsilosis,* 22% e 13%, respectivamente (VANINI *et al.,* 2022). Na Hungria, estudo restrospectivo entre 2008 a 2017 avaliou a prevalência de infecção urinária causada por *Candida* spp., ou candidúria, *C. albicans* foi o agente identificado entre 55% - 65% das amostras, seguida de *C. glabrata*, entre 20 – 30% dos isolados (GAJDÁCS *et al.,* 2019).

Por outro lado, as manifestações superficiais apresentam gravidade menor e acometimento restrito a tegumentos e estruturas anexas, como a mucosa oral e vaginal, e unhas (COLOMBO *et al.*, 2013). Dentre as infecções mucocutâneas, a candidíase vulvoginal (CVV) está associada a infecção vaginal presente em até ⅓ de mulheres no período reprodutivo,e ao contrário de outras infecções, manifesta-se predominantemente em mulheres hígidas e sem imunocomprometimento (SOBEL, 2007).

Nesse sentido, acompanhamento feito entre 2012 à 2017 com mulheres gregas, com 933 isolados, apresentou *C. albicans* foi o agente etiologico isolado em amostras de secreção vaginal em 75.6% do total de casos. Espécies *C.* não-*albicans* representaram 24.4% do total, em que 13.6% foram isolados de *C. glabrata*, 2.7% de *C. krusei*, e 0.9% de *C. parapsilosis* (MARAKI *et al*., 2019). No Brasil, a prevalência estimada de CVV é de 18%, sendo Norte e o Nordeste as regiões de maior prevalência identificada (16%), podendo ser maior (CARVALHO

et al., 2021).

2.1.2 Candidíase vulvovaginal

A CVV é uma manifestação inflamatória aguda oportunista da mucosa do trato genital feminino, demonstrada por eritema introital, vaginal e na vulva, além de fissuras e edema no local, podendo ser atribuída outras afecções vaginais, como tricomoníase, gonorreia e vaginose bacteriana pela inespecificidade dos sintomas (SOBEL, 2016; GONÇALVES *et al.*, 2015; FELIX *et al.*, 2020).

A prevalência de *C. albicans* representa até 92% como agente etiológico isolado, seguida por *C. glabrata*, e por outras *Candida* não-*albicans*, como *C. krusei*, *C. parapsilosis* e *C. tropicalis* (GONÇALVES *et al.*, 2015; CECCARANI *et al.* 2019; WILLEMS *et al.*, 2020).

Múltiplos fatores de risco são associados ao desenvolvimento de CVV em mulheres, como estresse emocional, atividades sexuais, diminuição da resposta imune, *diabetes mellitus* e uso de medicamentos para seu tratamento, como inibidores de cotransportador sódio-glicose 2 (SGLT2). Demais fatores relacionados a gestação, alterações nas populações que compõem a microbiota vaginal, uso de glicocorticoides, uso frequente de antimicrobianos, dieta favorecidapor carboidratos propiciam a infecção (SOBEL *et al.*, 1998; GONÇALVES *et al.*, 2015; DENING *et al.* 2018; KALIA; SINGH; KAUR, 2019). Tais condições favorecem casosrecidivantes de candidíase vulvovaginal (RCVV) ou CVV complicada (SOBEL, 2007; FOXMAN *et al.*, 2013; POWELL; NYIRJESY, 2014; GONÇALVES *et al.*, 2015; DENING *etal.* 2018; KALIA; SINGH; KAUR, 2019; ROSATI *et al*., 2020; WILLEMS *et al.*, 2020).

A RCVV é a condição multifatorial em que ocorrem mais de 3 episódios de CVV por ano, variando de 1 a 2 anos, ou em poucos casos, com 4 anos (FOXMAN *et al.*, 2013; POWELL; NYIRJESY, 2014; SOBEL, 2016; DENING *et al.* 2018; KALIA; SINGH; KAUR, 2019; ROSATI *et al*., 2020). Estimativa aponta prevalência de 3.871 casos de RCVV cada 100.000 mulheres, com maior presença (9%) na idade reprodutiva, idade entre 25 e 34 anos (DENING *et al.* 2018). A coinfecção entre *C. glabrata* e *C. albicans* pode ser uma das causas da RCVV, também relacionada à resistência intrínseca de *Candida* não-*albicans* a azois (GONÇALVES *et al.*, 2015, DENING *et al.* 2018).

2.2 Biofilme formados por espécies de *Candida*

O biofilme é um agrupamento formado pela justaposição entre células de microrganismos, de mesma espécie, ou de mesmo gênero, ou polimicrobianos. Essa configuração concede vantagens ecológicas estruturais e para a coordenação de funções voltadas à nutrição, metabolismo e proteção mútua contra agressores externos, quando comparado com a forma planctônica dos microrganismos (SARDI *et al.*, 2013; SILVA *et al.*, 2017).

A síntese do biofilme se inicia pela adesão de células fúngicas ou/e bacterianas junto a um substrato, constituído por uma superfície biótica ou abiótica. Apos a adesão, ocorre a fase da divisão e proliferação celular, e formação da matriz extracelular constituída, principalmente, por proteínas, carboidratos e hexoaminas. A maturação modifica a forma e representatividade das células presentes, alterando de leveduriforme, em menor quantidade, para a formação de hifas,em maior proporção. A dispersão decorre a partir da maturação, ocasionada pelo desagregamento e disperção de células sésseis (SARDI *et al.*, 2013; SILVA *et al.*, 2017; WALL *et al.*, 2019).

A presença de formas de resistência, tida como uma importante agravante na virulência, incluem a firme junção entre microrganismos característica do biofilme, fator que dificulta a penetração de substância nas camadas que formam a parte interna da estrutura (WALL *et al.*, 2019). Além desse fator, a presença de *persister cells*, bombas de efluxo, *quorum sensin*g entre microrganismos, múltiplas espécies ou gêneros formando a estrutura do biofilme podem atuar de modo concomitante no biofilme, tornando-o resistente a ações do ambiente e do sistema imune do hospedeiro (LAFLEUR *et al.*, 2006; HARRIOT *et al.*, 2010; RODRÍGUEZ-CERDEIRA *et al.,* 2019).

Espécies de *Candida* podem formar biofilme, onde a composição, a forma adotada, características genéticas e interações entre microrganismos proporcionam virulência que pode variar de acordo com a(s) espécie(s) que constitue(m) o biofilme. Segundo estudo de Harriott *et al.* (2010), *Candida albicans* apresenta capacidade de aderir a mucosa vaginal e formar biofilme *in vivo*, igualmente referenciada para *C. glabrata*, *C. parapsilosis*, *C. krusei* e *C. tropicalis* (RODRÍGUEZ-CERDEIRA *et al,* 2019).

Nesse sentido, a coexistência de multiespécies e gêneros na constituição do biofilme vaginal aparenta ser fator facilitador para processos infecciosos difusos, implicado diretamente na escolha racional do tratamento antimicrobiano, visto a diferença nos

respectivos tratamentos farmacológicos, que diferem de acordo com os múltiplos possíveis agentes etiológicos da infecção (LAFLEUR *et al.*, 2006; HARRIOT *et al.*, 2010; GONÇALVES *et al.*, 2015; RODRÍGUEZ-CERDEIRA *et al.* 2019).

2.3 Terapia medicamentosa no tratamento de infecções por *Candida*

O tratamento das infecções causadas por *Candida* spp., geralmente leva em consideração múltiplos fatores relacionados à epidemiologia, ao sítio anatômico, resposta imune do hospedeiro e gravidade das manifestações clínicas sistêmicas ou locais (COLOMBO *et al.*, 2013). A nível de evidência, o direcionamento do tratamento farmacológico deve ser guiado pela identificação da espécie, e a partir, individualizado considerando forma farmacêutica, segurança, espectro de ação e via de administração, baseado nos derivados de azóis, poliênicose equinocandinas para infecções sistêmicas (EGGIMANN; GARBINO; PITTET, 2003; COLOMBO *et al.*, 2013; SILVA *et al.*, 2017).

Atualmente, derivados de azóis, polienos, equinocandinas e fluoropirimidinas constituem o arsenal terapêutico para candidíase de maior gravidade, especialmente nas infecções sistêmicas. A indicação combinada ou isolada de formulações orais e/ou tópicas baseadas em azóis (imidazóis e triazóis), polienos e alilaminas (terbinafina) apresentam segurança e eficácia no tratamento de infecções mucocutâneas (COLOMBO *et al.*, 2013; PAPPAS *et al.*, 2016).

Para a CVV e RCVV, a escolha do tratamento dependente da gravidade e da recorrência das infecções, sendo geralmente, baseada na combinação de triazóis orais (fluconazol, itraconazol, posaconazol e voriconazol) e de imidazóis tópicos ou intravaginais (cetoconazol, clotrimazol, econazol, fenticonazol, miconazol, sulconazol e tioconazol) (SOBEL *et al.*, 1998, COLOMBO*et al.*, 2013; FELIX; ARAUJO; RÖDER; PEDROSO, 2020). Adicionalmente, formulações administradas por via intravaginal à base de ácido bórico, anfotericina B e flucitosina podem ser prescritas (POWELL *et al.*, 2014).

2.4 Resistência a antifúngicos

A gradativa resistência aos azóis demonstra um desafio para protocolos clínicos que sãoutilizados como profiláticos e de terapias prolongadas. Atualmente, essa resistência é associada à redução da incidência de infecções por *C. albicans* e o aumento significativo por espécies não-*Candida albicans,* como *C. glabrata* e *C. krusei* (COLOMBO *et al.*, 2013;

PAPPAS *et al*., 2015; DOI *et al*., 2016; MATTA; SOUZA; COLOMBO, 2017; CIUREA *et al.*, 2020).

Na América Latina, as taxas de resistência a fluconazol são menores que 3%, porém reforçam um aumento discreto nas taxas de resistência em isolados de *C. albicans, C. tropicalis*e *C. parapsilosis* (MATTA; SOUZA; COLOMBO, 2017). No Brasil, isolados de *C. albicans, C. tropicalis* e *C. Parapsilosis* de corrente sanguínea se mostraram suscetíveis a fluconazol, contudo 36% dos isolados de *C. glabrata* demonstraram resistência a azóis (DOI *et al.* 2016). Espécies como *C. krusei* e *C. glabrata* apresentam conhecida resistência aos triazóis (EGGIMANN; GARBINO; PITTET, 2003; COLOMBO *et al.*, 2013; MATTA; SOUZA; COLOMBO, 2017).

Acredita-se que a grande maioria dos casos de RVVC aconteça pela junção favorável de condições imunes, comportamentais e genéticas do hospedeiro, somada a fatores intrínsecosde espécies e aos aspectos do tratamento medicamentoso (WILLEMS *et al.*, 2020). Variados mecanismos moleculares e fenotípicos, garantem resistência a drogas antifúngicas para *Candida* spp., podendo ser diversos e combinados (EGGIMANN; GARBINO; PITTET, 2003; KALIA; SINGH; KAUR, 2019, ROSATI *et al.*, 2020).

A indicação inadequada de medicamentos, combinada ao uso de terapias prolongadas, configura potenciais condições seletoras de espécies de *Candida* não-*albicans* com reduzida susceptibilidade a antifúngicos, como visto por *C. glabrata* e *C. krusei* (SOBEL *et al.*, 1998). A classe dos azóis inibem a via enzimática da C14-α-lanosterol desmetilase, sintetizada pelo gene ERG 1, que nos casos de resistência fúngica sofre mutação e é expresso de forma aumentada. O aumento de bombas de efluxo e alterações na via de síntese do ergosterol são outros mecanismos de resistência a azóis (EGGIMANN; GARBINO; PITTET, 2003; SANGLARD; ODDS, 2002; CIUREA *et al.*, 2020).

A ação do estrogênio do hospedeiro e ação enzimática de proteinases aspartil secretoras (SAPs) e a da proteína candidalisina pode modificar a virulência do fungo associada a adesão,invasão e danos ao tecido, acionando a resposta imune produzidos por células epiteliais vaginais, contribuindo para a CVV ou sua recorrência (RCVV) (GONÇALVES *et al.*, 2015; KALIA; SINGH; KAUR, 2019; RODRÍGUEZ-CERDEIRA *et al.* 2019, ROSATTI *et al.*, 2020). Visto isso, o aumento da resistência e a diminuição da susceptibilidade às drogas também podem ser atribuídas ao biofilme, dada a sistematizada estrutura e funcionalidade, inerentes dobiofilme, além da capacidade de reunir diversos

mecanismos de resistência (RODRÍGUEZ- CERDEIRA *et al.* 2019).

2.5 Compostos naturais e o uso antifúngico de óleos essenciais

Plantas fazem parte do desenvolvimento histórico do homem enquanto sociedade, indoalém dos aspectos nutricionais, estabelecendo íntima relação nos processos de cura, tratamento e melhoria física e espiritual (SWAMY; AKHTAR; SINNIAH, 2016; NAZZARO *et al.*, 2017; KARPINSKI *et al.*, 2021). Das partes que compõem sua estrutura pode-se obter diferentes metabólitos ativos amplamente utilizados em preparações medicinais, terapêuticas e espirituais. Nesse contexto, o incentivo do uso desse saber tradicional foi fomentado pela Organização Mundial da Saúde (OMS) como prática complementar à saúde desde a década de 1970 (WORLDHEALTH ORGANIZATION, 1978). No Brasil, essa prática faz parte da Política Nacional de Práticas Integrativas e Complementares destinada ao Sistema Único de Saúde (SUS) implementada em 2006 (BRASIL, 2006).

Dentre esses produtos, o óleo essencial apresenta propriedades fitoquímicas que podemvariar de acordo com a estrutura anatômica da planta amplamente utilizada com potencial antioxidante, inseticida, antibacteriano, antimutagênico, anticancerígeno, antiviral, antiprotozoário, imunomodulatório e antifúngico, sendo empregados na indústria em formulações de produtos farmacêuticos, alimentícios e cosméticos (RAUT; KARUPPAYIL, 2014, MERTAS *et al.*, 2015; SWAMY; AKHTAR; SINNIAH, 2016; IBRAHIM *et al.*, 2017; PEDROSO *et al.*, 2019).

Atualmente, metodologias *in vitro* têm evidenciado a efetividade da ação antifúngica dos óleos essenciais de *Melaleuca alternifolia, C. semprevivens*, *Litsea cubeba* e *Citrus limon* usados isoladamente contra especies do gênero *Candida*. Em combinação,, os óleos essenciais dessas espécies podem apresentar interações complementares quanto a ação biológica pretendida, dentre elas exemplificada pelo sinergismo (RAUT; KARUPPAYIL, 2014; MERTAS *et al.*, 2015; IBRAHIM *et al.*, 2017; NIKOLIĆ *et al.*, 2017; ORCHARD, VAN VUUREN, 2017; ORCHARD; VAN VUUREN; VILJOE, 2019; THIELMANN; MURANYI, 2019; KLIMEK- SZCZYKUTOWICZ *et al.*, 2020).

2.5.1 Ação anti-*Candida* de óleos essenciais

2.5.1.1 Citrus limon

Considerada nativa da região asiática, o *Citrus limon* é uma espécie relevante dentro da família *Rutaceae* pela ampla quantidade de espécies híbridas, cultivares e variedades cultivadas para fins comerciais, alimentícios, farmacêuticos e industriais em diferentes continentes (MABBERLEY, 2004; KLIMEK-SZCZYKUTOWICZ *et al.*, 2020).

A composição química depende de múltiplos fatores detectando diferentes partes constituintes do fruto, onde terpenos têm sido extensivamente estudado para a espécie, incluindo monoterpenos como grupo de constituintes prevalentes, o limoneno, seguidos de β- pineno, p-cimeno, β-bisaboleno (NIKOLIĆ *et al.*, 2017; KLIMEK-SZCZYKUTOWICZ *et al.*,2020; SILVA *et al.*, 2021).

Foi demonstrado que preparações de *C. limon* na forma de suco, óleo essencial, extratoetanólico, aquoso e acetato podem apresentar atividade antitumoral, antioxidante, antiinflamatória, antimicrobiana, antiparasitária, anti-*Candida*, antialérgica, hepato-regenerativa,antidiabética, antiobesidade, e efeitos no sistema digestivo, cardiovascular, nervoso e esquelético (KLIMEK-SZCZYKUTOWICZ *et al.*, 2020).

O OE de *C. limon* demonstra uma alternativa favorável no tratamento de afecções e micoses superficiais (ORCHARD; VAN VUUREN, 2017). Em infecções mucocutâneas causada por *Candida* spp., combinações sinérgicas contendo o OE de *C. limon* foram capazes de inibir a forma planctônica (NAZZZARO *et al.*, 2017; NIKOLIĆ *et al.*, 2017; ORCHARD; VAN VUUREN, VILJOE, 2019; NUTA *et al.*, 2021), além de inibir etapas do desenvolvimento do biofilme de *C. albicans*, *C. glabrata*, *C. krusei*, *C. orthopsilosis*, *C. parapsilosis* e *C. tropicalis* (RAUT *et al.*, 2013; PEDROSO *et al.*, 2019; NUTA *et al.*, 2021).

2.5.1.2 Cupressus sempervirens

O *Cupressus sempervirens* (Cupressaceae) é uma espécie ornamental cultivada na América do Sul, mediterrâneo e norte da África (IBRAHIM *et al.*, 2017). Variados compostos podem ser identificados a partir de OE extraídodas partes aéreas, que incluem os sesquiterpenos, (+) - cedrol, α- pineno e timol (IBRAHIM *et al.*, 2017), sabineno, citral, terpinen-4-ol, α-pineno (PEDROSO *et al.*, 2019). Tradicionalmente, as folhas são utilizadas no tratamento de dores no estômago, afecções inflamatórias, diabetes, contra variadas infecções e como contraceptivo (SELIM *et al.*, 2014), e em afecções da pele (ORCHAND; VANVUURREN, 2017).

A atividade antibacteriana, antiviral e antifúngica do óleo essencial extraído de *C. sempervirens* é amplamente referenciada (ORCHAND; VAN VUURREN, 2017), o que incluisua ação como agente isolado sobre o gênero *Candida* spp. (IBRAHIM *et al.*, 2017; PEDROSO*et al.*, 2019; SILVA *et al.*, 2021) ou combinado a outros óleos essenciais (ORCHAND; VAN VUURREN, 2017). Além disso, o OE de *C. sempervirens* foi capaz de inibir etapas do desenvolvimento inicial e em biofilmes formados *C. albicans*, *C. glabrata*, *C. krusei*, *C. orthopsilosis*, *C. parapsilosis* e *C. tropicalis* (PEDROSO *etal.*, 2019).

2.5.1.3 Litsea cubeba

Litsea cubeba espécie pertencente à família Lauraceae originária do sudeste asiático. Dos compostos comuns presentes nos OE extraídos das folhas, casca do caule e folhas, identifica-se o 1,8-cineol, limoneno, sabineno, terpinen-4-ol, hidrato de trans-sabineno, α-pineno, α-terpineol, α-tujeno e β-pineno (THIELMANN; MURANYI, 2019) e referenciada ação anti-*Candida* frente a forma planctônica e sobre o biofilme, particularmente contra *C. krusei* (PEDROSO *et al.*, 2019).

2.5.1.4 Melaleuca alternifolia

O óleo obtido da espécie *Melaleuca alternifolia* (*tea tree*), planta da família *Myrtaceae* originária da Austrália, apresentam predominantemente o terpinen-4-ol, 1,8-cineol (eucaliptol), γ-terpineno, α-terpineno e terpinoleno (SWAMY; AKHTAR; SINNIAH, 2016).

A atividade antimicrobiana do óleo essencial de melaleuca pode ser atribuída a danos na membrana celular, inibição de bombas de efluxo e sobre biofilmes em espécies de *Candida* (NAZZARO *et al.*, 2017; PEDROSO *et al.*, 2019). Estudos têm mostrado a combinação sinérgica de OE de *M. alternifolia* com outros óleos essenciais, inclusive com *C. Limon*, sobre *C. albicans, C. tropicalis* e *C. parapsilosis* (NIKOLIC *et al.*, 2017; ORCHARD; VAN VUUREN; VILJOEN, 2019).

2.6 Avaliação da toxicidade aguda de óleos essenciais *in vivo* em modelos alternativos

A predição dos possíveis efeitos adversos manifestados quando da exposição humanaa determinada substância química tem sido o objetivo dos estudos relacionados à saúde humana(HERNANDO-ORTIZ *et al.*, 2020). O Brasil dispõe desde 2017 da Rede Nacional de MétodosAlternativos – Renama, criada como via especializada na validação de novos métodos de uso animal, sobretudo com modelos animais alternativos (BRASIL,

2017). Alguns modelos são capazes de substituir mamíferos e/ou outros animais vertebrados por outros organismos em testes de predição toxicológica, no estudo de mecanismos de virulência e patogenicidade causada por microrganismos patogênicos e eficácia de drogas. Os principais modelos incluem as espécies *Galleria mellonella*, murinos, *Danio rerio*, *Drosophila melanogaster, Caenorhabditis elegans* (SCORZONI et al., 2016; ELKABTI; ISSI; RAO, 2018; SEGAL, FRENKEL, 2018; KEAN *et al.*, 2020).

O nematódeo *Caenorhabditis elegans* apresenta vasta aplicabilidade para avaliação da toxicidade *in vivo*. A similaridade com modelos vertebrados, equivalente a DL_{50} por camundongo, ou seja, a dose letal capaz de matar 50% da população avaliada (HUNT *et al.*, 2017), têm sido amplamente utilizados em protocolos de triagem para produtos de interesse, sendo avaliado a neurotoxicidade, ecotoxicidade de produtos químicos e pesticidas, bem como a toxicidade aguda, estudos de drogas pré-clínicas, e infectividade na relação hospedeiro-patógeno nos últimos anos (SCORZONI *et al.*, 2013; HUNT *et al.*, 2017; SEGAL; FRENKEL,2018). O baixo custo, fácil cultivo (1 mm de comprimento) e ciclo de vida curto (do ovo a adulto em 3,5 dias) indicam aplicabilidade preditiva para substâncias. Nesse sentido, apresentaalta similaridade com mecanismos fisiológicos de vertebrados, como sistema imune capaz de reconhecer e eliminar patógenos e pela expressão da maioria dos genes humanos, custos laboratoriais reduzidos e cultivo facilitado, tornando-o um modelo alternativo de escolha nos últimos anos (SCORZONI *et al.*, 2013; CORSI, WIGHTMAN, CHALFIE, 2015; SEGAL; FRENKEL, 2018).

Por estas características, o modelo *Caenorhabditis elegans* apresenta potencialidades naavaliação do processo infeccioso na dinâmica hospedeiro-patógeno e da relação com produtos naturais, incluindo óleos essenciais (OKOLI *et al.*, 2009; BREGER *et al.*, 2007; SCORZONI *et al.*, 2013; ELKABTI; ISSI; RAO, 2018; PEDROSO *et al.*, 2019; HERNANDO-ORTIZ *et al.*, 2020).

3 JUSTIFICATIVA

Óleos essenciais desempenham uma alternativa viável para a obtenção de derivadosativos contra microrganismos de relevância epidemiológica em diversas áreas. Estudos que avaliam a atividade biológica destes metabólitos secundários, em sua maioria, investigam apenas um, não aprofundando no potencial sinérgico pela ação entre óleos essenciais (BAKKALI *et al.*, 2008; RAUT; KARUPPAYIL, 2014; CANNAS *et al.*, 2016; SWAMY; AKHTAR; SINNIAH; 2016).

É demonstrado que o sinergismo entre óleos essenciais, ou de substâncias presentes nesses óleos em combinação com outros agentes antifúngicos, podem potencializar a eficácia contra microrganismos, reduzindo a concentração inibitória mínima necessária para inibir o crescimento fungos (RAUT; KARUPPAYIL, 2014; MERTAS *et al.*, 2015; NIKOLIĆ *et al.*, 2017). Os trabalhos que avaliam o sinergismo, determinando as concentrações efetivas, e a toxicidade das combinações destes derivados de produtos naturais ainda são pouco exploradospela literatura científica diante da grande variabilidade de óleos essenciais disponíveis comercialmente.

Diante disso e da crescente resistência e virulência dos isolados no contexto atual, há a necessidade de avaliar a atividade antifúngica e a toxicidade de óleos essenciais isolados e combinados como uma forma alternativa e complementar à terapia antifúngica convencional, utilizada para tratamento de infecções fúngicas humanas, inclusive na candidíase vulvovaginal.

Desta forma, como hipótese norteadora desta pesquisa está a determinação da atividade antifúngica sinérgica de óleos essenciais de *Cupressus sempervirens, Citrus limon*, *Litsea cubeba* e *Melaleuca alternifolia* frente a *C. albicans*, *C. glabrata*, *C. krusei* e *C. parapsilosis*, além da avaliação da capacidade de inibir ou erradicar o biofilme formado por essas espécies. Por fim, investigar a toxicidade *in vitro* dos compostos utilizando o modelo *in vivo C. elegans*.

4 OBJETIVOS

4.1 Objetivo geral

Avaliar a atividade antifúngica e a toxicidade aguda de óleos essenciais isolados e combinados sobre *Candida* spp. e no modelo *in vivo Caenorhabditis elegans*, respectivamente.

4.2 Objetivos específicos

- Determinar a concentração inibitória mínima e a concentração fungicida mínima dos óleosessenciais de *Cupressus sempervirens, Citrus limon*, *Litsea cubeba* e *Melaleuca alternifolia* sobre espécies de *Candida*;

- Realizar o teste de combinação dos óleos essenciais para caracterização da interação com maior atividade anti-*Candida* nas menores concentrações individuais, pelo método do tabuleiro de xadrez (*Checkerboard*) em combinação com óleos essenciais e Clotrimazol;

- Determinar as concentrações inibitória mínimas para a formação do biofilme e de erradicação do biofilme em combinação com óleos essenciais e Clotrimazol;

- Avaliar a toxicidade *in vivo* dos óleos essenciais isolados e em combinações que apresentaram ação sinérgica promissora contra espécies de *Candida* utilizando o modelo *in vivo Caenorhabditis elegans*.

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Article

Combining Essential Oils with Each Other and with Clotrimazole Prevents the Formation of *Candida* Biofilms and Eradicates Mature Biofilms

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Abstract: Fungal infections by *Candida* spp. are opportunistic and most often occur in individuals with some predisposing factor. Essential oils (EO) have anti-*Candida* potential, being a therapeutic alternative to be explored, especially for superficial and mucosal candidiasis. The objective was to analyze the synergistic potential between the EO of *Citrus limon*, *Cupressus sempervirens*, *Litsea cubeba* and *Melaleuca alternifolia*, and each of them with clotrimazole, to inhibit in vitro the formation and eradication of *Candida* spp. biofilms. Added to this, the survival of *Caenorhabditis elegans* was evaluated after exposure to EO, clotrimazole and their synergistic combinations. Anti-*Candida* activity was determined by microdilution for the substances alone and in EO–EO and EO–clotrimazole combinations. The combinations were performed by the checkerboard method, and the reduction in the metabolic activity of biofilms was determined by the viability of MTT/menadione. *C. elegans* larvae survival was evaluated after 24 h of exposure to EO, clotrimazole and synergistic combinations. The minimum inhibitory concentration (MIC) of EO ranged from 500 to >4000 μg/mL. The lowest MIC (500 μg/mL) was for *C. sempervirens* and *L. cubeba* on a *C. krusei* isolate; for clotrimazole, the MIC ranged from 0.015 to 0.5 μg/mL. Biofilm inhibition and eradication both ranged from 1000 to >4000 μg/mL. The lethal concentration (LC_{50}) of *C. limon*, *L. cubeba* and *M. alternifolia* was 2000 μg/mL for *C. elegans*, while for *C. sempervirens* and clotrimazole, it was not determined within the concentration limits tested. In combination, more than 85% of the larvae survived *M. alternifolia*–clotrimazole, *M. alternifolia*–*L. cubeba*, *C. sempervirens*–clotrimazole and *C. sempervirens*–*C. limon* combinations. This study is the first, to our knowledge, to present a synergistic relationship of EO–EO and EO–clotrimazole combinations on *Candida* spp. biofilms.

Keywords: essential oils; clotrimazole; *Candida* spp.; synergy; biofilm; toxicity; *Caenorhabditis elegans*

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1. Introduction

Infections caused by species of the genus *Candida* are opportunistic and more severe in immunocompromised, hospitalized individuals, using invasive devices and with comorbidities [1]. Superficial candidiasis affects the oral and vaginal mucosa, skin and

nails; and factors external to the individual, such as climatic conditions in tropical and subtropical regions, and factors inherent to it, such as local humidity, use of immunosuppressive or antibacterial drugs and some comorbidities, such as diabetes, facilitate the development of the disease [2]. In superficial infections, the most frequent *Candida* species is *C. albicans*; however, in recent years, *Candida* non-*albicans* species have shown relevance among the causative agents of vulvovaginal candidiasis (VVC) and recurrent VVC (RVVC), including the species *C. glabrata*, *C. krusei* and *C. tropicalis* [2–4].

The azole antifungals have been one of the therapeutic options for the treatment of superficial mycoses since the 1960s–1970s [5,6]. In this sense, topical formulations containing azoles are attractive for VVC and RVVC due to the lower incidence of adverse effects compared to the same drug class for oral use and systemic action [3]. Clotrimazole has a cure rate of between 73% and 100% of infections, similar to other topical antifungals such as nystatin [6,7].

In the last decades, the report of new *Candida* species and in vitro resistant isolates to traditional antifungals has been an incentive for the search and development of new ways of managing these infections [8]. Resistance is a result of multiple factors that include structural changes in the drug target and the ability of *Candida* spp. to form biofilms [9–11]. In this sense, the community structure and firm adherence between the microorganisms of the biofilm allow a barrier condition that makes penetration of drugs difficult and consequently reduces the effectiveness of the treatment [8,10,11].

Essential oils are plant-derived products with potential activity against microorganisms, attributable to the complex mixture of chemotypes [12–14]. Recently, combinations of multiple agents have optimized antifungal activity against clinically relevant fungi. Thus, a new therapeutic approach combining conventional antifungal drugs, such as clotrimazole, and natural products with antifungal activity may have the potential for clinical use [15–18].

The *in vivo* screening of compounds with proven in vitro antimicrobial action is one of the necessary steps within the current safety context to identify the toxicity of new anti-infective agents [19]. In this context, in vivo studies using alternative animal models such as *Drosophila melanogaster*, *Galleria mellonella* and *Caenorhabditis elegans* have been proposed to assess the preliminary toxicity of new health products [20,21]. Thus, the free-living nematode *C. elegans* can be an alternative predictive model option, being of low cost, fast cultivation and not very complex laboratory handling, and lending itself to the evaluation and screening of acute toxicity for use in animals, including humans, and contamination of the environment [11,19,22]. In this sense, the evident anti-*Candida* action of isolated essential oils could mean they present lower inhibitory values when combined with other essential oils or antifungal substances, such as clotrimazole, and its acute toxic repercussions.

Thus, in this study, the in vitro inhibitory activity of the essential oils of *Cupressus sempervirens*, *Citrus limon*, *Litsea cubeba* and *Melaleuca alternifolia*, alone and in combination, and associated with clotrimazole, against *Candida* species biofilms were analyzed. Furthermore, the in vivo toxicity of these essential oils against *C. elegans* was also evaluated.

2. Materials and Methods

2.1. Essential Oils and Candida Species

The essential oils (EO) of *Citrus limon*, *Cupressus sempervirens*, *Litsea cubeba* and *Melaleuca alternifolia* (FERQUIMA®; Vargem Grande Paulista, SP, Brazil) were included in this study. The analysis of the EO, carried out by chromatography, was informed by the supplier company and is shown in Table 1. Four reference strains, *Candida albicans* ATCC 90028, *Candida glabrata* ATCC 2001, *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019, and four clinical isolates from the vaginal mucosa (*Candida albicans* SV 01, *Candida glabrata* SV 02, *Candida krusei* SV 03 and *Candida parapsilosis* SV 04) obtained from

previous studies were included in this study [23]. All microorganisms were stored in brain heart infusion (BHI)–glycerol broth at −20 °C and subcultured on Sabouraud dextrose agar (SDA; Disco, Detroit, MI, USA) and CHROMagar *Candida* medium (Becton Dickinson and Company, Sparks, MD, USA), to evaluate the viability and purity, and even to confirm the identification of the species.

Table 1. Main components of essential oils from *Citrus limon*, *Cupressus sempervirens*, *Litsea cubeba* and *Melaleuca alternifolia*, according to the supplier company.

Essential Oil	Part of the Plant	Extraction Method	Main Components
C. limon	Fruit	Cold pressing	Limonene (65.6%), β-pinene (15.06%), γ-terpinene (7.93%), α-pinene (2.34%), sabinene (1.76%) and myrcene (1.55%)
C. sempervirens	Leaf	NI	α-Pinene (52.4%), δ-3-carene (22%), limonene (3.5%), terpinolene (3.4%), myrcene (2.4%), terpenyl acetate (1.7%), cedrol (1.4%), β-pinene (1.2%) and terpinen-4-ol (1%)
L. cubeba	Fruit	Steam distillation	Geranyl acetate (42%), neral (30%) and limonene (13%)
M. alternifolia	Leaf	Steam distillation	Terpinen-4-ol (41%), γ-terpinene (20.5%), α-terpinene (9.63%), α-terpinolene (3.37%), α-terpineol (2.78%), α-pinene (2.59%), ϱ-cymene (2.39%), aromadendrene (2%), vidiflorene (1.81%), δ-cadinene (1.54%) and 1,8-cineol (1.50%)

NI: not informed. Chemical analysis of the oils by chromatography was provided by the supplier company.

2.2. Determination of the Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC)

To determine the MIC of EO and antifungal agents, the broth microdilution method was used [24], with some adaptations. Flat-bottomed 96-well plates (Kasvi, PR, Brazil) and RPMI-1640 broth with glutamine and without sodium bicarbonate (Corning Incorporated, Corning, NY, USA) were used, plus 18 g/L of glucose (Sigma-Aldrich, St. Louis, MO, USA), buffered with MOPS at pH 7 (Sigma-Aldrich, St. Louis, MO, USA) as a culture medium, and yeast suspension at a resulting final concentration of 0.5–2.5 × 10^3 CFU/mL. The concentration ranges varied from 7.81 to 4000 μg/mL for EO, from 0.03 to 16 μg/mL for amphotericin B (Sigma-Aldrich, St. Louis, MO, USA) and from 0.125 to 64 μg/mL for fluconazole and clotrimazole (Sigma-Aldrich, St. Louis, MO, USA). Amphotericin B and fluconazole were used as controls [24].

EO and amphotericin B were solubilized in DMSO (dimethyl sulfoxide; 2%), fluconazole and clotrimazole in water and later diluted in RPMI. *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 strains were used to validate the tests [24]. The MIC was determined in a spectrophotometer at 490 nm [24]. The cut-off point for defining susceptibility was set at 80% inhibition of fungal growth compared to azole-free growth and 90% inhibition for amphotericin B [24] and EO [25]; cultures were incubated for 24 h at 35 °C. The tests were performed in triplicate.

The MFC was determined by transferring 5 μL of cell suspension from each well to a plate containing SDA, followed by incubation for 48 h at 30 °C. The MFC was the one corresponding to the concentration of the well where the growth of yeast colonies was no longer evident [26]. The ratio of the MFC and MIC of EO and clotrimazole was used to

interpret the results, defining the drug as fungistatic (MFC/MIC: > 4) or fungicidal (MFC/MIC: ≤ 4) [27].

2.3. Evaluation of the Activity of EO and Clotrimazole against Candida spp. Biofilms

2.3.1. Determination of the Minimum Biofilm-Inhibiting Concentration (MBIC)

Inhibition of biofilm formation was determined in 96-well, flat-bottomed plates [28], to which 100 μL of cell suspension in RPMI-1640 was added (1 to 5 × 10^6 cells/mL, adjusted to a turbidity equivalent to 0.5 McFarland scale), as well as 100 μL of the drug (EO and/or clotrimazole), at concentrations of 4 × MIC, 2 × MIC, 1 × MIC, 0.5 × MIC and 0.25 × MIC. The culture was incubated at 35 °C for 48 h. Then, non-adherent cells were removed, and the wells were washed three times with PBS (10 mM phosphate buffer, 2.7 mM potassium chloride, 137 mM sodium chloride, pH 7.4). Then, 100 μL of MTT solution (5 mg/mL; 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide; Sigma-Aldrich, St. Louis, MO, USA) plus 10 μL of phytomenadione (1 μM; 2-methyl-3-[(E,7R,11R)-3,7,11,15-tetramethylhexadec-2-enyl]naphthalene-1, 4-dione; Sigma-Aldrich, St. Louis, MO, USA) was added to each well. The plate was incubated at 35 °C for 24 h in the dark. Subsequently, the supernatant was removed, and 100 μL of DMSO was added to each well, and the plate was incubated at 35 °C for 15 min, protected from light. Then, 80 μL of solvent was removed from each well and transferred to another plate, and the reading was performed at 490 nm [29]. Growth and sterility controls were included for each plate in the experiment. The tests were performed in triplicate.

2.3.2. Determination of the Minimum Biofilm-Eradication Concentration (MBEC)

The biofilm was previously formed in 96-well, flat-bottomed plates. Two hundred microliters of the yeast cell suspension were added to each well (1 to 5 × 10^6 cells/mL, adjusted to 0.5 McFarland in RPMI-1640), and the plates were incubated at 35 °C for 48 h [28]. Then, non-adherent cells were removed, and the wells were washed three times with PBS. Then, 100 μL of the drug (EO and/or clotrimazole) was added at concentrations of 4 × MIC, 2 × MIC, 1 × MIC, 0.5 × MIC and 0.25 × MIC and incubated at 35 °C for 24 h in the dark. The procedures for revealing the biofilm were the same as described in the previous item.

2.4. Evaluation of the Synergistic Potential of EO–EO and EO–Clotrimazole Associations against Planktonic Growth and on Biofilms

To evaluate the combined effect of EO and clotrimazole on planktonic cells, the checkerboard technique was used [30,31]. The synergistic potential of the combination of the EO of *C. sempervirens*, *C. limon*, *L. cubeba* and *M. alternifolia* among themselves and of each one of them with clotrimazole on the *Candida* biofilm was made according to the results obtained for the MIC of the planktonic cells, as provided in the Supplementary Material (Table S1). Concentrations of 0.25 × MIC, 0.5 × MIC, 1 × MIC, 2 × MIC and 4 × MIC were used for drug testing. Then, 100 μL of drug A (EO) horizontal and 100 μL of drug B (EO/clotrimazole) vertical, and 100 μL of cell suspension (1 to 5 × 10^3 cells/mL) were added to all wells of a 96-well flat-bottomed plate. Growth (containing drug-free yeast suspension) and sterility (RPMI-1640) controls were included in each plate. The plates were incubated at 35 °C for 48 h, and the reading was performed at 490 nm; results were considered capable of reducing ≥ 90% of optical density (OD) in relation to the control free of EO and clotrimazole [30]. The results were interpreted according to the fractional inhibitory concentration index (FICI), determined as follows:

$$\text{FICI: } \left(\frac{\text{MIC “A” combined}}{\text{MIC “A” isolated}}\right) + \left(\frac{\text{MIC “B” combined}}{\text{MIC “B” isolated}}\right) \quad (1)$$

The interpretation was conducted according to the classification of the substance interaction score, where antagonism was considered when the score was greater than 4.0,

indifference at a score greater than 1, additivity at a score between 0.5 and 1.0, and synergism at a score less than 0.5 [31]. One hundred microliters of the combined solution "A" (EO) and "B" (EO or clotrimazole) was added to each well, starting from column 11 and column 2. Briefly, the first and last wells received the highest concentrations of each of the two compounds evaluated, resulting in decreasing concentrations from one end of the plate to the other. Growth and sterility controls were included in each of the plates, and each experiment was conducted in triplicate.

2.5. Toxicity Assay for Caenorhabditis elegans

The toxicity test was performed by exposing *C. elegans* larvae (AU37 [glp-4 (bn2) I; mutant strain sek-1 (km4) X) to EO and clotrimazole [25].

C. elegans larvae were transferred to nematode growth medium (NGM), contained in Petri dishes, which contained a previous mat of *Escherichia coli* OP50 (*E. coli*). The plates were incubated at 16 °C for 72 h. Then, synchronization of the larvae in stage L2 was performed by treating the larvae with sodium hypochlorite. Then, the larvae were transferred to another plate containing NGM medium without *E. coli* OP50 and incubated at 16 °C for 24 h [20,32].

For the experiment, a solution medium, composed of 40% BHI broth, plus cholesterol (10 µg/mL), kanamycin (90 µg/mL), ampicillin (200 µg/mL) and 60% 50 mM NaCl, was used. The assay was performed using 96-well flat-bottomed plates. Then, 180 µL of solution medium and 20 µL of the suspension of synchronized larvae in stage L4 were added to each well of the plate so that 10 to 20 *C. elegans* larvae were placed in each well, evaluated in final serial concentrations ranging from 4000 to 250 µg/mL diluted in solution medium. As a survival control, solution medium plus the larvae, without drug, was used, and as a test control, solution medium and DMSO were used. The plates were incubated for 24 h at 35 °C in a humid chamber.

The results were interpreted considering the survival rate of the larvae and the 50% lethal dose (LD_{50}), determined by the concentration of the drug that was able to kill 50% of the larvae [33,34]. Each experiment was performed twice in triplicate.

3. Results

3.1. Determination of the MIC and MFC of Essential and Antifungal Oils against Planktonic Growth

The lowest MIC (500 µg/mL) found was for *C. krusei* SV 03, with the EO of *C. sempervirens* and *L. cubeba*. The MIC of the EO ranged from 500 to > 4000 µg/mL, considering the different oils and the eight isolates tested. The EO of *C. limon* presented an MIC that ranged from 1000 to 4000 µg/mL, *C. sempervirens* from 500 to > 4000 µg/mL, *L. cubeba* from 500 to 2000 µg/mL and *M. alternifolia* from 1000 to 2000 µg/mL. For clotrimazole, the MIC ranged from 0.015 to 0.5 µg/mL (Table 1). The MIC of fluconazole and amphotericin B (test validation controls) were, respectively, 32 and 1 µg/mL; those for *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 2019 were 0.25 and 0.25 µg/mL, respectively.

The lowest fungicidal concentrations (1000 µg/mL) were found for the EO of *C. limon* against *C. albicans* ATCC 90028, *C. sempervirens* against *C. krusei* SV 03 and *L. cubeba* against *C. albicans* ATCC 90028 and *C. krusei* SV 03; for clotrimazole, the lowest fungicidal concentration (0.030 µg/mL) was found for the isolate of *C. glabrata* ATCC 2001.

Evaluation of the fungicidal activity (MFC/MIC: ≤ 4) showed that the EO of *L. cubeba* and the antifungal clotrimazole were fungicidal for all the tested isolates; however, all the other EO evaluated presented fungicidal activity dependent on the isolate, as can be seen in Table 2. Thus, fungicidal activity was found for the EO of *C. limon*, *M. alternifolia* and *C. sempervirens*, respectively, for four, five and six isolates.

Table 2. Minimum inhibitory concentration (µg/mL) and minimum fungicidal concentration (µg/mL) of essential oils and clotrimazole tested with *Candida* species.

***Candida* spp. Isolates**	***C. limon***		***C. sempervirens***		***L. cubeba***		***M. alternifolia***		**Clotrimazole**	
	MIC	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**	**MIC**	**MFC**
C. albicans ATCC 90028	**2000**	1000	**2000**	4000	**1000**	1000	**2000**	4000	**0.25**	0.25
C. albicans SV 01	4000	>4000	4000	>4000	**2000**	4000	4000	>4000	**0.125**	0.25
C. glabrata ATCC 2001	**4000**	4000	**2000**	4000	**2000**	4000	**4000**	4000	**0.015**	0.030
C. glabrata SV 02	4000	>4000	**1000**	4000	**2000**	4000	4000	>4000	**0.25**	0.5
C. krusei ATCC 6258	4000	>4000	**2000**	4000	**1000**	2000	**4000**	4000	**0.5**	1
C. krusei SV 03	**1000**	4000	**500**	1000	**500**	1000	**2000**	4000	**0.5**	1
C. parapsilosis ATCC 22019	**1000**	4000	**4000**	4000	**1000**	4000	**4000**	4000	**0.25**	0.5
C. parapsilosis SV 04	4000	>4000	>4000	>4000	**2000**	4000	4000	>4000	**0.125**	0.25

MIC: minimum inhibitory concentration (µg/mL); MFC: minimum fungicidal concentration (µg/mL). Fungicide: (MFC/MIC: < 4) in **bold**.

3.2. Assessment of the Development of Candida spp. Biofilms

The activity of EO and clotrimazole to inhibit (MBIC) and eradicate (MBEC) the biofilm formed by *Candida* species is shown in Table 3. Most EO presented an MBIC greater than or equal to 4000 µg/mL. The lowest MBIC was 1000 µg/mL, found for *C. sempervirens* (*C. krusei* SV 03), *L. cubeba* (*C. albicans* ATCC 90028) and *M. alternifolia* (*C. krusei* SV 03). The lowest MBEC was 1000 µg/mL for *L. cubeba* (*C. albicans* ATCC 90028). Clotrimazole demonstrated MBIC and MBEC values ranging from 0.125 to 2 µg/mL and 0.25 to 4 µg/mL, respectively.

Table 3. Activity of essential oils and clotrimazole against the formation of biofilms and preformed biofilms of *Candida* species.

Species	***C. limon***			***C. sempervirens***			***L. cubeba***			***M. alternifolia***			***Clotrimazole***		
	MIC	**MBIC**	**MBEC**	**MIC**	**MBIC**	**MBEC**	**MIC**	**MBIC**	**MBEC**	**MIC**	**MBIC**	**MBEC**	**MIC**	**MBIC**	**MBEC**
C. albicans ATCC 90028	2000	4000	4000	2000	4000	>4000	**1000**	**1000**	**1000**	2000	2000	4000	0.25	1	1
C. albicans SV 01	4000	>4000	>4000	4000	4000	4000	2000	2000	4000	4000	4000	4000	0.125	0.5	1
C. glabrata ATCC 2001	4000	>4000	>4000	2000	>4000	4000	2000	2000	>4000	4000	4000	>4000	0.015	0.125	0.25
C. glabrata SV 02	4000	>4000	>4000	1000	>4000	>4000	2000	>4000	>4000	4000	4000	>4000	0.25	0.5	1
C. krusei ATCC 6258	4000	>4000	>4000	2000	2000	2000	**1000**	>4000	>4000	2000	4000	>4000	0.5	1	2
C. krusei SV 03	**1000**	2000	4000	**500**	**1000**	>4000	**500**	4000	>4000	**1000**	**1000**	>4000	0.5	1	1
C. parapsilosis ATCC 22019	**1000**	>4000	>4000	4000	4000	>4000	**1000**	4000	>4000	4000	4000	>4000	0.25	2	4
C. parapsilosis SV 04	4000	>4000	>4000	>4000	4000	>4000	2000	>4000	>4000	4000	>4000	>4000	0.125	0.25	0.5

Isolated MBIC and MBEC (µg/mL): capable of reducing ≥ 90% of optical density (OD) compared to control free of EO and clotrimazole. Results in **bold**: ≤ 1000 µg/mL.

3.3. Evaluation of Synergism of EO and Clotrimazole

The tests of EO–EO and EO–clotrimazole combinations resulted in 80 combinations; of these, 13 (16.25%) showed antagonism, 42 (52.5%) showed indifference, 17 (21.25%) had an additive effect and 8 (10%) showed synergism. The OE–OE and OE–clotrimazole combinations performed and their results related to inhibition of *Candida* spp. are provided in the Supplementary Materials (Table S1). The synergistic effect was variable, depending on the combination (EO–EO or EO–clotrimazole) and the *Candida* strain. The EO–EO and EO–clotrimazole combinations that showed synergism in the evaluation of MIC were selected for evaluation of inhibition (MBIC) and eradication (MBEC) of biofilms (Table 4).

Table 4. Minimum inhibitory, biofilm-inhibitory and biofilm-eradication concentrations of EO–EO and EO–clotrimazole combinations against *Candida* species.

Species	Combination	MIC (μg/mL)		Biofilm (μg/mL)			
		Isolated MIC *	Combined MIC **	Isolated MBIC *	Combined MBIC **	Isolated MBEC *	Combined MBEC **
C. albicans ATCC 90028	*M. alternifolia*	2000	250	2000	62.5	4000	62.5
	Clotrimazole	0.25	0.063	1	0.25	1	0.25
C. albicans SV 01	*L. cubeba*	2000	250	2000	125	4000	250
	M. alternifolia	4000	1000	4000	2000	4000	1000
C. glabrata ATCC 2001	*L. cubeba*	2000	500	2000	2000	>4000	2000
	C. limon	4000	1000	>4000	250	>4000	250
C. glabrata SV 02	*L. cubeba*	2000	250	>4000	1000	>4000	>1000
	M. alternifolia	4000	1000	4000	250	>4000	>250
	C. limon	4000	1000	>4000	4000	>4000	4000
	M. alternifolia	4000	1000	4000	250	>4000	250
C. krusei ATCC 6258	*C. sempervirens*	2000	1000	2000	4000	2000	>4000
	C. limon	4000	250	>4000	62.5	>4000	>250
	C. limon	1000	1000	>4000	4000	>4000	2000
	M. alternifolia	2000	1000	4000	250	>4000	500
C. parapsilosis SV 04	*C. sempervirens*	>4000	250	4000	500	>4000	125
	Clotrimazole	0.125	0.032	0.25	0.015	0.5	0.063

Isolated MBIC and MBEC (μg/mL): able to reduce by ≥ 90% optical density (OD) compared to control free of EO and clotrimazole. * Isolated: only one substance (EO or clotrimazole). ** Combined: MIC of the combination (EO–EO or EO–clotrimazole) that resulted in synergism.

3.4. In Vivo Assay in *Caenorhabditis elegans*

The test of acute toxicity of EO and clotrimazole against *C. elegans* showed average survival greater than 90% of larvae for the concentration of 250 μg/mL of all tested EO; and for clotrimazole, 100% of *C. elegans* larvae survived at all concentrations within the evaluated range (0.125–4 μg/mL). It was not possible to determine the LC_{50} of *C. sempervirens* because at all concentrations evaluated, survival occurred in more than 90% of the larvae; the LC_{50} for the EO of *M. alternifolia* was 2000 μg/mL and for *C. limon* and *L. cubeba* it was 4000 μg/mL (Figure 1; Table 5). The test controls showed that DMSO concentrations ≤ 5% did not affect the survival of *C. elegans* larvae, and the untreated control showed a mean survival of 96% at 24 h.

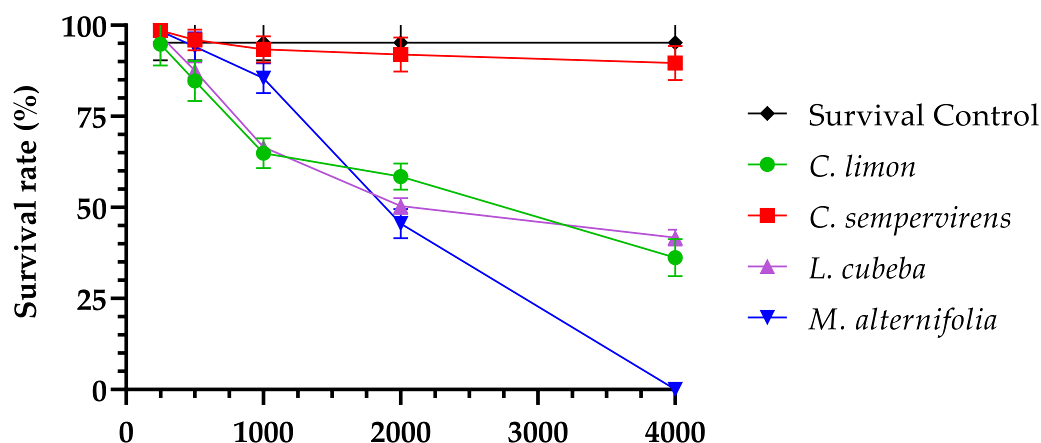

Figure 1. Survival rate (%) of *C. elegans* larvae in 24 h, evaluated at different concentrations of essential oils of *C. limon*, *C. sempervirens*, *L. cubeba* and *M. alternifolia*.

Table 5. Mean survival rate (%) of *C. elegans* tested at different concentrations of essential oils from *C. limon*, *C. sempervirens*, *L. cubeba* and *M. alternifolia*.

Concentration (µg/mL)	*C. limon*	*C. sempervirens*	*L. cubeba*	*M. alternifolia*
250	95.83	97.83	97.83	97.82
500	85.96	95.35	87.76	94.00
1000	64.58	93.48	67.50	85.11
2000	58.82	92.86	51.11 *	46.15 *
4000	37.25 *	88.64	40.43	0

* LC_{50}: lethal concentration responsible for the mortality of 50% of *C. elegans* larvae.

The EO–EO and EO–clotrimazole combinations that showed synergism in the evaluation of the MIC were selected to evaluate the survival of *C. elegans* larvae. Overall, the combinations showed a mean survival of 90% of the larvae for the combinations *L. cubeba*–*M. alternifolia*, *C. sempervirens*–*C. limon*, *M. alternifolia*–clotrimazole and *C. sempervirens*–clotrimazole (Table 6). Survival of less than 20% of larvae, demonstrating greater acute toxicity, was found for the combinations *C. limon*–*M. alternifolia* and *L. cubeba*–*C. limon*.

Table 6. Survival rate (%) of *C. elegans* subjected to combinations of essential oil and clotrimazole after 24 h of exposure.

Compound "A"	Concentration (µg/mL)	Compound "B"	Concentration (µg/mL)	Survival (% Average)
M. alternifolia	250	Clotrimazole	0.063	88.89
L. cubeba	250	*M. alternifolia*	1000	93.3
C. sempervirens	250	Clotrimazole	0.032	100.00
C. sempervirens	1000	*C. limon*	250	90.00
C. limon	1000	*M. alternifolia*	1000	18.52
L. cubeba	500	*C. limon*	1000	13.79

4. Discussion

EO have been extensively studied nowadays and can be a complementary alternative for the treatment of infections caused by *Candida* species, especially mucocutaneous infections. This study investigated the activity of the EO of *C. limon*, *C. sempervirens*, *L. cubeba* and *M. alternifolia*, alone and in combination with each other and with clotrimazole,

on four species of the genus *Candida*, to determine in vitro the MIC, MBIC and MBEC; in addition to this, an in vivo toxicity assessment for the nematode *C. elegans* was performed.

The EO extracted from plants of the studied species are products that have a complex chemical composition and may have more than 20 identified compounds [12–15,18]. Our study used EO that presented limonene (65.6%), α-pinene (52.4%), terpinen-4-ol (41%) and geranyl acetate (42%) as the main component, respectively, for *C. limon*, *C. sempervirens*, *M. alternifolia* and *L. cubeba*. These constituents are like those described for EO of these plants in other studies [15,18,29,35–41]. Terpene derivatives, a class that includes the mentioned constituents, are closely related to the antimicrobial biological action of these EO, as already demonstrated for *Candida* spp. in other studies [13,15,16,41–43].

In the present study, the MIC varied according to the isolate and according to the EO, but the EO of *C. sempervirens* and *L. cubeba* presented the lowest MIC (500 µg/mL) for the same species (*C. krusei* SV 03), while *M. alternifolia* and clotrimazole combined (62.5–0.25 µg/mL) inhibited *C. albicans* ATCC 90028 at lower concentrations than in isolation. In this sense, the ranges of results for the EO showed the effectiveness of plant-derived products in inhibiting microorganisms [42,43], a significant finding for the genus, given the recognized adaptive antifungal arsenal associated with *C. albicans* and the intrinsic fluconazole resistance of *C. krusei* [9,20,44].

This variability of MIC can be observed in the literature [9,18] and is due to the characteristics of each isolate, which may be related to virulence factors and the origin of the isolate (blood, feces, respiratory tract or environment). In addition to this, storage and the constant activation and reactivation of cells, which occur in repeated cultures, may have generated adaptive changes in the phenotypic profile of the reference strains [45].

The MFC were, on average, 2 × MIC for most isolates and EO, but for some, it was not possible to make this determination, as the values were greater than 4000 µg/mL, that is, greater than the limits of concentrations tested. Still, MIC and MFC of 2000 and 1000 µg/mL, respectively, were observed for *C. albicans* ATCC 90028 when evaluated for the EO of *C. limon*. This fact may be explained if the growth curve of cells in contact with this EO is evaluated, as it is possible that the fungicidal effect occurs through mechanisms that involve the depletion of some essential intracellular constituent for growth, such as ergosterol reserves, associated with other mechanisms of enzymatic inhibition, or action on the membrane or cell wall [46] which is time-dependent, but other assays need to be performed to elucidate this finding.

The anti-*Candida* activity of EO may be a direct result of the interaction of the various chemical components present and the association of different mechanisms, which may explain the fungistatic and fungicidal effects. The characteristics common to EO, such as lipophilicity and ability to cause damage to vital structures, membrane, and cell wall, result in increased membrane permeability and release of intracellular contents, with consequent death of *Candida* spp. cells [13,37].

The fungistatic action of clotrimazole at low concentrations is due to structural changes in ergosterol; at high concentrations, it has a fungicidal effect [6]. Thus, it can be assumed that the potentiation of the effects, demonstrated by the synergism observed in the association of clotrimazole with different proportions of EO, is the result of the multiplicity of mechanisms resulting from the various constituents of the EO, leading to the fungicidal effect [47]. The activity of EO against biofilm [10,13,17,25,26,48–51] is another factor that contributes to the need for studies that evaluate the combination of other drugs and a greater number of EO [26,47,51,52].

The application of a product with simultaneous inhibition of microbial growth and biofilm is advantageous since it allows for more efficient satisfactory results in different structures of *Candida* spp. The present study demonstrated that there was inhibition of biofilm formation and a reduction in the viability of the cells of previously formed biofilm, with MIC up to five times lower for the synergistic combination when compared to the same MIC found for the drugs evaluated alone. This study is the first, to our knowledge,

to present a synergistic relationship of EO–EO and EO–clotrimazole on *Candida* spp., evaluating their action on biofilms.

The initial assessment of a substance, such as toxicity and antifungal activity, is a preliminary step in the design of new drug and health product candidates [32]. Our study sought to evaluate the safety of EO and clotrimazole alone, as well as in combinations, exposed for 24 h to the in *vivo* model *C. elegans*. It was observed that more than 80% of *C. elegans* larvae survived at concentrations of 500 μg/mL for three of the evaluated EO. For *C. sempervirens*, 80% of the larvae survived at the concentration of 4000 μg/mL, and for clotrimazole, the survival of 100% of the larvae was observed at all concentrations.

Among the EO evaluated, the biological activity of the EO of *C. limon* and *M. alternifolia* is better known when compared with those of *L. cubeba* and *C. sempervirens* [18]. As observed in Table 4, the LC_{50} was not determined for the EO of *C. sempervirens* (LC_{50}: > 4000 μg/mL), suggesting that it is the least toxic for *C. elegans* larvae among the four evaluated. Our study demonstrated that lethal toxicity of *L. cubeba* EO against *C. elegans* larvae was at 2000 μg/mL; however, lower concentrations such as 0.120–0.525 mg/mL (120–525 μg/mL) were found previously for the nematode *Bursaphelenchus xylophilus* [37]. In our study, we found lower toxicity of *C. sempervirens* EO alone; however, it was moderate and high for other combinations (OE–OE and OE–clotrimazole). Some studies have provided other models for assessing toxicity by evaluating different cell cultures, showing that in vitro inhibitory concentrations (IC_{50}) for MCF-7 and MDA-MB-231 mammary tumor cells were lower than 34.5 and 65.2 μg/mL, respectively [53], and that *C. sempervirens* is lethal at higher concentrations in human promyelocytic leukemia strains (HL-60 and NB4) (LC_{50}: 333.79 to 365.41 μg/mL) [38] and in experimental animal Ehrlich ascitic carcinoma (LC_{50}: 372.43 μg/mL) [38]. In the larvae of *Culex quinquefasciatus*, a non-vertebrate model and vector of filariasis, the LC_{50} was 16.1 μg/mL after 24 h of exposure [39].

The complexity of factors intrinsic to EO, such as the variability and concentration of chemotypes, which can vary in the same plant species according to the part of the plant used for extraction, region of cultivation and stage of development, may be, in part, responsible for the different results obtained in the same toxicity model used. In different models, this variability of constituents can be even greater, as can be seen in some studies [12,15,18,41,49,53]. Thus, it is suggested that toxicity is evaluated in different models to obtain evidence of greater safety and definition of the best drug concentrations that may have biological action and an absence or reduction of damage.

Our study focused on the preliminary assessment of EO–EO and EO–clotrimazole combinations, using concentration ranges applied predictively to planktonic cells and subsequently to biofilm and *C. elegans* after 24 h. Therefore, the totality of combinations that the checkerboard provides for the biofilm was not explored, nor was the influence of different exposure times of the substances for inhibition, eradication and toxicity. Our study used evaluation in the *C. elegans* model; therefore, it is important to evaluate correlation with the results in other models for a better understanding of the mechanism related to toxicity, including the use of EO in biocompatible pharmaceutical applications in nanosystems to improve aspects of physicochemical and biological agents against *Candida* spp.

The complexity of the composition of EO allows wide use in alternative and complementary medicine. The exploration of antimicrobial activity may enable new strategies and therapeutic alternatives for infectious diseases, especially mucocutaneous ones, where topical application is possible. The association of EO makes it possible for some constituents, even though they are not in the majority, to interact, enhancing or evidencing biological effects and reducing toxicity. In this context, studies still need to be carried out to determine the practical relevance of the combinations, better concentrations of each one of them, and the economic and market viability, in addition to the advantages over existing products.

5. Conclusions

The EO–EO and EO–clotrimazole combinations showed synergistic activity in vitro, dependent on the isolate and on the *Candida* species, and of the combined drugs, when evaluating the inhibition of planktonic growth in vitro and the inhibition of biofilm formation and eradication. The combinations *M. alternifolia*–clotrimazole, *L. cubeba*–*M. alternifolia*, *C. sempervirens*–clotrimazole and *C. sempervirens*–*C. limon* were the most efficient against planktonic cells and biofilm. In addition, they demonstrated low or negligible toxicity to *C. elegans* larvae. Thus, our results suggest that the drug combinations evaluated here show promising activity in the control and treatment of vaginal infections caused by *Candida* species, for topical application through different devices, for example, local nanorelease systems, such as mucoadhesive formulations.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Table S1: Combinations between OE–OE and OE–clotrimazole in *Candida* species.

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Supplementary Materials: Combining essential oils with each other and with clotrimazole prevents the formation of *Candida* biofilms and eradicates mature biofilms

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Table S1. Combinations between OE-OE and OE-clotrimazole in *Candida* species.

Species	Compound "A"	Compound "B"	Isolated MIC "A"*	Isolated MIC "B"*	MIC "A" combined**	MIC "B" combined**	FICI	Result
C. albicans ATCC 90028	*L. cubeba*	*M. alternifolia*	1000	4000	500	2000	1	Additive
C. albicans ATCC 90028	*C. limon*	Clotrimazole	2000	0,25	4000	1	6	Antagonism
C. albicans ATCC 90028	*C. sempervirens*	*C. limon*	2000	2000	4000	4000	4	Indifference
C. albicans ATCC 90028	*C. sempervirens*	*L. cubeba*	2000	1000	1000	1000	1,5	Indifference
C. albicans ATCC 90028	*C. sempervirens*	*M. alternifolia*	2000	4000	4000	4000	3	Indifference
C. albicans ATCC 90028	*L. cubeba*	*C. limon*	1000	2000	2000	2000	3	Indifference
C. albicans ATCC 90028	*C. limon*	*M. alternifolia*	2000	4000	4000	4000	3	Indifference
C. albicans ATCC 90028	*C. sempervirens*	Clotrimazole	2000	0,25	250	0,5	2,125	Indifference
C. albicans ATCC 90028	*L. cubeba*	Clotrimazole	1000	0,25	250	0,5	2,25	Indifference
C. albicans ATCC 90028	*M. alternifolia*	Clotrimazole	4000	0,25	250	0,063	0,3145	Synergism
C. albicans SV01	*C. sempervirens*	*L. cubeba*	4000	2000	2000	1000	1	Additive
C. albicans SV01	*C. sempervirens*	*M. alternifolia*	4000	4000	2000	1000	0,75	Additive
C. albicans SV01	*C. sempervirens*	Clotrimazole	4000	0,125	500	0,063	0,629	Additive
C. albicans SV01	*M. alternifolia*	Clotrimazole	4000	0,125	250	0,063	0,5665	Additive
C. albicans SV01	*C. sempervirens*	*C. limon*	4000	4000	4000	4000	2	Indifference

C. albicans SV01	*L. cubeba*	*C. limon*	2000	4000	2000	2000	1,5	Indifference
C. albicans SV01	*C. limon*	*M. alternifolia*	4000	4000	4000	4000	2	Indifference
C. albicans SV01	*C. limon*	Clotrimazole	4000	0,125	4000	0,25	3	Indifference
C. albicans SV01	*L. cubeba*	Clotrimazole	2000	0,125	2000	0,25	3	Indifference
C. albicans SV01	*L. cubeba*	*M. alternifolia*	2000	4000	500	1000	0,5	Synergism
C. glabrata ATCC 2001	*C. sempervirens*	*L. cubeba*	2000	2000	1000	500	0,75	Additive
C. glabrata ATCC 2001	*L. cubeba*	*M. alternifolia*	2000	4000	1000	1000	0,75	Additive
C. glabrata ATCC 2001	*C. limon*	*M. alternifolia*	4000	4000	2000	2000	1	Additive
C. glabrata ATCC 2001	*C. sempervirens*	Clotrimazole	2000	0,015	4000	0,06	6	Antagonism
C. glabrata ATCC 2001	*C. limon*	Clotrimazole	4000	0,015	4000	0,06	5	Antagonism
C. glabrata ATCC 2001	*M. alternifolia*	Clotrimazole	4000	0,015	4000	0,06	5	Antagonism
C. glabrata ATCC 2001	*C. sempervirens*	*C. limon*	2000	4000	2000	250	1,0625	Indifference
C. glabrata ATCC 2001	*C. sempervirens*	*M. alternifolia*	2000	4000	2000	250	1,0625	Indifference
C. glabrata ATCC 2001	*L. cubeba*	Clotrimazole	2000	0,015	2000	0,015	2	Indifference
C. glabrata ATCC 2001	*L. cubeba*	*C. limon*	2000	4000	500	1000	0,5	Synergism
C. glabrata SV 02	*C. sempervirens*	*M. alternifolia*	1000	4000	500	1000	0,75	Additive
C. glabrata SV 02	*L. cubeba*	*C. limon*	2000	4000	500	2000	0,75	Additive
C. glabrata SV 02	*C. sempervirens*	*C. limon*	1000	4000	4000	4000	5	Antagonism
C. glabrata SV 02	*C. limon*	Clotrimazole	4000	0,25	4000	1	5	Antagonism
C. glabrata SV 02	*M. alternifolia*	Clotrimazole	4000	0,25	4000	1	5	Antagonism
C. glabrata SV 02	*C. sempervirens*	*L. cubeba*	1000	2000	1000	1000	1,5	Indifference
C. glabrata SV 02	*C. sempervirens*	Clotrimazole	1000	0,25	1000	0,5	3	Indifference
C. glabrata SV 02	*L. cubeba*	Clotrimazole	2000	0,25	2000	0,5	3	Indifference
C. glabrata SV 02	*L. cubeba*	*M. alternifolia*	2000	4000	250	1000	0,375	Synergism
C. glabrata SV 02	*C. limon*	*M. alternifolia*	4000	4000	1000	1000	0,5	Synergism
C. krusei ATCC 6258	*C. sempervirens*	*L. cubeba*	2000	1000	500	500	0,75	Additive
C. krusei ATCC 6258	*C. sempervirens*	*M. alternifolia*	2000	4000	1000	1000	0,75	Additive

C. krusei ATCC 6258	*M. alternifolia*	Clotrimazole	4000	0,5	2000	0,125	0,75	Additive
C. krusei ATCC 6258	*C. limon*	Clotrimazole	4000	0,5	4000	2	5	Antagonism
C. krusei ATCC 6258	*L. cubeba*	*M. alternifolia*	1000	4000	2000	250	2,0625	Indifference
C. krusei ATCC 6258	*C. sempervirens*	Clotrimazole	2000	0,5	2000	0,125	1,25	Indifference
C. krusei ATCC 6258	*L. cubeba*	*C. limon*	1000	0,5	2000	0,25	2,5	Indifference
C. krusei ATCC 6258	*L. cubeba*	Clotrimazole	1000	0,5	2000	0,25	2,5	Indifference
C. krusei ATCC 6258	*C. sempervirens*	*C. limon*	4000	4000	1000	250	0,3125	Synergism
C. krusei ATCC 6258	*C. limon*	*M. alternifolia*	4000	4000	1000	1000	0,5	Synergism
C. krusei SV 03	*C. sempervirens*	Clotrimazole	500	0,5	2000	0,125	4,25	Antagonism
C. krusei SV 03	*C. limon*	Clotrimazole	1000	0,5	4000	2	8	Antagonism
C. krusei SV 03	*C. sempervirens*	*C. limon*	500	1000	1000	1000	3	Indifference
C. krusei SV 03	*C. sempervirens*	*L. cubeba*	500	500	500	250	1,5	Indifference
C. krusei SV 03	*C. sempervirens*	*M. alternifolia*	500	2000	1000	250	2,125	Indifference
C. krusei SV 03	*L. cubeba*	*C. limon*	500	1000	1000	250	2,25	Indifference
C. krusei SV 03	*L. cubeba*	*M. alternifolia*	500	2000	1000	250	2,125	Indifference
C. krusei SV 03	*C. limon*	*M. alternifolia*	1000	2000	2000	2000	3	Indifference
C. krusei SV 03	*L. cubeba*	Clotrimazole	500	0,5	500	0,5	2	Indifference
C. krusei SV 03	*M. alternifolia*	Clotrimazole	2000	0,5	2000	0,125	1,25	Indifference
C. parapsilosis ATCC 22019	*L. cubeba*	*M. alternifolia*	1000	4000	500	2000	1	Additive
C. parapsilosis ATCC 22019	*C. sempervirens*	*C. limon*	4000	1000	4000	4000	5	Antagonism
C. parapsilosis ATCC 22019	*C. limon*	*M. alternifolia*	1000	4000	4000	4000	5	Antagonism
C. parapsilosis ATCC 22019	*L. cubeba*	Clotrimazole	1000	0,25	4000	1	8	Antagonism
C. parapsilosis ATCC 22019	*C. sempervirens*	*L. cubeba*	4000	1000	1000	1000	1,25	Indifference
C. parapsilosis ATCC 22019	*C. sempervirens*	*M. alternifolia*	4000	4000	4000	4000	2	Indifference
C. parapsilosis ATCC 22019	*L. cubeba*	*C. limon*	1000	1000	1000	1000	2	Indifference
C. parapsilosis ATCC 22019	*C. sempervirens*	Clotrimazole	4000	0,25	250	0,5	2,0625	Indifference
C. parapsilosis ATCC 22019	*M. alternifolia*	Clotrimazole	4000	0,25	250	0,25	1,0625	Indifference

C. parapsilosis ATCC 22019	*C. limon*	Clotrimazole	1000	0,25	1000	0,125	1,5	Indifference
C. parapsilosis SV 04	*C. sempervirens*	*C. limon*	4000	4000	2000	250	0,5625	Additive
C. parapsilosis SV 04	*C. sempervirens*	*L. cubeba*	4000	2000	1000	1000	0,75	Additive
C. parapsilosis SV 04	*C. sempervirens*	*M. alternifolia*	4000	4000	2000	250	0,5625	Additive
C. parapsilosis SV 04	*L. cubeba*	*C. limon*	2000	4000	2000	2000	1,5	Indifference
C. parapsilosis SV 04	*L. cubeba*	*M. alternifolia*	2000	4000	2000	250	1,0625	Indifference
C. parapsilosis SV 04	*C. limon*	*M. alternifolia*	4000	4000	4000	4000	2	Indifference
C. parapsilosis SV 04	*L. cubeba*	Clotrimazole	2000	0,125	2000	0,25	3	Indifference
C. parapsilosis SV 04	*C. sempervirens*	Clotrimazole	4000	0,125	250	0,032	0,3185	Synergism
C. parapsilosis SV 110	*C. limon*	Clotrimazole	4000	0,125	2000	0,125	1,5	Indifference
C. parapsilosis SV 110	*M. alternifolia*	Clotrimazole	4000	0,125	2000	0,125	1,5	Indifference

MIC: µg/mL (able to reduce by ≥ 90% optical density (OD) compared to control free of EO and clotrimazole). *Isolated MIC: Only 1 substance (EO or clotrimazole). **MIC Combined: MIC of the combination (EO-EO or EO-clotrimazole). FICI: fractional inhibitory concentration index.

As publicações geradas durante o mestrado estão apresentadas no Apêndice 1 – artigo intitulado "Essential Oils of Melaleuca, Citrus, Cupressus, and Litsea for the Management of Infections Caused by *Candida* Species: a systematic review" e Apêndice 2 - capítulo de livro intitulado " O óleo essencial de *Citrus limon* como alternativa para o tratamento de candidíase".

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Review

Essential Oils of *Melaleuca*, *Citrus*, *Cupressus*, and *Litsea* for the Management of Infections Caused by *Candida* Species: A Systematic Review

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Abstract: *Candida* is a common agent of infection in humans, which has a wide distribution and is a colonizer fungus of the body, occasionally assuming the role of a pathogen. The type of treatment depends on the site of infection and the clinical condition of the patient. Superficial infections, such as mucosal infections, can be treated with topical medications. So-called alternative therapies have rarely been studied, although the literature records the effectiveness of some treatments, especially as complementary therapy. The aims of this review were to analyze evidence of the anti-*Candida* inhibitory activity of essential oils of the *Citrus*, *Cupressus*, *Litsea*, and *Melaleuca* species; in addition to addressing the chemical composition, probable mechanisms of antifungal action and studies of toxicity, cytotoxicity, and genotoxicity were included. The literature from Medline/PubMed, Science Direct, Scopus, Web of Science, and the Brazilian database Periodic Capes was reviewed. Thirty-eight articles were selected, which included two articles on *Litsea* spp., seven on *Cupressus* spp., thirteen articles on *Citrus* spp., and twenty-one articles on *Melaleuca* spp. In conclusion, this study showed in vitro evidence for the use of essential oils of the plant species evaluated for the treatment of infections caused by different *Candida* species.

Keywords: essential oils; anti-*Candida* activity; alternative therapy

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1. Introduction

Mycoses caused by *Candida* species are the most frequent opportunistic fungal infections affecting humans. The clinical manifestations are the most varied, from superficial and subcutaneous to deep and disseminated infections [1]. More serious infections occur in hospitalized patients, who are often immunocompromised, undergoing invasive procedures, or using antibacterial drugs [2]. The most frequent species include *C. albicans* and others, often referred to as non-*C. albicans* species, such as *C. tropicalis*, *C. parapsilosis*, *C. glabrata*, and *C. krusei* [3].

Non-invasive infections include those that affect the oral cavity, vagina, penis, and other parts of the body. Oral candidiasis is the most common, affecting the oral mucosa, tongue, and throat, followed by vulvovaginal candidiasis, causing vaginal discharge and other signs and symptoms. Penile infection, on the other hand, is less frequent [1].

Candida species resistance to some antifungal agents has been known for decades (for example, the intrinsic or acquired resistance, respectively, of *C. krusei* and *C. glabrata* to fluconazole). This resistance increases the need for new alternative treatment proposals [4]. Currently, the emergence of *C. auris*, a *Candida* species that has shown resistance to most of the available antifungal drugs, has aroused interest in the search for new therapeutic alternatives [5].

The search for new drugs with an antifungal effect, a wider spectrum, or different from the existing ones can minimize the impact of the dissemination of resistant isolates. Natural products, including those obtained from plants, have shown a considerable diversity of chemical constituents that have in vitro antimicrobial activity, with potential for clinical use [6–8].

Essential oils (EOs) include natural products obtained from plants that are widely used in the industry and have potential as agents with antimicrobial activity, meaning that they can be explored for the treatment of human and animal infections. Antimicrobial activity is often attributed to the association of major components present in EOs [9,10]. The proposed mechanisms of action are diverse, including a direct action on the microbial cell, the interaction with the host's immune system, and others. These general mechanisms try to define which chemical components are responsible for the antifungal effect [8].

According to previous studies, the EOs of species of the *Litsea, Citrus*, and *Cupressus* have anti-*Candida* effects in vitro [11]. In addition, *Melaleuca alternifolia* has been reported by its antimicrobial activity for a long time [7,12–16]. All of the EOs are interesting options for the alternative and complementary treatment of clinically relevant microorganisms, such as *Candida* species, the main cause of superficial mycoses in humans.

In this way, the aim of this review was to analyze evidence of the anti-*Candida* inhibitory effect of essential oils from the species of *Citrus, Cupressus, Litsea*, and *Melaleuca*, in addition to addressing the phytochemical composition, possible mechanisms of antifungal action and toxicity, cytotoxicity, and genotoxicity studies.

2. Materials and Methods

2.1. Study Design

This review was carried out through a systematic literature search addressing the anti-*Candida* inhibitory effect of essential oils from *Citrus, Cupressus, Litsea*, and *Melaleuca*. The research was registered in PROSPERO (No. CRD42020188918). Independently, the assessment of the risk of bias for each included article was performed by two reviewers, and disagreements were resolved by discussing until reaching a consensus with a third reviewer.

2.2. Search Strategy

The research was conducted in the Medline/PubMed, Science Direct, Scopus, Web of Science, and Brazilian database Periodic Capes using the terms ("anticandidal" OR "antifungal") AND ("fungal" OR "*Candida*") AND ("volatile oil" OR "essential oil") AND ("mycoses" OR "candidiasis" OR "infections") AND ("*Citrus*" OR "*Melaleuca*" OR "*Cupressus*" OR "*Litsea*"). For Science Direct: ("anticandidal" OR "antifungal") AND ("fungal" OR "*Candida*") AND ("volatile oil" OR "essential oil") AND ("candidiasis" OR "infections") AND ("*Citrus*" OR "*Melaleuca*" OR "*Cupressus*" OR "*Litsea*") and ("anticandidal" OR "antifungal") AND ("fungal" OR "*Candida*") AND ("volatile oil" OR "essential oil") AND ("mycoses" " OR "infections") AND ("*Citrus*" OR "*Melaleuca*" OR "*Cupressus*" OR "*Litsea*").

2.3. Selection of Articles, Inclusion, and Exclusion Criteria

The publications considered for inclusion in this review were those published from 2011 to 2020, containing the following information: (I) Biological activity: antifungal activity involving *Candida* species; (II) Plants and derivatives: essential oils only; and (III) Study design: Experimental in vitro, laboratory studies using the broth dilution assay (CLSI—Clinical and Laboratory Standards Institute and EUCAST—The European Committee on Antimicrobial Susceptibility Testing, and adaptations), agar diffusion disk (Kirby–Bauer and adaptations), and agar dilution assay, preclinical studies, case reports, randomized clinical trials, cross-sectional studies, and prospective studies. The exclusion criteria were the lack of access to the full content of the published article.

2.4. Study Analysis

The information collected in the articles was descriptively evaluated and grouped according to the essential oil (EO), *Candida* genus and/or species, chemical constitution, minimum inhibitory concentration (MIC), minimum fungicidal concentration (MFC), minimal biofilm inhibitory concentration (MBIC), and minimal biofilm eradication concentration (MBEC), in addition to information on synergism with antifungals or EOs. Experimental toxicity in vitro and in vivo, such as mean inhibitory concentration (IC_{50}), mean lethal concentration (LC_{50}), mean lethal dose (LD_{50}), and genotoxicity, was also evaluated.

3. Results

3.1. Characteristics of the Studies

The search of databases identified 881 studies; after analysis according to the inclusion and exclusion criteria, 32 publications were eligible, including two articles on *Litsea* spp., seven on *Cupressus* spp., thirteen articles on *Citrus* spp., and twenty-one articles on *Melaleuca* spp. Figure 1 shows the flow of articles included in this study.

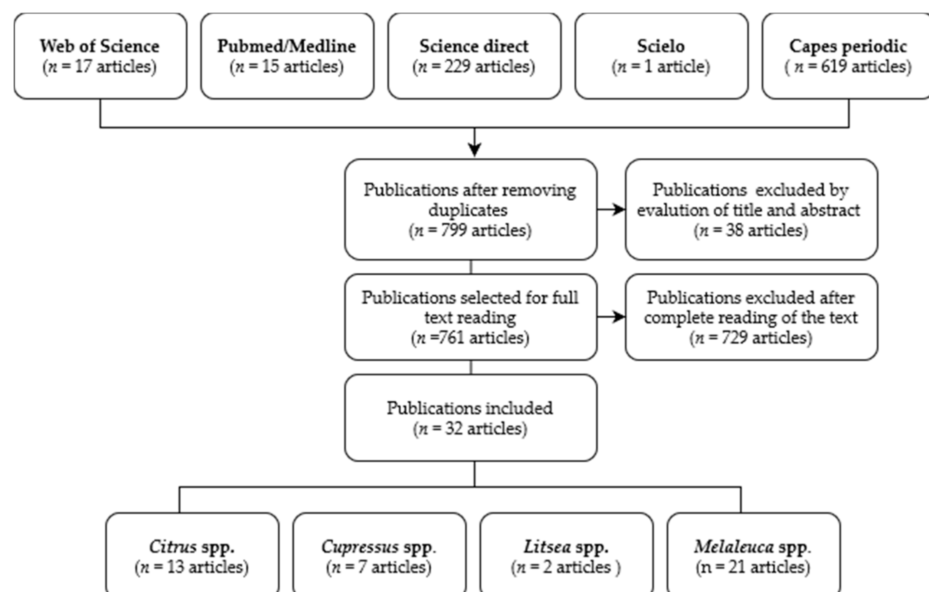

Figure 1. Flow diagram of retrieved, selected, included, and excluded studies.

Most studies related the in vitro antifungal effect against *Candida* species isolates and used different methodologies and techniques, with many adaptations and variations from those recommended by the CLSI and EUCAST, and based on other research. Accordingly, in vitro studies showed antifungal susceptibility testing by macro- and microdilution methodologies in broth (with results expressed in µg/mL, µL/mL, and in percent—% *v/v*), which exhibit the minimum inhibitory concentration, and still, some others reported the minimum fungicidal concentration. In addition, other studies used the agar diffusion methodology from the well or the disk (results expressed in mm). Studies that addressed other techniques or technologies, or even activities on biofilm, are reported and discussed in the text and shown in Tables and in Supplementary Materials. A list of *Melaleuca* spp., *Citrus* spp., *Cupressus* spp., and *Litsea* spp. essential oils and their main components related in the text, as well as a summary of the most important results, are provided in the Supplementary Materials.

The origin of EO was also the cause of the differences observed between the susceptibilities of different isolates and species, in addition to other variations observed between different studies.

3.2. *Melaleuca spp.*

The genus *Melaleuca* includes plants belonging to the Myrtaceae family, such as the species *M. linariifolia*, *M. viridiflora*, *M. dissitiflora*, M. *leucadendra*, *M. acacioides*, *M. ericifolia*, *M. alsophila*, and *M. alternifolia* [17–19]. Among these species, the EO of *M. alternifolia*, also known as Tea tree, is the most widespread globally. The product of the distillation of the leaves and branches of *M. alternifolia* is traditionally known by native peoples of Oceania for its anti-infective and anti-inflammatory properties in medicinal preparations [19]. Tea tree EO is used in topical antimicrobial formulations in the pharmaceutical and cosmetic industries and has been used as a flavoring in the food industry [12,17–20].

3.2.1. Chemical Composition of Essential Oils from *Melaleuca* spp.

Tea tree EO presents a diversity of constituents (Supplementary Materials), which varies according to the origin and region in which the plant was cultivated [17]. The constituent variation is even greater when different species of *Melaleuca* are considered [20–22].

The main components described in the EO of *M. alternifolia* leaves are terpinen-4-ol (35.93–47.5%), γ-terpinene (17.8–23.58%), α-terpinene (6.84–11.91%), and limonene (1–19.79%) [13,14,22,23].

In the leaves, bark, fruits, and in tips of branches of *M. leucadendra*, the following were identified: α-eudesmol (13.7–30.7%), guaiol (7.3–12.5%), (E)-caryophyllene (3.8–7%), 1,8-cineole (0.2–5.2%), linalool (1.4–5.1%), and bulnesol (2.2–5.3%) [15]. The analysis of the species *M. quinquenervia* revealed 1,8-cineol (40.3%), carveol (27.15%), and myrtenol (9.43%) to be the most frequent constituents [21].

3.2.2. Anti-*Candida* Activity of *Melaleuca* spp. Essential Oil

Tea tree EO has shown antifungal effects in vitro against several *Candida* species, with suitable efficacy when used topically in the treatment of oral candidiasis [24]. The EO of *Melaleuca* at a concentration of 0.5% (*v*/*v*) (equal to MIC_{50}) acts by inhibiting both the initial adhesion and the subsequent stages of *C. albicans* biofilm formation [24]. The mechanism of action is associated with changes in the structure of the fungus cell membrane, making it permeable [12] (Table 1).

The in vitro antifungal effect has been described in different studies, regardless of the methodology and technique used, whether by disk diffusion in agar, macrodilution and microdilution in broth, or agar dilution. This was demonstrated in studies using the EO of *M. alternifolia*, showing inhibitory effects against different species of *Candida* spp. [7,25,26]. By disk diffusion in agar, the EO of *M. alternifolia* showed inhibitory effects against *C. albicans* [14,22] and also for the species *C. kefyr, C. dublinensis, C. lusitaniae*, and *C. parapsilosis* [20] (Table 1).

C. albicans is the most studied species, possibly because it is the species most related to human infections. The MIC for the EO of *M. alternifolia*, expressed in % (*v*/*v*), varies according to the study, with values as low as 0.12–4% (*v*/*v*) [7,15,23,25–27]. Moreover, other studies reported an MIC (*m*/*v*) of 0.0097–5 mg/mL (which is the same as 9.7–5000 µg/mL) [8,11,14,21] (Table 1).

In *C. albicans*, the isolates susceptible and resistant in vitro to fluconazole are evaluated [7] with an MIC reported to range from 0.06–0.5% (*v*/*v*), and 5–20 mg/mL (equivalent to 5000–20,000 µg/mL), respectively, and no differences between the MICs of the two groups of isolates were found [13] (Table 1).

The studies that included the evaluation of tea tree EO activity on *C. glabrata* also showed an in vitro inhibitory effect. Other MIC values range from 0.156–4% (expressed in *v*/*v*) [4,16,27,28], and have also been seen in the range of 9.7–625 µg/mL (equivalent to 0.0097–0.625 mg/mL) [14]. On the other hand, values greater than 2000 µg/mL have also been reported [11].

For *C. parapsilosis*, MICs greater than 2000 µg/mL [11] and 5 mg/mL [14] (equivalent to 5000 µg/mL) have been reported. Other studies have found results ranging from 0.312–1% (*v*/*v*) for *C. krusei* isolates [4,27,29] (Table 1).

An isolate of *C. tropicalis* resistant to nystatin, fluconazole, and voriconazole in vitro presented an MIC of 8% (*v*/*v*) [27]. Other studies found MICs equal to 1 mg/mL (1000 µg/mL) [21], while MIC values were found up to the limit of 2000 µg/mL, which was the highest concentration used in the study [11] (Table 1).

Other *Candida* species have also shown inhibition by EO of *Melaleuca*, as demonstrated for the species *C. boidinni*, *C. colliculosa, C. dubliniensis, C. famata, C. lusitaniae, C. pelliculosa*, and *C. rugosa* [14,27], with an MIC less than or equal to 1% (*v*/*v*), and including isolates resistant to the drugs nystatin, fluconazole, and voriconazole [27] (Table 1).

The EO of *M. alternifolia* shows inhibitory activity against biofilm formation of *C. albicans* [24,26]. For this species, no concentration capable of inhibiting biofilm formation was observed within the limits of those tested (up to 8% *v*/*v*), *C. albicans* isolates, whose MIC was 0.2% (*v*/*v*) [26]. On the other hand, the concentration of 1% EO (*v*/*v*) was able to inhibit the development of biofilm in cases where the MIC was 0.5% (*v*/*v*) [24], while 12.5% (*v*/*v*) could eradicate biofilms formed by *C. albicans* [15]. The EO of *M. alternifolia*, when incorporated into nanoparticles, showed a greater antibiofilm inhibitory effect in vitro when compared to the EO at a concentration of 15.6%, which was able to inhibit more than 70% of the biofilm formed by *C. glabrata* [30] (Table 1).

Other species of *Melaleuca* have also been evaluated for their in vitro inhibitory activity against *C. albicans*. The EO of *M. quinquenervia* had an MIC of 4 mg/mL (equivalent to 4000 µg/mL) [21], and the EO of *M. leucadendra*, extracted from the bark, leaves, and fruits, had an MIC of 64, 128, and 256 µg/mL, respectively [20] (Table 1).

In vivo antifungal activity after the use of the EO *of M. alternifolia* showed a reduction of colonization by *C. albicans* in the oral cavity in different sensitivity profiles [13,15]. An in vivo study in mice showed that 4% (*v*/*v*) EO had a protective action after two days of treatment against oral candidiasis induced by *C. albicans* isolates that were susceptible and resistant to fluconazole [13], while there was still the presence of tissue lesions characteristic of candidiasis in the oral cavity tissue of mice after 24 h of treatment, even when treated with 12.5% (*v*/*v*) [15].

Table 1. In vitro antifungal activities of *Melaleuca* spp. essential oils tested against *Candida* species according to different methods.

***Melaleuca* Species**	**Method of Antifungal Susceptibility**	**Species of *Candida* (Number of Strains Tested)**	**Agar Diffusion * or MIC ****	**Reference**
M. alternifolia	Disk diffusion	*C. albicans* (19)	12–25 mm	[14,22]
		C. atlântica (1)	21.1 mm	[14]
		C. dublinenensis (1)	15 mm	[14]
		C. famata (1)	20.66 mm	[14]
		C. glabrata (3)	11.66–14.33 mm	[14]
		C. intermedia (1)	20 mm	[14]
		C. kefyr (2)	19.33–25.3 mm	[14]
		C. lusitaniae (1)	15.33 mm	[14]
		C. marítima (1)	24.66 mm	[14]
		C. parapsilosis (1)	14.66 mm	[14]
		C. sake (1)	16.33 mm	[14]

Table 1. *Cont.*

Melaleuca Species	Method of Antifungal Susceptibility	Species of *Candida* (Number of Strains Tested)	Agar Diffusion * or MIC **	Reference
M. alternifolia	Broth microdilution	*C. albicans* (207)	0.125–4% (*v*/*v*)	[4,7,15,23,24,26,27]
		C. boidinii (3)	0.12–0.25% (*v*/*v*)	[27]
		C. colliculosa (1)	0.25% (*v*/*v*)	[27]
		C. famata (2)	0.25–0.5% (*v*/*v*)	[27]
		C. glabrata (52)	0.156–4% (*v*/*v*)	[4,16,27,28]
		C. krusei (13)	0.12–0.625% (*v*/*v*)	[4,27,29]
		C. lusitaniae (5)	0.25–1.0% (*v*/*v*)	[27]
		C. pelliculosa (1)	0.5% (*v*/*v*)	[27]
		C. rugosa (1)	0.12% (*v*/*v*)	[27]
		C. tropicalis (1)	8% (*v*/*v*)	[27]
M. alternifolia	Broth microdilution	*C. albicans* (20)	0.0097–20 mg/mL	[13,14,22]
		C. atlântica (1)	0.0097 mg/mL	[14]
		C. dublinenensis (1)	0.0195 mg/mL	[14]
		C. famata (1)	0.0097 mg/mL	[14]
		C. glabrata (3)	0.0097–0.625 mg/mL	[14]
		C. intermedia (1)	0.0097 mg/mL	[14]
		C. kefyr (2)	0.0097 mg/mL	[14]
		C. lusitaniae (1)	0.0097 mg/mL	[14]
		C. maritima (1)	0.0097 mg/mL	[14]
		C. parapsilosis (1)	5 mg/mL	[14]
		C. sake (1)	0.0097 mg/mL	[14]
M. alternifolia	Broth microdilution	*C. albicans* (2)	625 to >2000 µg/mL	[8,17]
		C. glabrata (1)	>2000 µg/mL	[11]
		C. krusei (1)	2000 µg/mL	[11]
		C. orthopsilosis (1)	>2000 µg/mL	[11]
		C. parapsilosis (1)	>2000 µg/mL	[11]
		C. tropicalis (1)	>2000 µg/mL	[11]
M. leucadendra	Broth microdilution	*C. albicans* (1)	64–256 µg/mL	[20]
M. quinquenervia	Broth microdilution	*C. albicans* (2)	1–4 mg/mL	[11]
M. quinquenervia		*C. tropicalis* (1)	1 mg/mL	[11]

* Agar diffusion in mm. ** MIC: Minimum inhibitory concentration (expressed as µg/mL or mg/mL or % (*v*/*v*).

3.2.3. Other Biological Activity of Essential Oils of *Melaleuca* spp.

In vitro studies showed that *M. alternifolia* EO has potent antioxidant activity, the ability to reduce and eliminate superoxide anion radicals [14], and the ability to reduce infectivity against Herpes simplex type 1 (HSV-1) and Herpes simplex type 2 (HSV-2) [21,25].

The EO of *M. alternifolia* has been evaluated for its toxicity to different cell lines and its influence on mediators of the inflammatory process. In vitro studies using MCF-7 and MDA-MB-231 cells derived from breast tumors showed that concentrations greater than 100 µg/mL were toxic [8]. In OKF6-TERT2 cells originating from the oral epithelium, 0.25% (*v*/*v*) demonstrated both a cytotoxic effect and the ability to inhibit the expression of the cytokine IL-8 [24]. In in vivo models using mice with pneumonia induced by *C. albicans*, there was a reduction in the pro-inflammatory mediators IL-1β and TNF-α, as well as a

decrease in the recruitment of leukocytes and neutrophils, when inhalable nanoemulsions containing EO of *M. alternifolia* were administered [31]. The *M. leucadendra* EO showed acute toxicity to *Aedes aegypti* and *Cx. quinquefasciatus* larvae, showing repellent potential [20].

3.3. *Citrus spp.*

The genus *Citrus* originates from Southeast Asia and includes about 40 species. It is one of the most important genera of the Rutaceae family and is cultivated in several countries with warm climates [32,33]. Some factors contribute to the known and extensive biological activity of the species of *Citrus*, such as the part of the plant used, plant growth conditions, and the developmental stage at the time of extraction in the case of the fruit, among others [33–36]. EO can be extracted from the fruit, leaf, and peel, and is used in the composition of fragrances, in cooking, and in the pharmaceutical and cosmetic industries [32,34].

3.3.1. Chemical Composition of Essential Oils from Species of *Citrus* spp.

The main constituents of the EO of *Citrus* spp. described in most publications are limonene, β and α-pinene isomers, and linalool (Supplementary Materials) [8,11,21,34,36–38]. The main constituent, limonene, is present in a greater proportion, reaching concentrations of 75.43–90% in the EO of *C. grandis, C. reticulata, C. sinensis, C. paradisi*, and *C. hystrix* [8,21,37]. Intermediate concentrations, however, ranging from 51.09–51.46%, were found for the EO of *C. aurantifolia* [8] and *C. latifolia,* respectively [37]. Other species, such as *C. reticulata* var. Blanco and *C. bergamia*, had concentrations of 34.6% and 37.5%, respectively [8,16].

Other species had different major constituents with varying concentrations. *C. limonum* was the species with the greatest variation in limonene concentration, ranging from 22.4–63.27% [8,11,34]. However, citral was reported to be the main component, reaching a proportion of 53.85% among the constituents, while the proportion of limonene was 5.29% [38]. *C. grandis* had citronellol as the major constituent in the EO extracted from the leaves, which ranged from 30.87–34.54% [36], while the proportion of borneol in the bark was 42.24% [21]. In *C. aurantium*, the major constituents were linalyl acetate and linalool, at levels of 51.5% and 25.4%, respectively [8,39].

3.3.2. Anti-*Candida* Activity of *Citrus* spp. Essential Oil

In many industrial processes involving species of *Citrus*, the peel is not considered, even though it represents about 50% of the fruit [33,36]; however, it is from the peel that EO can be extracted. *Citrus* EO is a potent antimicrobial agent against microorganisms that have considerable importance for human health, such as Gram-negative and Gram-positive bacteria [21], and yeasts such as *Candida* spp. [8,11,28,34].

EOs from different *Citrus* species have been evaluated in vitro against *Candida* species [4,11,21,34,36–39]. These EOs have a wide spectrum of action against *C. albicans*, in which the in vitro inhibitory activity is variable. The EO of *C. sinensis* and *C. latifolia* showed low action, forming inhibitory halos of 5.51 and 9.46 mm, respectively, when assayed by well diffusion in agar [37]. Still, other findings for *C. sinensis* described an MIC of 625 μg/mL [8] and values greater than 2000 μg/mL [11]. The EO for *C. aurantium* showed an MIC equal to those reported for *C. sinensis,* with an MIC ranging from 0.15–0.31% (*v*/*v*) [8,11,39]. The EO action of *C. hystix* and *C. grandis* was inhibitory against *C. albicans* [21,36]. MICs ranging from 1000–4000 μg/mL and 4000 μg/mL, respectively, for *C. hystix* and *C. grandis* EOs [21], while values from 0.116–0.121% (*v*/*v*) were found for *C. grandis* [36] (Table 2).

EOs from other *Citrus* species have also shown in vitro inhibitory activity against *C. albicans* isolates. MIC variations found according to the studies ranged from 0.0097–3.0% (*v*/*v*) by cylinder-plate diffusion [4,34], and concentrations lower than 0.043–31.325 mg/mL by microdilution in broth [38]. The EO of *C. limon* presented an MIC equal to 500 μg/mL [11] and 625 μg/mL [8]. A similar MIC (625 μg/mL) was observed for the EO of *C. bergamia* and *C. aurantifolia* [8]. The MIC of the EO of *C. reticulata* showed the widest range of variation,

from 300 µg/mL to greater than 2000 µg/mL [8,11,21] (Table 2). Meanwhile, MICs of 2000 µg/mL have been related to the EO of *C. nobilis* [11], while there was variation in the MIC for the EO of *C. paradisi* [23] from 0.125–0.25% (*v*/*v*) and an MIC of 313 µg/mL [8].

C. glabrata is also susceptible in vitro to *Citrus* EO. For *C. paradisi* EO, the variation in MIC was from 0.0024–1% (*v*/*v*) [4,28]. For *C. limonum*, the range of MICs was lower than 0.043–5.33 mg/mL [38], also demonstrating the concentration-dependent inhibitory activity of *C. limonum* through the cylinder-plate diffusion method against *C. glabrata* (halo formation ranging from 44.6–45 mm) [34]. The EOs of *C. sinensis* and *C. latifolia* showed a halo of 5.78 and 8.52 mm, respectively [37]. MIC ranging from 250 µg/mL to greater than 2000 µg/mL have been reported for EO of *C. limon, C. reticulata, C. nobilis, C. aurantium*, and *C. sinensis* [11] (Table 2).

The EOs of *C. sinensis* and *C. latifolia* inhibited 50% of the growth of *Candida* species, including *C. lusitaniae* (2.00 and 8.06 mm, respectively) and *C. guilliermondii* (only *C. latifolia* was active, 8.94 mm) [37] (Table 2). *C. parapsilosis* and *C. orthopsilosis* were not inhibited in vitro at concentrations of up to 2000 µg/mL with *C. nobilis* and *C. reticulata* EOs, as shown in a previous study [11] (Table 2).

C. krusei was inhibited with an MIC between 0.0024 and 0.0019 (% *v*/*v*) when tested in vitro with *C. limonum* EO [4]. However, the EOs of *C. limon, C. sinensis, C. reticulata, C. aurantium*, and *C. nobilis* have shown inhibition ranging from 250 µg/mL to values greater than 2000 µg/mL for *C. krusei* [11] (Table 2).

The EO of *C. limon* presented an MIC of 500 µL/mL for *C. tropicalis* [40], while there were inhibition halos reported between 15.3 and 16.3 mm when they tested *C. limonum* by cylinder-plate diffusion [34]; in contrast, there were inhibition halos ranging from 4.44 to 10.87 mm when using EO of *C. sinensis* and *C. latifolia* by agar diffusion [37] (Table 2). EOs of *C. reticulata, C. aurantium, C. nobilis, C. sinensis, C. hystix*, and *C. grandis* have shown an MIC ranging from 1,000 µg/mL to values greater than 4,000 µg/mL by microdilution in broth [11,21].

Citrus EOs have also been evaluated for their ability to inhibit and eradicate preformed biofilms. The EO of *C. limon* eradicated 70% or more of the *C. tropicalis* biofilm at concentrations starting from 0.125 x MIC (MIC equal to 500 µL/mL) [40]. Other reports show that 125 µg/mL and 250 µg/mL of the EO of *C. limon*, respectively, were able to inhibit and eradicate the biofilm of *C. krusei* [11].

The EO of *C. limonum* showed the best MIC range for *C. krusei*, from 0.0024–0.0097% (*v*/*v*), for *C. glabrata* from 0.0024–0.1565% (*v*/*v*), and for *C. albicans* from 0.0097–0.312% (*v*/*v*); according to the authors of [4], all isolates were resistant to fluconazole, while there was an MIC of 0.005 and 0.312% (*v*/*v*), respectively, for *C. glabrata* and *C. albicans* [38].

The mechanisms by which the different EOs show inhibitory activity on *Candida* spp. are complex and depend on the chemical constitution and concentration of the major constituents, but usually involve damage to the cell membrane, leading to changes in permeability; however, other cellular activities, such as the disruption of proton pumps, the coagulation of cell contents, leakage of intracellular contents, and consequent apoptosis, necrosis and cell death, have also been reported [38].

Table 2. In vitro antifungal activities of *Citrus* spp. essential oils tested against *Candida* species according to different methods.

Citrus Species	Method of Antifungal Susceptibility	Species of *Candida* (Number of Strains Tested)	Agar Diffusion * or MIC **	Reference
C. aurantifolia	Broth microdilution	*C. albicans* (1)	625 µL/mL	[8]
C. aurantium		*C. albicans* (1)	625 µL/mL	
C. aurantium	Broth microdilution	*C. albicans* (1)	>2000 µg/mL	[11]
		C. glabrata (1)	>2000 µg/mL	
		C. krusei (1)	>2000 µg/mL	
		C. orthopsilosis (1)	>2000 µg/mL	
		C. parapsilosis (1)	>2000 µg/mL	
		C. tropicalis (1)	>2000 µg/mL	
C. aurantium	Disk diffusion	*C. albicans* (2)	19–25.3 mm	[39]
	Broth microdilution	*C. albicans* (2)	0.15–0.31% (*v*/*v*)	
C. bergamia	Broth microdilution	*C. albicans* (1)	625 µL/mL	[8]
C. grandis	Broth microdilution	*C. albicans* (1)	4 mg/mL	[21]
		C. tropicalis (1)	4 mg/mL	
C. grandis	Broth microdilution	*C. albicans* (1)	0.116–0121% (*v*/*v*)	[36]
C. hystix	Broth microdilution	*C. albicans* (1)	1–4 mg/mL	[21]
		C. tropicalis (1)	2 mg/mL	
C. latifolia	Disk diffusion	*C. albicans* (1)	9.46 mm	[37]
		C. glabrata (1)	8.52 mm	
		C. guilliermondii (1)	8.94 mm	
		C. lusitaniae (1)	8.06 mm	
		C. tropicalis (1)	10.87 mm	
C. limon	Broth microdilution	*C. albicans* (1)	625 µL/mL	[8]
C. limon	Broth microdilution	*C. albicans* (1)	500 µg/mL	[11]
		C. glabrata (1)	250 µg/mL	
		C. krusei (1)	500 µg/mL	
		C. orthopsilosis (1)	500 µg/mL	
		C. parapsilosis (1)	500 µg/mL	
		C. tropicalis (1)	250 µg/mL	
C. limonum	Broth microdilution	*C. albicans* (20)	0.0097–0.312% (*v*/*v*)	[4]
		C. glabrata (14)	0.0024–0.1565 (*v*/*v*)	
		C. krusei (10)	0.0024–0.0097% (*v*/*v*)	
C. limonum	Broth microdilution	*C. albicans* (183)	<0.043 to >21.325 mg/mL	[38]
		C. glabrata (76)	<0.044 to 5.331 mg/mL	
C. limonum	Cylinder-plate diffusion	*C. albicans* (1)	0 mm	[34]
		C. glabrata (1)	44.8–45 mm	
		C. tropicalis (1)	0 mm	

Table 2. *Cont.*

Citrus Species	Method of Antifungal Susceptibility	Species of *Candida* (Number of Strains Tested)	Agar Diffusion * or MIC **	Reference
C. limonum	Cylinder-plate diffusion	*C. albicans* (1)	44.8–45 mm	[34]
		C. glabrata (1)	0 mm	
		C. tropicalis (1)	15.3–16.3 mm	
	Cylinder-plate diffusion	*C. albicans* (1)	23–45.0 mm	[34]
		C. glabrata (1)	44.6–44.8 mm	
		C. tropicalis (1)	0 mm	
	Cylinder-plate diffusion	*C. albicans* (1)	0 mm	[34]
		C. glabrata (1)	0 mm	
		C. tropicalis (1)	0 mm	
	Cylinder-plate diffusion	*C. albicans* (1)	0 mm	[34]
		C. glabrata (1)	0 mm	
		C. tropicalis (1)	0 mm	
C. nobilis	Broth microdilution	*C. albicans* (1)	2000 µg/mL	[11]
		C. glabrata (1)	2000 µg/mL	
		C. krusei (1)	>2000 µg/mL	
		C. orthopsilosis (1)	>2000 µg/mL	
		C. parapsilosis (1)	>2000 µg/mL	
		C. tropicalis (1)	>2000 µg/mL	
C. paradisi	Broth microdilution	*C. albicans* (1)	313 µL/mL	[8]
C. paradisi	Broth microdilution	*C. albicans* (30)	0.0039–1% (*v*/*v*)	[23]
C. paradisi	Broth microdilution	*C. glabrata* (30)	0.007–1% (*v*/*v*)	[28]
C. reticulata	Broth microdilution	*C. albicans* (1)	625 µL/mL	[8]
C. reticulata	Broth microdilution	*C. albicans* (1)	2000 µg/mL	[11]
		C. krusei (1)	250 µg/mL	
		C. glabrata (1)	1000 µg/mL	
		C. parapsilosis (1)	1000 µg/mL	
		C. orthopsilosis (1)	250 µg/mL	
		C. tropicalis (1)	1,000 µg/mL	
C. reticulata var. Blanco	Broth microdilution	*C. albicans* (1)	1.00 to > 2000 µg/mL	[11]
		C. krusei (1)	500 to >2000 µg/mL	
		C. glabrata (1)	1000–2000 µg/mL	
		C. parapsilosis (1)	1000–2000 µg/mL	
		C. orthopsilosis (1)	1000–2000 µg/mL	
		C. tropicalis (1)	2.00 to > 2000 µg/mL	

Table 2. *Cont.*

Citrus Species	Method of Antifungal Susceptibility	Species of *Candida* (Number of Strains Tested)	Agar Diffusion * or MIC **	Reference
C. reticulata Blanco var. *cravo*	Broth microdilution	*C. albicans* (1)	2.00 to > 2000 µg/mL	[11]
		C. krusei (1)	>2000 µg/mL	
		C. glabrata (1)	>2000 µg/mL	
		C. parapsilosis (1)	>2000 µg/mL	
		C. orthopsilosis (1)	>2000 µg/mL	
		C. tropicalis (1)	2.00 to >2000 µg/mL	
C. reticulata var. Blanco	Broth microdilution	*C. albicans* (1)	0.3–4 mg/mL	[21]
		C. tropicalis (1)	2 mg/mL	
C. sinensis	Disk diffusion	*C. albicans* (1)	5.51 mm	[37]
		C. glabrata (1)	5.78	
		C. lusitaniae (1)	2.00	
		C. tropicalis (1)	4.44 mm	
C. sinensis	Broth microdilution	*C. albicans* (1)	>2000 µg/mL	[11]
		C. krusei (1)	>2000 µg/mL	
		C. glabrata (1)	>2000 µg/mL	
		C. parapsilosis (1)	>2000 µg/mL	
		C. orthopsilosis (1)	>2000 µg/mL	
		C. tropicalis (1)	>2000 µg/mL	

* Agar diffusion in mm. ** MIC: Minimum inhibitory concentration (expressed as µg/mL or mg/mL or % (*v*/*v*).

3.3.3. Other Biological Activity of Essential Oils of *Citrus* spp.

Citrus EOs have other biological activities, as shown by in vitro studies, such as antioxidant, anti-inflammatory, and anti-pigmentation. These activities are present in the EO of *C. grandis*, which make it an option for the development of dermatological products, in which in vitro studies have shown effectiveness at concentrations lower than 0.05% (*v*/*v*) [33,36].

By computational modeling (in silico) [39], the potential use of *C. aurantium* EO as an antimicrobial agent has been suggested in in vivo models of infection, such as *Caenorhabditis elegans* [11]. On the other hand, the EO of *C. limon* showed toxicity to larvae of *C. elegans*, even at the same concentration as that which was effective in vitro against *C. tropicalis* [11].

The in vitro toxicity of *Citrus* EOs varied according to the different cells assayed, such as human breast cancer cell lines and human oral epithelium [8,28,37]. Most EOs tested showed toxicity above 50 µg/mL in MDA-MB-231 and MCF-7 breast cancer cells [8], and 21.8 µg/mL for *C. latifolia* in human oral epithelial cells [37].

3.4. *Cupressus spp.*

The genus *Cupressus* is native to the northern hemisphere and includes more than ten species and variants [41]. Plants of this genus are cultivated in a temperate climate, which is attractive for ornamental purposes and wood extraction, and are distributed in commercial plantations all over the world [42]. A wide spectrum of biological activities has been attributed to substances present in its aerial parts, including in the EO [43]. In folk medicine, cypress EO acts as an antispasmodic for coughing, as a diuretic, and in the improvement of affections of the venous and renal circulation, in addition to acting on inflammatory processes and against infectious microorganisms [10,41,42,44].

3.4.1. Chemical Composition of Essential Oils from Species of *Cupressus* spp.

Cupressus EO is usually extracted from aerial parts and leaves, and the chemical composition varies according to the species and study. The main component is α-pinene, found in *C. arizonica* (26.53–29.76%) [42], *C. lusitanica* (13.8–35.7%) [44], *C. macrocarpa* (63.2%) [21], and in *C. sempervirens* (4.6–49.7%) [8,10,11]. Other constituents, such as δ-3-carene, terpinen-4-ol, limonene, sabinene, umbellulone, α-thujene, and cedrol, appear in smaller proportions and vary according to species [8,21] (Supplementary Materials).

As in all essential oils in general, the factors that influence the different proportions of constituents include the location/region of cultivation, the part of the plant collected, the period of plant development in the EO extraction, and varieties of the species [6,8,10,11,42,44].

3.4.2. Anti-*Candida* Activity of *Cupressus* spp. Essential Oil

Cupressus EOs have an anti-*Candida* inhibitory effect demonstrated by in vitro studies, which vary according to the yeast species but also according to the plant species [6,42], (Table 3). The evaluation of the inhibitory effect of EOs against *C. albicans*, according to the methodology used, showed that the species *C. arizonica*, *C. sempervirens, C. lusitanica*, and *C. macrocarpa* have inhibitory activities in some way by different concentrations of EO. Evaluating the same *Candida* species by microdilution, the inhibitory activity of EO of *C. arizonica* was expressed by an MIC of 0.05 µL/mL [42] and was also expressed at 0.42 ± 0.027 µL/mL for the EO of *C. sempervirens* [10]. Other studies found an MIC of 625 µg/mL for *C. sempervirens* [8] and 2000 µg/mL for *C. macrocarpa* [21]. For the EO of *C. lusitanica* against *C. albicans*, both MIC and CFM were equal to 0.16% (*v*/*v*) [6]. By using the agar diffusion disk technique, a 13.0 mm halo was produced when using a 10 µL/100% (*v*/*v*) *C. lusitanica* EO disk [6]; for this same species, there were inhibition halos of 7.5 to 8.5 mm when 1.5 µL of EO/disks of *C. lusitanica* were placed [44] (Table 3).

For *C. glabrata*, the EO of *C. arizonica* presented an MIC ranging between 0.01 and 0.05 µL/mL [42], the EO of *C. lusitanica* presented an MIC of 1.25% (*v*/*v*) [6], and the EO of *C. sempervirens* presented an MIC of 31.25µg/mL [11].

C. krusei was tested with EOs of *C. sempervirens* and *C. lusitanica*. The MIC for *C. sempervirens* was 62.5 µg/mL [11], and for *C. lusitanica* it was 1.25% (*v*/*v*); the halos were 10 mm when using disks containing 10 µL of the EO [6].

Cupressus species showed variable results for *C. parapsilosis*. This *Candida* species was inhibited by an MIC ranging from 0.01–0.05 µL/mL when assayed with the EO of *C. arizonica* [42]. Using the disk diffusion technique, the EO of *C. lusitanica* obtained an MIC of 1.25% (*v*/*v*) and halos of 7.0 mm [6]. Assessing *C. parapsilosis* and *C. orthopsilosis*, the MICs were found to be 62.5 µg/mL and 31.25 µg/mL, respectively [11] (Table 3).

For *C. tropicalis*, *C. arizonica*, and varieties, the MIC ranged from 0.001–0.01 µL/mL [42], for *C. sempervirens* it was 250 µg/mL [11], and for *C. macrocarpa* it was 2000 µg/mL [21]. The EO of *C. lusitanica* inhibited *C. arizonica* at a concentration of 1.25% (*v*/*v*) and presented halos of 14.0 mm, when they used disks containing 10 µL of the EO [6].

For *C. lusitaniae*, the EO of *C. lusitanica* presented an MIC of 0.62 µg/mL and halos of 13.0 mm in disks containing 10 µL of the EO [6]. Other *Candida* species, such as *C. bracarensis* and *C. dubliniensis*, were inhibited by concentrations ranging from 0.01–0.05 µL/mL when the EO of *C. arizonica* and varieties were evaluated [42] (Table 3).

3.4.3. Biological Activity of Essential Oils of *Cupressus* spp.

In vitro studies have reported different activities of *Cupressus* EOs, as reported for the antioxidant and anti-inflammatory activity of *C. lusitanica* [44]. An in vivo study using a murine model (Swiss mice and albino Wistar rats) showed a lethal dose of 6.33 g/kg [6].

The toxicity evaluation of *C. sempervirens* EO using human breast cancer cell lines (MCF-7 and MDA-MB-231) showed a 50% inhibition of cell viability at concentrations of 34.5 µg/mL and 65.2 µg/mL, respectively, for MCF-7 and MDA-MB-231 lineages [8]. In another study, 60% of *C. elegans* larvae infected with *C. glabrata* survived after four days of exposure to *C. sempervirens* EO at a concentration of 62.5 µg/mL [11].

3.5. *Litsea spp.*

About 400 species of *Litsea* have been described around the world; *L. cubeba* is one of the most well-studied, due to its antimicrobial, anti-inflammatory, and immunomodulatory activities [45], but also for its commercial value, with the countries India, Taiwan, Japan, and China being the largest producers and exporters of *L. cubeba* EO worldwide [46]. In general, EOs of *Litsea* have a fresh, sweet, citrus aroma, are insoluble in water, and are widely used in traditional medicine [45,46].

3.5.1. Chemical Composition of *Litsea* Species

The composition of *Litsea* EOs varies, as for all EOs from different plants, according to species of the plant, the part of the plant from which they are extracted, and the region and country of origin. Two species of *Litsea* included in this study had their chemical composition detailed: *L. cubeba* and *L. viridis* (Supplementary Materials).

The composition of the EO of *L. viridis*, extracted from the leaves of the plant collected in Vietnam, includes bicyclogermacrene (25.5%), decanal (14.4%), α-pinene (11.1%), β-pinene (8.3%), and aromadendrene (3%) as the most frequent compounds [47].

In Brazil, limonene (37%), neral (31.4%), and citral (12%) were the most frequent compounds in the EO extracted from the fruits of *L. cubeba* [11]. However, other review studies on the EO of *L. cubeba* extracted from plants cultivated in other countries revealed a diverse chemical composition, with a predominance of 1,8-cineole, sabinene, and α-pinene in the leaves [9], and citral, citronellol, citronellal, geranial, limonene, linalool, neral, α-pinene, and β-pinene in the EO extracted from fruits [46] (Table 3).

3.5.2. Anti-*Candida* Activity of *Litsea* spp. Essential Oil

The in vitro inhibitory effect of *L. viridis* EO showed an MIC of 128 μg/mL for *C. albicans* [46], and that of *L. cubeba* showed an MIC equal to 500 μg/mL for *C. albicans* [11] (Table 3).

Table 3. In vitro antifungal activities of *Cupressus* spp. and *Litsea* spp. essential oils tested against *Candida* species according to different methods.

***Melaleuca* Species**	**Method of Antifungal Susceptibility**	**Species of *Candida* (Number of Strains Tested)**	**Agar Diffusion * or MIC ****	**Reference**
C. lusitanica	Disk diffusion	*C. albicans* (2)	6–13 mm	[6,44]
		C. glabrata (1)	6 mm	[6]
		C. krusei (1)	6–10 mm	[6]
		C. lusitaniae (1)	6–13 mm	[6]
		C. parapsilosis (1)	6–7 mm	[6]
		C. tropicalis (1)	6–14 mm	[6]
	Macrowell dilution	*C. albicans* (1)	0.16% (*v*/*v*)	[6]
		C. glabrata (1)	1.25% (*v*/*v*)	[6]
		C. krusei (1)	1.25% (*v*/*v*)	[6]
		C. lusitaniae (1)	0.62% (*v*/*v*)	[6]
		C. parapsilosis (1)	1.25% (*v*/*v*)	[6]
		C. tropicalis (1)	1.25% (*v*/*v*)	[6]

Table 3. *Cont.*

Melaleuca Species	Method of Antifungal Susceptibility	Species of *Candida* (Number of Strains Tested)	Agar Diffusion * or MIC **	Reference
C. arizonica var. *glabra*	Broth mcrodilution	*C. albicans* (1)	0.05 µL/mL	[42]
		C. dublinenensis (1)	0.01 µL/mL	[42]
		C. glabrata (1)	0.05 µL/mL	[42]
		C. parapsilosis (1)	0.05 µL/mL	[42]
		C. tropicalis (1)	0.001 µL/mL	[42]
C. arizonica var. *arizonica*	Broth mcrodilution	*C. albicans* (1)	0.05 µL/mL	[42]
		C. dublinenensis (1)	0.01 µL/mL	[42]
		C. glabrata (1)	0.01 µL/mL	[42]
		C. parapsilosis (1)	0.01 µL/mL	[42]
		C. tropicalis (1)	0.01 µL/mL	[42]
C. sempervirens	Broth microdilution	*C. albicans* (1)	0.42 ± 0.027 µL/mL	[10]
		C. glabrata (1)	<64 µL/mL	[10]
		C. krusei (1)	<64 µL/mL	[10]
		C. parapsilosis (1)	0.757 ± 0.067 µL/mL	[10]
C. sempervirens	Broth microdilution	*C. albicans* (2)	250–625 µg/mL	[8,11]
		C. glabrata (1)	31.25 µg/mL	[11]
		C. krusei (1)	62.5 µg/mL	[11]
		C. orthopsilosis (1)	31.25 µg/mL	[11]
		C. parapsilosis (1)	62.5 µg/mL	[11]
		C. tropicalis (1)	250 µg/mL	[11]
C. macrocarpa	Broth microdilution	*C. albicans* (2)	1–2 mg/mL	[21]
		C. tropicalis (1)	2 mg/mL	[21]
L. viridis	Broth microdilution	*C. albicans* (1)	128 µg/mL	[47]
L. cubeba	Broth microdilution	*C. albicans* (1)	500 µg/mL	[11]
		C. krusei (1)	62.5 µg/mL	[11]
		C. glabrata (1)	250 µg/mL	[11]
		C. orthopsilosis (1)	250 µg/mL	[11]
		C. parapsilosis (1)	500 µg/mL	[11]
		C. tropicalis (1)	1000 µg/mL	[11]

* Agar diffusion in mm. ** MIC: Minimum inhibitory concentration (expressed as µg/mL or mg/mL or % (*v*/*v*).

The in vitro inhibitory effect of *L. cubeba* EO was evaluated in biofilm formation and performed biofilm eradication for *C. albicans* and non-*albicans Candida* species such as *C. glabrata*, *C. orthopsilosis*, and *C. tropicalis* [11]. Thus, they found that EOs at concentrations of 2000 and 1000 µg/mL were able to, respectively, inhibit biofilm formation and eliminate biofilms for most of the species. For *C. parapsilosis*, both MBIC and MBEC were 1000 µg/mL, whereas the MBIC and MBEC for *C. krusei* were 250 and 1000 µg/mL, respectively [11].

3.5.3. Other Biological Activity of Essential Oils of *Litsea* spp.

The toxicity of *Litsea cubeba* EO was evaluated in an in vivo *C. elegans* model and showed no toxic effects at concentrations up to 125 µg/mL following 24 h of exposure [11].

4. Discussion

This systematic review presented an evaluation of the in vitro anti-*Candida* inhibitory effect of essential oils from *Melaleuca, Citrus*, *Litsea*, and *Cupressus*. Several factors interfere with the chemical composition of the EO, including the origin of the plant, as well as the location and growing conditions, seasonal variation, phenotypic variation, and the part of the plant from which the EO was extracted. This variation is even greater when comparing the EO from different species of the same genus. In addition, the predominance of certain chemical constituents in the EO can determine its greater or lesser effectiveness [33–36,48].

The in vitro determination of antifungal inhibitory effect is performed by different techniques. According to the publications analyzed, there was a predominance of the broth dilution methodology, using techniques whose results were expressed as % (v/v), µg/mL, and µL/mL. In recent years, the use of more sensitive methodologies in the evaluation of potential antimicrobial agents has shown that techniques based on agar diffusion have been replaced by microdilution in broth [49,50]. For this reason, a comparison of the results between studies was one limitation due to the lack of standardization of the methodologies used. The diversity of methodologies compromises an accurate analysis of the results, often allowing evidence of in vitro antimicrobial activity, without analyzing to any extent the reason why EO from different origins and different studies show variable results. Thus, it is not defined which factors can influence the results of in vitro tests and how much, such as the origin and chemical composition of the oil, the particularity of the tested isolates, technical conditions whose tests were performed, and the solvent used to dilute the EO [6,44].

The antifungal activity of EO of *Melaleuca*, mainly *M. alternifolia*, has been extensively studied for *Candida* species, and there seem to be no major differences in responses for EOs of *Melaleuca* for different isolates, regardless of the EO origin [8,11,15,21,23–25,27]. In addition, other species of *Melaleuca* have shown the potential inhibition of *Candida* spp., especially for EOs extracted from leaves and aerial parts. This may expand to the EOs extracted from other parts of the plant, which requires further investigation [20].

On the other hand, *Citrus* presents an extensive variability of EO-producing according to species. This allows the comparison of inhibitory activity against *Candida* spp. and also enables the evaluation of anti-*Candida* activity among EO extracted from different parts of the plant [8,11,21,37].

Cupressus EOs were evaluated by different methodologies and showed antifungal effects against many of the *Candida* species. The techniques employed in the in vitro evaluations of EOs can also, in addition to determining the in vitro susceptibility of fungi to antifungal drug candidates, be considered for the research and development of new strategies of use, such as for evaluating the synergism between different natural products and between them and the already known antifungal drugs [7,38,39,51].

The EOs of the two *Litsea* species have been evaluated. They showed in vitro anti-*Candida* activity, including on biofilm (*L. cubeba*), in a study that tested *C. glabrata* and *C. krusei*, species with limited susceptibility or resistance to fluconazole, one of the azole drugs that is most commonly used for the treatment of *Candida* spp. [11,45].

Thus, considering the great diversity of *Litsea* species, it will be of substantial importance to explore the EOs of the other species, grown in different regions throughout the world, in determining the chemical constitution and performing biological studies, including searches for antifungal activities [44,46].

The increasing discoveries in the field of natural products, and the development and improvement of technologies in the pharmaceutical field, which enable the incorporation of drugs into nanoparticles and nanodispersions, can promote the optimization of the activity profile of several drugs. Effective and safe nanodispersion technologies can circumvent the limitations of hydrophobicity, volatility, and other therapeutic adversities attributed to the loss of physical–chemical stability in formulations containing EOs, mainly applied in formulations aimed at the treatment of superficial candidiasis [4,22,27,30].

In recent years, it has become clear that there is still much to be studied regarding EO. Ethnobotany and ethnopharmacology can contribute considerably to this field. The chemical composition of oils, both qualitative and quantitative, is very variable, and the combination of different molecules, from different classes, in a single oil can result in characteristics that act differently in biological systems. Thus, an understanding of the associated antioxidant, anti-inflammatory, antimicrobial properties, in addition to others involving the field of aromatherapy study, may contribute substantially to the treatment of problems that affect humans, such as infections caused by *Candida* species.

5. Conclusions

Infections caused by *Candida* spp. mainly involve patients with comorbidities. The increasing number of patients in immunocompromised conditions or with bacterial and viral coinfections or other opportunistic fungi has made treatment with conventional antifungal agents a challenge. Therefore, innovative research is being developed to understand which EO molecules have relevant biological activity for application in the treatment of fungal infections. Technologies that enable the incorporation of EOs in pharmaceutical formulations can improve the active release profile. Thus, this is a field with growing potential for future studies. In conclusion, this study showed in vitro evidence for the use of *Melaleuca, Cupressus, Citrus*, and *Litsea* EOs for the treatment of infections caused by different *Candida* species.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/pharmaceutics13101700/s1: Table S1: Anti-*Candida* Activity of *Melaleuca* spp. essential oils and their main components, Table S2: Anti-*Candida* Activity of *Citrus* spp. essential oils and their main components, Table S3: Anti-*Candida* Activity of *Cupressus* spp. essential oils and their main components, Table S4: Anti-*Candida* Activity of *Litsea* spp. essential oils and their main components.

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Supplementary Materials: Essential Oils of *Melaleuca, Citrus*, *Cupressus*, and *Litsea* for the Management of Infections Caused by *Candida* Species: A Systematic Review

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Table S1. Anti-*Candida* Activity of *Melaleuca* spp. essential oils and their main components.

Melaleuca Species	Part of the Plant	Antifungal Activity					Major Components (>1%)	Reference
		Method of Antifungal Susceptibility	Species of *Candida*	Agar Diffusion or MIC *	MFC **	Comparison Drug Results		
M. alternifolia	Leaves	Disk difusion	*C. albicans* (RCMB 05035)	20.3 ± 0.44 mm	-	Amphotericin B (5 µg/mL/disk): 21.9 ± 0.12 mm	Terpinen-4-ol (44.41%), γ-terpinene (21.89%), α-terpinene (6.84%), α-terpineol (6.46%), α-pinene (2.87%), limonene (2.86%), aromadendrene (2.86%), o-cymene (o-cymol) (2.81%), Terpinolene (2.45%), eucalyptol (1,8-cineole) (1.31%).	[1]
M. alternifolia *M. alternifolia*	Leaves	Disk difusion	*C. albicans* (ATCC 90028)	(30 mg/10 µL/ disk): 19.33 ± 0.57 mm	-	Amphotericin B (10 µg/disk): 11 mm	Terpinen-4-ol (40.44%), γ-terpinene (19.54%), α-terpinene (7.69%), 1,8-cineole (5.20%), *þ* -cymene (4.74%), α-terpineol (3.31%), α-terpinolene (3.09%), α-pinene (2.67%).	[2]
			C. albicans (ATCC 2091)	(30 mg/10 µL/ disk): 21 ±1 mm	-	Amphotericin B (10 µg/disk): 14.66 ± 0.57 mm		
			C. albicans	(30 mg/10 µL/ disk): 12–25 mm	-	Amphotericin B (10 µg/disk): 9.66–11.66 mm		
			C. atlantica (CECT 1435)	(30 mg/10 µL/ disk): 21 ± 1 mm	-	Amphotericin B (10 µg/disk): 11 ± 0 mm		
			C. dublinenensis (CECT 11455)	(30 mg/10 µL/ disk): 15 ± 0 mm	-	Amphotericin B (10 µg/disk): 11.33±0.57 mm		
			C. famata (CECT 11957)	(30 mg/10 µL/	-	Amphotericin B		

				disk): 20.66 ± 0.57 mm		(10 µg/disk): 12.33±0.57 mm
			C. glabrata (ATCC 90030)	(30 mg/10 µL/ disk): 14.33 ± 0.57 mm	-	Amphotericin B (10 µg/disk): 14.33 ± 0.57 mm
			C. glabrata	(30 mg/10 µL/ disk): 11.66–12 mm	-	Amphotericin B (10 µg/disk): 10.66 ± 10.33 mm
			C. intermedia (CECT 11869)	(30 mg/10 µL/ disk): 20 ± 0 mm	-	Amphotericin B (10 µg/disk): 12 ± 0 mm
			C. kefyr (CECT 1017)	(30 mg/10 µL/ disk): 19.33 ± 1.15 mm	-	Amphotericin B (10 µg/disk): 10.66 ± 1.15 mm
			C. kefyr	(30 mg/10 µL/ disk): 25.3 ± 0.57 mm	-	Amphotericin B (10 µg/disk): 9.66 ± 0.57 mm
			C lusitaniae (CECT 11455)	(30 mg/10 µL/ disk): 15.33 ± 0.57 mm	-	Amphotericin B (10 µg/disk): 12.33 ± 1.15 mm
			C. maritima (CECT 1435)	(30 mg/10 µL/ disk): 24.66 ± 0.57 mm	-	Amphotericin B (10 µg/disk): 12.66 ± 1.15 mm
			C. parapsilosis	(30 mg/10 µL/ disk): 14.66 ± 0.57 mm	-	Amphotericin B (10 µg/disk): 11 ± 1 mm
			C. sake (CECT 1044)	(30 mg/ 10 µL/disk): 16.33±0.57 mm	-	Amphotericin B (10 µg/disk): 12 ± 1 mm
M. alternifolia	Leaves	Broth microdilution	*C. albicans* (ATCC 90028)	0.312 mg/mL	>10 mg/mL	Amphotericin B (MIC): 0.781 mg/mL
			C. albicans (ATCC 2091)	0.0097 mg/mL	>10 mg/mL	Amphotericin B (MIC): 0.781 mg/mL
			C. albicans	0.0097–5 mg/mL	5 to > 10 mg/mL	Amphotericin B (MIC): 0.04–1.562 mg/mL
			C. atlantica CECT 1435	0.0097 mg/mL	10 mg/mL	Amphotericin B (MIC): 0.0097 mg/mL
			C. dublinensis	0.0195 mg/mL	5 mg/mL	Amphotericin B (MIC):

						0.0097 mg/mL		
			C. famata CECT 11957	0.0097 mg/mL	>10 mg/mL	Amphotericin B (MIC): 0.0097 mg/mL		
			C. glabrata (ATCC 90030)	0.625 mg/mL	10 mg/mL	Amphotericin B (MIC): 1.562 mg/mL		
			C. glabrata	0.0097 – 0.0195 mg/mL	2.5–10 mg/mL	Amphotericin B (MIC): 0.195–0.39 mg/mL		
			C. intermedia CECT 11869	0.0097 mg/mL	> 10 mg/mL	Amphotericin B (MIC): 0.0097 mg/mL		
			C. *kefyr* (CECT 1017)	0.0097 mg/mL	5 mg/mL	Amphotericin B (MIC): 0.39 mg/mL		
			C. kefyr	0.0097 mg/mL	10 mg/mL	Amphotericin B (MIC): 1.562 mg/mL		
			C. lusitaniae	0.0097 mg/mL	>10 mg/mL	Amphotericin B (MIC): 0.0097 mg/mL		
			C. maritima CECT 1435	0.0097 mg/mL	10 mg/mL	Amphotericin B (MIC): 0.0097 mg/mL		
			C. parapsilosis	5 mg/mL	>10 mg/mL	Amphotericin B (MIC): 0.195 mg/mL		
			C. sake CECT 1044	0.0097 mg/mL	10 mg/mL	Amphotericin B (MIC): 0.0097 mg/mL		
M. alternifolia	Leaves	Broth microdilution	*C. albicans* (ATCC 10231)	0.125% (*v*/*v*)	0.25% (*v*/*v*)	Fluconazole (MIC): 256 µg/mL; (MFC): 256 µg/mL	α-Pinene (2.5%), sabinene (0.1%), α-terpinene (8.1%), limonene (1%), þ -cymene (4.4%), 1,8-cineole (2.8%), γ-terpinene (19.6%), terpinolene (3.2%), terpinen-4-ol (41%), α-terpineol (3%), aromadendrene (1.3%).	[3]
			C. albicans	0.19% (*v*/*v*)	0.37% (*v*/*v*)	Fluconazole (MIC): 244 µg/mL; (MFC): 254.48 µg/mL		
M. alternifolia	-	Broth microdilution	*C. albicans*	0.25–2.0% (*v*/*v*)	-	-	Terpinen-4-ol (41.9%), γ-terpinene (17.8%), α-terpinene (8%), þ -cymene (4.6%), 1,8-cineole (4.4%), α-terpineol (3.8%), α-terpinolene (3%), α-pinene (2.4%), limonene (1.8%), ledene (1.2%).	[4]
			C. albicans – Fluconazole Resistant	0.5% (*v*/*v*)	-	-		
			C. albicans – Fluconazole, voriconzol Resistant	0.12–1.0% (*v*/*v*)	-	-		
			C. albicans - Fluconazol, Nystatin and Voriconazol Resistant	1% (*v*/*v*)	-	-		
			C. boidinii - Fluconazole Resistent	0.12% (*v*/*v*)	-	-		

			C. boidinii	0.12–0.25% (*v*/*v*)	-	-		
			C. colliculosa	0.25% (*v*/*v*)	-	-		
			C. famata	0.25–0.5% (*v*/*v*)	-	-		
			C. glabrata - Fluconazole, voriconazol Resistant	0.25% (*v*/*v*)	-	-		
			C. krusei	0.12–0.25% (*v*/*v*)	-	-		
			C. lusitaniae	0.25–1.0% (*v*/*v*)	-	-		
			C. pelliculosa - Nystatin Resistant	0.5% (*v*/*v*)	-	-		
			C. rugosa – Fluconazole Resistant	0.12% (*v*/*v*)	-	-		
			C. tropicalis - Fluconazol, Nystatin and Voriconazol Re-sistent	8% (*v*/*v*)	-	-		
M. alternifolia	-	Broth microdilution	*C. albicans* (ATCC 18804)	625 µg/mL	-	-	Terpinen-4-ol (47.5%), γ -terpinene (20.2%), α-terpinene (8.6%).	[5]
M. alternifolia	Leaves and twigs	Broth microdilution	*C. albicans* (NYCY 1363)	0.2% (*v*/*v*) / 1.8 (g/L)	-	-	-	[6]
			C. albicans (135 BM2/94)	0.2% (*v*/*v*) /1.8 (g/L)	-	-		
M. alternifolia	-	Broth microdilution	*C. albicans* (ATCC 10231)	0.5% (*v*/*v*)	-	-	Terpinen-4-ol (41.9%), γ-terpinene (17.8%), α-terpinene (8%), *p* -cymene (4.6%), 1,8-cineole (4.4%), α-terpineol (3.8%), α-terpinolene (3%), α-pinene (2.4%), limo-nene (1.8%), ledene (1.2%).	[7]
M. alternifolia	-	Broth microdilution	*C. albicans* (ATCC 18804)	0.195% (*v*/*v*) (1.95 mg/mL)	-	-	Terpinen-4-ol (42.8%), γ-terpinene (20.4%), *p* -cymene (9.6%), α-terpinene (7.9%), 1,8-cineole (3%), α-terpineol, (2.8%), α-pinene (2.4%).	[8]
M. alternifolia	-	Broth microdilution	*C. albicans* (ATCC 14053)	2–4% (*v*/*v*)	-	Clotrimazole (MIC): >64 µg/mL Fluconazole (MIC): >64 µg/mL Itraconazole (MIC): >64 µg/mL	α-Terpinen (8.35%), 1,8-cineole (3.39%), α-terpineole (2.7%), α-pinene (2.63 %), aromadendrene (1.65%).	[9]

M. alternifolia	-	Broth microdilution	*C. albicans*	0.50–1% (*v*/*v*)	-	-	-	[10]
M. alternifolia	-	Broth microdilution	*C. albicans* (TIMM 3163)	20 mg/mL (20,000 µg/mL)	-	Fluconazole (MIC): 1 µg/mL	Terpinen-4-ol (37.7%), γ-terpinene (21.25%), α-terpinene (10.5%), terpinolene (3.65%), 1–8 cineole (3.65%), α-terpinenol (2.75%), α-pinene (2.65%), *p* -cymene (2.3%).	[11]
			C. albicans (TIMM 2640) - Fluconazole Resistant	5 mg/mL (5000 µg/mL)	-	Fluconazole (MIC): >64 µg/mL		
M. alternifolia	-	Broth microdilution	*C. albicans* – Fluconazole resistant	0.156–1.25% (*v*/*v*)	0.312–1.25% (*v*/*v*)	-	-	[12]
			C. glabrata – Fluconazole resistant.	0.156–1.25% (*v*/*v*)	0.625–1.25% (*v*/*v*)	-		
			C. krusei– Fluconazole resistant.	0.312–0.625% (*v*/*v*)	0.312–1.25% (*v*/*v*)	-		
M. alternifolia	-	Broth microdilution	*C. glabrata* (ATCC 2950)	0.75–2.5% (*v*/*v*)	-	Amphotericin B (MIC): 0.0018% (*v*/*v*)	Terpinen-4-ol (40–50% (*v*/*v*)); γ-terpinene (15–25% (*v*/*v*)); α-terpinene (5–15% (*v*/*v*)); Limonene + 1,8-cineole (5–10% (*v*/*v*)); α-pinene (0–10% (*v*/*v*)), α-terpinen-4-ol (0–10% (*v*/*v*)); p -cymene (5–<10% (*v*/*v*)); terpinolene (0–<5% (*v*/*v*)).	[13]
M. alternifolia	-	Broth microdilution	*C. glabrata* (ATCC 15126)	-	-	Clotrimazole (MIC): 8 µg/mL Fluconazole (MIC): 8 µg/mL Itraconazole (MIC): 1 µg/mL	-	[14]
M. alternifolia	-	Broth microdilution	*C. glabrata*	4% (*v*/*v*)	-	Clotrimazole (MIC): 0.062–8 µg/mL Fluconazole (MIC): 2–>64 µg/mL Itraconazole (MIC): 0.125–>64 µg/mL		
M. alternifolia	-	Broth microdilution	*C. krusei*	0.5% (*v*/*v*)	0.5% (*v*/*v*)	-	-	[15]
M. alternifolia	-	Broth microdilution	*C. albicans* (SC 5314)	>2000 µg/mL	-	-	-	[16]
			C. glabrata (ATCC 2001)	>2000 µg/mL	-	-		

			C. krusei (ATCC 62580)	2000 μg/mL	-	-		
			C. orthopsilosis (ATCC 96141)	>2000 μg/mL	-	-		
			C. parapsilosis (ATCC 22019)	>2000 μg/mL	-	-		
			C. parapsilosis (ATCC 96141)	>2000 μg/mL	-	-		
			C. tropicalis (ATCC 13803)	>2000 μg/mL	-	-		
M. leucadendra	Young Leaf	Broth microdilution	*C. albicans* (ATCC 10231)	-	-	Nystatin (MIC): 8 μg/mL	α-Eudesmol (21.2%), guaiol (12.5%), bulnesol (5.3%), Linalool (4.9%), *p* -cymene (3.9%), γ-eudesmol (3.9%), (E)-caryophyllene (3.8%), cis-cadin-4-en-7-ol (3.5%), eremoligenol (3.4%), terpinolene (3%), α-humulene (2.8%), β-selinene (2.4%), γ-terpinene (2.2%), α-selinene (2.1%), selina-6-en-4β-ol (2%), selin-11-en-4α-ol (1.9%), caryo-phyllene oxide (1.8%), *p* -cymen-8-ol (1%), δ-selinene (1%), hinesol (1%).	
M. leucadendra	Old Leaf	Broth microdilution	*C. albicans* (ATCC 10231	128 μg/mL	-	Nystatin (MIC): 8 μg/mL	α-eudesmol (17.6%), guaiol (10.9%), (E)-caryophyllene (7%), 1,8-cineole (5.2%), linalool (5.1%), α-humulene (4.4%), β-selinene (3.7%), α-selinene (3.7%), bulnesol (3.6%), eremoligenol (3.4%), cis-cadin-4-en-7-ol (3%), γ-eudesmol (2.8%), caryophyllene oxide (2.3%), α-terpineol (1.8%), *p* -cymene (1.7%), terpinolene (1.6%), selina-6-en-4β-ol (1.6%), δ-selinene (1.6%), selin-11-en-4α-ol (1.5%), γ-terpinene (1.3%), α-amorphene (1.2%), γ-gurjunene (1.1%).	[17]
M. leucadendra	Stem Bark	Broth microdilution	*C. albicans* (ATCC 10231	64 μg/mL	-	Nystatin (MIC): 8 μg/mL	α-eudesmol (24.1%), guaiol (11.3%), (E)-caryophyllene (5.5%), eremoligenol (4.9%), β-selinene (4.8%), α-selinene (3.6%), α-humulene (3.5%), γ-eudesmol (3.5%), bulnesol (3.3%), cis-cadin-4-en-7-ol (3.3%), caryophyllene oxide (3.3%), 1,8-cineole	

							(1.8%), selina-6-en-4β-ol (1.7%), α-amorphene (1.5%), humulene epoxide II (1.5%), linalool (1.4%), limonene (1.4%), *p* -cymene (1.3%), selin-11-en-4α-ol (1.3%), hinesol (1.2%), γ-gurjunene (1.1%),	
M. leucadendra	Fruit	Broth microdilution	*C. albicans* (ATCC 10231	256 μg/mL	-	Nystatin (MIC): 8 μg/mL	α-eudesmol (30.7%), guaiol (10.4%), eremoligenol (6.5%), γ-eudesmol (5.3%), bulnesol (4.4%), (E)-caryophyllene (4.3%), cis-cadin-4-en-7-ol (3.5%), caryophyllene oxide (3.2%), β-selinene (3.1%), α-humulene (2.8%), α-selinene (2.5%), selina-6-en-4β-ol (2.2%), selin-11-en-4α-ol (1.6%), hinesol (1.6%), humulene epoxide II (1.3%).	
M. leucadendra	Branch Tips	Broth microdilution	*C. albicans* (ATCC 10231	-	-	Nystatin (MIC): 8 μg/mL	α-Eudesmol (13.7%), *p* -cymene (8.7%), guaiol (7.3%), (E)-caryophyllene (5.7%), terpinolene (4.4%), β-selinene (4.2%), linalool (4.2%), α-selinene (4.1%), caryophyllene oxide (4%), α-humulene (3.7%), γ-terpinene (3.3%), eremoligenol (2.7%), bulnesol (2.2%), cis-cadin-4-en-7-ol (2.2%), γ-eudesmol (1.9%), humulene epoxide II (1.6%), γ-gurjunene (1.4%), α-pinene (1.4%), δ-selinene (1.3%), α-thujene (1.2%), selina-6-en-4β-ol (1.2%), α-amorphene (1.2%), *p* -cymen-8-ol (1.2%), terpinen-4-ol (1.1%), selin-11-en-4α-ol (1%).	
M. quinquenervia	Leaves	Broth microdilution	*C. albicans* (ATCC 10231)	1 mg/mL (1000 μg/mL)	-	Amphotericin B (MIC): 4 mg/mL (4000 μg/mL) Nystatin (MIC): 2 mg/mL (2000 μg/mL)	1,8-Cineole (40.3%), carveol (27.15%), myrtenol (9.43%), eucarvone (5.7%), camphor (5.66%), nopinone (1.9%), sabinene hydrate (1.7%).	[18]

C. albicans	4 mg/mL (4000 μg/mL)	-	Amphotericin B (MIC): 4 mg/mL (4000 μg/mL) Nystatin (MIC): 2 mg/mL (2000 μg/mL)
C. tropicalis (ATCC 750)	1 mg/mL (1000 μg/mL)	-	Amphotericin B (MIC): 4 mg/mL (4000 μg/mL) Nystatin (MIC): 2 mg/mL (2000 μg/mL)

Note: Diameter of inhibition zones includes diameter of disks (6 mm). * MIC: Minimum inhibitory concentration. ** MFC: Minimum fungicidal concentration. ATCC: American Type Culture Collection.

Table S2. Anti-*Candida* activity of *Citrus* spp. essential oils and their main components.

Citrus Species	Part of the Plant	Antifungal Activity					Major Components (>1%)	Reference
		Method of Antifungal Susceptibility	Species of *Candida*	Agar Diffusion or MIC *	MFC **	Comparison Drug Results		
C. aurantifolia	-	Broth micro-dilution	*C. albicans* (ATCC 18804)	625 µL/mL	-	-	Limonene (51.9%), β-pinene (18.8%), γ-terpinene (8.1%).	[5]
C. aurantium	-	Broth micro-dilution	*C. albicans* (ATCC 18804)	625 µL/mL	-	-	Linalyl acetate (51.5%), linalool (25.4%).	
C. aurantium	Peel	Broth micro-dilution	*C. albicans* (SC 5314)	>2000 µg/mL	-	-	-	[16]
			C. glabrata (ATCC 2001)	>2000 µg/mL	-	-		
			C. krusei (ATCC 62580)	>2000 µg/mL	-	-		
			C. orthopsilosis (ATCC 96141)	>2000 µg/mL	-	-		
			C. parapsilosis (ATCC 22019)	>2000 µg/mL				
			C. tropicalis (ATCC 13803)	>2000 µg/mL	-	-		
C. aurantium	Young Leaf	Disk difusion	*C. albicans* (ATCC 90028)	25.3 ± 0.47 mm	-	Fluconazole (20 µg/disk): 18 ± 0.2 mm Amphotericin B (20 µg/disk): 21 ± 0.5 mm	2-βpinene (100%), δ-3 carene (84%), D-limonene (28%), β -myrcene (18.22%), citronella (11.6%), L-linalool (11.2%), γ -terpinene (9.08%), 3-cyclohexen-1-ol (8.74%), sabinene (5.93 %), citronellyl acetate (5.45 %).	[19]
			C. albicans (MTCC 277)	19 ± 0.02 mm	-	Fluconazole (20 µg/disk): 18 ± 0.2 mm Amphotericin B (20 µg/disk): 21 ± 0.5 mm		

C. aurantium	Young Leaf	Broth microdilution	*C. albicans* (ATCC 90028)	0.15% (*v*/*v*)	0.15% (*v*/*v*)	Fluconazole (MIC): 0.15% (*v*/*v*); (MFC): 0.15% (*v*/*v*) Amphotericin B (MIC): 0.62% (*v*/*v*); (MFC): 0.31% (*v*/*v*)	2-βpinene (100%), δ-3 carene (84%), D-limonene (28%), β -myrcene (18.22%), citronella (11.6%), L-linalool (11.2%), γ -terpinene (9.08), 3-cyclohexen-1-ol (8.74%), sabinene (5.93 %), citronellyl acetate (5.45 %).	[19]
			C. albicans (MTCC 277)	0.31% (*v*/*v*)	0.31% (*v*/*v*)	Fluconazole (MIC): 0.62% (*v*/*v*); (MFC): 0.62% (*v*/*v*) Amphotericin B (MIC): 0.62% (*v*/*v*); (MFC): 0.31% (*v*/*v*)		
C. bergamia	-	Broth microdilution	*C. albicans* (ATCC 18804)	625 µL/mL	-	-	Limonene (34.6%), linalyl acetate (34.3%), linalool (12.7%), γ -terpinene (6.6%), β-pinene (5.6%).	[5]
C. grandis	Leaves	Broth microdilution	*C. albicans* (ATCC 10231)	4 mg/ mL (4000 µg/mL)	-	Amphotericin B (20 µg/disk): 12.05 mm Nystatin (20 µg/disk): 12.05 mm	Borneol (42.24%), linalool (26.01%), linalyl acetate (19.89%), terpinen-4-ol (5.24%), fenchyl acetate (4.69%).	[18]
C. grandis	Peel	Broth microdilution	*C. albicans* (ATCC 10231)	4 mg/ mL (4000 µg/mL)	-	Amphotericin B: (MIC): 4 mg/mL (4000 µg/mL) Nystatin (MIC): 2 mg/mL (2000 µg/mL)	Limonene (75.43%), β -myrcene (3.52%), cycloisosativene (2.73%), terpinen-4-ol (2.35%), germacrene (2.09%), α -terpinene (2.05%), α -pinene (1.59%), β -pinene (1.57%).	[18]
			C. albicans	-	-	Amphotericin B (MIC): 4 mg/mL (4000 µg/mL) Nystatin (MIC): 2 mg/mL (2000 µg/mL)		

C. grandis	Peel	Broth microdilution	*C. tropicalis* (ATCC 750)	4 mg/ mL (4000 μg/mL)	-	Amphotericin B: (MIC): 4 mg/mL (4000 μg/mL) Nystatin (MIC): 2 mg/mL (2000 μg/mL)	Limonene (75.43%), β -myrcene (3.52%), cycloisosativene (2.73%), terpinen-4-ol (2.35%), germacrene (2.09%), α -terpinene (2.05%), α -pinene (1.59%), β -pinene (1.57%).	[18]
C. grandis	Leaves	Broth microdilution	*C. albicans* (ATCC 10231)	0.116 ± 0.004% (*v*/*v*)	-	-	Steam distillation (SD-EO): Citronellal (34.54%), citronellol (16.17%), citronellyl butyrate (10%), β-caryophyllene (8.22%), (−)-β-pinene (7.33%), trans-β-ocimene (6.8%), 3,7,11-tetramethyl bicyclo [8.1.0]2,6-undecadiene (3.43%), phytol (1.65%) e α-farnesene (1.57%), Sabinene (1.43%), dipentene (1.08%).	[20]
			C. albicans (ATCC 10231)	0.121 ± 0.007% (*v*/*v*)	-	-	Solvent-Free Microwave Extraction (SFME-EO): Citronellal (30.87%), citronellol (28.95%), (−)-β-pinene (9.58%), α-ocimene (6.38%), citronellyl butyrate (5.7%), Sabinene (3.01%), β-caryophyllene (2.76%), caryophyllene oxide (2.21%), dipentene (1.93%). spathulenol (1.02%).	[20]
C. hystix	Riped Fruits	Broth microdilution	*C. albicans* (ATCC 10231)	1 mg/mL (1000 μg/mL)	-	Amphotericin B: (MIC): 4 mg/mL (4000 μg/mL) Nystatin (MIC): 2 mg/mL (2000 μg/mL)	Limonene (83.89%), α-Pinene (3.02%).	[18]

			C. albicans	4 mg/mL (4000 μg/mL)	-	Amphotericin B: (MIC): 4 mg/mL (4000 μg/mL) Nystatin (MIC): 2 mg/mL (2000 μg/mL)		
			C. tropicalis (ATCC 750)	2 mg/mL (2000 μg/mL)	-	Amphotericin B: (MIC): 4 mg/mL (4000 μg/mL) Nystatin (MIC): 2 mg/mL (2000 μg/mL)		
C. latifolia	Peel	Agar difusion	*C. albicans*	0.4 μg/50 mL: 9.46 mm.	-	Amphotericin B (20 μg/disk): 12.05 mm	*d*-limonene (51.64%), β -thujene (14.85%), γ -terpinene (12.80%), β -pinene (12.79%), α -pinene (2.17%), α -myrcene (1.43%).	[21]
			C. glabrata	0.22 μg/50 mL: 8.52 mm.	-	Amphotericin B (20 μg/disk): 12.05 mm		
			C. guilliermondii	0.19 μg/50 mL: 8.94 mm.	-	Amphotericin B (20 μg/disk): 12.05 mm		
C. latifolia	Peel	Agar difusion	*C. lusitaniae*	1.09 μg/50 mL: 8.06 mm.	-	Amphotericin B (20 μg/disk): 12.05 mm	*d*-limonene (51.64%), β -thujene (14.85%), γ -terpinene (12.80%), β -pinene (12.79%), α -pinene (2.17%), α -myrcene (1.43%).	[21]
			C. tropicalis	1.3 μg/50 mL: 10.87 mm.	-	Amphotericin B (20 μg/disk): 12.05 mm		
C. limon	-	Broth micro-dilution	*C. albicans* (ATCC 18804)	625 μL/mL	-	-	Limonene (56.1%), β-pinene (15.8%), γ -terpinene (10.5%).	[5]
C. limon	Leaves	Broth micro-	*C. albicans* (SC 5314)	500 μg/mL	-	-	-	[16]

		dilution	*C. glabrata* (ATCC 2001)	250 µg/mL	-	-		
			C. krusei (ATCC 62580)	500 µg/mL	-	-		
			C. orthopsilosis (ATCC 96141)	500 µg/mL	-	-		
			C. parapsilosis (ATCC 22019)	500 µg/mL	-	-		
			C. tropicalis (ATCC 13803)	250 µg/mL				
C. limonum	-	Broth micro-dilution	*C. albicans* - Fluconazole Resistent	0.0097–0.312% (*v*/*v*)	0.0097–0.312% (*v*/*v*)	-	-	[12]
			C. glabrata- Fluconazole Resistent	0.0024–0.1565 (*v*/*v*)	0.0024–0.156% (*v*/*v*)	-		
			C. krusei- Fluconazole Resistent	0.0024–0.0097% (*v*/*v*)	0.0024–0.019% (v/v)	-		
C. limonum		Broth microdilution	*C. albicans*	<0.043 to >21.325 mg/mL	<0.043–>21.325 mg/mL	0.06 mg/mL	Citral (53.85%), others (27.18%), limonene (5.29%), terpinolene (4.57%), terpinene (4.49%).	[22]
			C. glabrata	<0.044 to 5.331 mg/mL	<0.043–5.331 mg/mL	-		
C. limonum	-	Cylinder-plate diffusion	*C. albicans*	(0.6–3.0%: 0 mm)	-	-	Limonene (48.27%), β-pinene (15.14%), α-pinene (11.06%), trans-citral (7.14%), c-terpinene (4.85%), cis-citral (4.3%), β-myrcene (4.11%), terpinolene (1.19%), geranyl acetate (0.91%).	[23]
			C. glabrata	(2.6%: 45 mm) (3.0%: 44.8 mm)				
			C. tropicalis	(0.6–3.0%: 0 mm)	-	-		
C. limonum	-	Cylinder-plate diffusion	*C. albicans*	(0.6%: 44.8 mm) (1.0%: 44.8 mm) (1.6%: 44.8 mm) (2.0%: 44.8 mm) (2.6%: 45.0 mm) (3.0%: 45.0 mm)	-	-	Limonene (23.39%), cis-citral (19.41%), trans-citral (15.52%), β-pinene (8.93%), α-caryophyllene (6.09%), c-terpinene (5.87%), cis-geraniol (4.96%), farnesol (4.48%), trans-geraniol (3.39%), β-myrcene (1.46%), α-pinene (1.44%).	

			C. glabrata	(0.6–3.0%: 0 mm)				
			C. tropicalis	(2.6%: 15.3 mm) (3.0%: 16.3 mm)	-	-		
C. limonum	-	Cylinder-plate diffusion	*C. albicans*	(0.6%: 23 mm) (1.0%: 25 mm) (1.6%: 44.8 mm) (2.0%: 44.8 mm) (2.6%: 44.8 mm) (3.0%: 45.0 mm)	-	-	Limonene (42.03%), β-pinene (15.15%), carvone (7.28%), 1-terpinen-4-ol (5.57%), pinocarveol (5.44%), verbenol (4.9%), α-pinene (3.42%), trans-carveol (3.36%), caryophyllene oxide (3.2%), α-bergamotene (2.31%), trans-p-2,8- (1.84%), trans-geraniol (1.58%), trans-verbenol (1.5%).	
			C. glabrata	(2.6%: 44.6 mm) (3.0%: 44.8 mm)				
			C. tropicalis	(0.6–3.0%: 0 mm)	-	-		
C. limonum	-	Cylinder-plate diffusion	*C. albicans*	(3.0%: 17.4 mm)	-	-	Isopropyl myristate (42.78%), limonene (22.42%), bergamol (9.53%), β-ionene (7.81%), hydrocinnamic acid (6.54%), farnesol (6.12%), β-pinene (2.21%).	
			C. glabrata	(0.6–3.0%: 0 mm)				
			C. tropicalis	(0.6–3.0%: 0 mm)	-	-		
C. limonum	-	Cylinder-plate diffusion	*C. albicans*	(0.6–3.0%: 0 mm)	-	-	Limonene (63.2%), β-pinene (14.31%), β-myrcene (6.94%), c-terpinene (4.78%), α-pinene (3.61%), linalool (1.73%), citral (mix of isomers) (1.06%).	
			C. glabrata	(0.6–3.0%: 0 mm)				
			C. tropicalis	(0.6–3.0%: 0 mm)	-	-		
C. limonum	-	Cylinder-plate diffusion	*C. albicans*	(0.6–3.0%: 0 mm)	-	-	Limonene (38.5%), β-pinene (19.98%), β-felandrene (15.03%), α-pinene (5.56%), β-myrcene (5.42%), citral (mix of isomers) (2.63%), β-bisabolene (1.82%), cis-citral (1.54%), β-bergamotene (1.54%), Linalool (1.51%), 3-thujene (1.25%), neryl acetate (1.14%), caryophyllene (1.11%).	[23]
			C. glabrata	(0.6–3.0%: 0 mm)	-	-		
			C. tropicalis	(0.6–3.0%: 0 mm)				

C. nobilis	Peel	Broth microdilution	*C. albicans* (SC 5314)	2000 µg/mL	-	-		[16]
			C. glabrata (ATCC 2001)	2000 µg/mL	-	-		
			C. krusei (ATCC 62580)	>2000 µg/mL	-	-		
			C. orthopsilosis (ATCC 96141)	>2000 µg/mL	-	-		
			C. parapsilosis (ATCC 22019)	>2000 µg/mL				
			C. tropicalis (ATCC 13803)	>2000 µg/mL	-	-		
C. paradisi	-	Broth microdilution	*C. albicans* (ATCC 18804)	313 µL/mL	-	-	Limonene (91.3%).	[5]
C. paradisi	-	Broth microdilution	*C. albicans*	0.0039–1% (*v*/*v*)	-	Clotrimazole (MIC): >64 µg/mL Fluconazole (MIC): >64 µg/mL Itraconazole (MIC): >64 µg/mL	α-Copaene (0.1 %), α-pinen (0.55 %), β-caryophyllene (0.39 %), β-cubenene (0.09 %).	[9]
C. paradisi	-	Broth microdilution	*C. glabrata* (ATCC 15126)	0.007–1% (*v*/*v*)	-	Clotrimazole (MIC): 8 µg/mL Fluconazole (MIC): 8 µg/mL) Itraconazole (MIC): 1 µg/mL	-	[14]
C. paradisi	-	Broth microdilution	*C. glabrata*	-	-	Clotrimazole (MIC): 0.062 - 8 µg/mL Fluconazole (MIC): 2 - >64 µg/mL Itraconazole (MIC): 0.125 - >64 µg/mL	-	[14]

C. reticulata	-	Broth microdilution	*C. albicans* (ATCC 18804)	625 µL/mL	-	-	Limonene (91.3%).	[5]
C. reticulata	Peel	Broth microdilution	*C. albicans* (SC 5314)	2000 µg/mL	-	-	-	[16]
			C. glabrata (ATCC 2001)	1000 µg/mL	-	-		
			C. krusei (ATCC 62580)	250 µg/mL				
			C. orthopsilosis (ATCC 96141)	250 µg/mL				
			C. parapsilosis (ATCC 22019)	1000 µg/mL	-	-		
			C. tropicalis (ATCC 13803)	1000 µg/mL	-	-		
C. reticulata var. *Blanco*	Dry Leaves	Broth microdilution	*C. albicans* (SC 5314)	>2000 µg/mL	-	-	-	[16]
			C. glabrata (ATCC 2001)	2000 µg/mL	-	-		
			C. krusei (ATCC 62580)	>2000 µg/mL	-	-		
			C. orthopsilosis (ATCC 96141)	2000 µg/mL	-	-		
			C. parapsilosis (ATCC 22019)	2000 µg/mL	-	-		
			C. tropicalis (ATCC 13803)	>2000 µg/mL	-	-		
C. reticulata var. *Blanco*	Peels	Broth microdilution	*C. albicans* (SC 5314)	1000 µg/mL	-	-	-	[16]
			C. glabrata (ATCC 2001)	1000 µg/mL	-	-		
			C. krusei (ATCC 62580)	500 µg/mL	-	-		
			C. orthopsilosis (ATCC 96141)	1000 µg/mL	-	-		
			C. parapsilosis (ATCC 22019)	1000 µg/mL	-	-		

			C. tropicalis (ATCC 13803)	2000 µg/mL				
C. reticulata Blanco var *cravo*	Peels	Broth microdilution	*C. albicans* (SC 5314)	>2000 µg/mL	-	-	-	[16]
			C. glabrata (ATCC 2001)	2000 µg/mL	-	-		
			C. krusei (ATCC 62580)	>2000 µg/mL	-	-		
			C. orthopsilosis (ATCC 96141)	2000 µg/mL	-	-		
			C. parapsilosis (ATCC 22019)	>2000 µg/mL				
			C. tropicalis (ATCC 13803)	>2000 µg/mL	-	-		
C. reticulata Blanco var *cravo*	Fresh leaves	Broth microdilution	*C. albicans* (SC 5314)	2000 µg/mL	-	-	-	[16]
			C. glabrata (ATCC 2001)	>2000 µg/mL	-	-		
			C. krusei (ATCC 62580)	>2000 µg/mL	-	-		
			C. orthopsilosis (ATCC 96141)	>2000 µg/mL	-	-		
			C. parapsilosis (ATCC 22019)	>2000 µg/mL	-	-		
			C. tropicalis (ATCC 13803)	2000 µg/mL				
C. reticulata var *Blanco*	Riped Fruits	Broth microdilution	*C. albicans* (ATCC 10231)	0.3 mg/mL (300 µg/mL)	-	Amphotericin B (MIC): 4 mg/mL (4000 µg/mL) Nystatin (MIC): 2 mg/mL (2000 µg/mL)	Limonene (37.55%), β -pinene (13.24%), γ -terpinene (9.11%), β -myrcene (6.85%), terpinen-4-ol (6.32%), cymene (5.21%), α -pinene (4.7%), α -3-carene (3.11%), linoleic acid ethyl ester (2.44%), α -terpineol (1.05%).	[16]
			C. albicans	4 mg/mL (4000 µg/mL)	-	Amphotericin B (MIC): 4 mg/mL (4000 µg/mL) Nystatin (MIC): 2 mg/mL (2000 µg/mL)		
			C. tropicalis (ATCC 750)	2 mg/mL (2000 µg/mL)	-	Amphotericin B (MIC): 4 mg/mL (4000 µg/mL)		

						Nystatin (MIC): 2 mg/mL (2000 μg/mL)		
C. sinensis	Peel	Agar diffusion	*C. albicans*	1.68 μg/50 mL: 5.51 mm.	-	Amphotericin B (20 μg/disk): 12.05 mm	*d*-limonene (96.046%), *α* -myrcene (2.796%).	[21]
			C. glabrata	0.42 μg/50 mL: 5.78 mm.	-	Amphotericin B (20 μg/disk): 12.05 mm		
			C. guilliermondii	-	-	Amphotericin B (20 μg/disk): 12.05 mm		
			C. lusitaniae	3.71 μg/50 mL: 2.00 mm	-	Amphotericin B (20 μg/disk): 12.05 mm		
			C. tropicalis	0.72 μg/50 mL: 4.44 mm.	-	Amphotericin B (20 μg/disk): 12.05 mm		
C. sinensis	Peel	Broth microdilution	*C. albicans* (SC 5314)	>2000 μg/mL	-	-	-	[16]
			C. glabrata (ATCC 2001)	>2000 μg/mL	-	-		
			C. krusei (ATCC 62580)	>2000 μg/mL	-	-		
			C. orthopsilosis (ATCC 96141)	>2000 μg/mL	-	-		
			C. parapsilosis (ATCC 22019)	>2000 μg/mL	-	-		
			C. tropicalis (ATCC 13803)	>2000 μg/mL				

Note: Diameter of inhibition zones includes diameter of disks (6 mm). * MIC: Minimum inhibitory concentration. ** MFC: Minimum fungicidal concentration. ATCC: American Type Culture Collection.

Table S3. Anti-*Candida* activity *Cupressus* spp. essential oils and their main components.

Cupressus Species	Part of the Plant	Antifungal Activity: Method of Antifungal Susceptibility	Species of *Candida*	Agar diffusion or MIC *	MFC **	Comparison Drug Results	Major Components (>1%)	Reference
C. lusitanica	Aerial parts	Agar disk diffusion	*C. albicans* (ATCC 1098) Collection date: 17 January	7.5 ± 0.0 mm	-	Amphotericin B (10 μg/mL/disk): 13–15 ± 0.0 mm	Limonene (19.5%), sabinene (17.7%), δ-3-carene (16.8%), α-pinene (13.8%), β-myrcene (2.9%), terpinen-4-ol (2.3%), cadina-3,5-diene (2.3%), γ-terpinene (2%), terpinolene (2%), β-phellandrene (1.9%), α-terpinene (1.3%), trans-pinocarveol (1.3%), cis-thujopsene (1.1%), camphene (1%).	[24]
			C. albicans (ATCC 1098) Collection date: 17 February	8.5 ± 0.0 mm			α-Pinene (35.7%), sabinene (13.4%), limonene (7.7%), δ-3-carene (7.6%), β-phellandrene (4.6%), β-myrcene (4.4%), umbellulone (3.2%), terpinen-4-ol (2.9%), terpinolene (2.5%), γ-terpinene (2.3%), α-terpinene (2%), α-thujene (1.6%), cadina-3,5-diene (1.4%).	
C. lusitanica	Leaves -non-flowering	Agar disk diffusion	*C. albicans* (ATCC 9002)	100%, 10%, 1% (*v/v*): respectively, 13 mm, 7 mm, 6 mm	-	Nystatin (10 μg/disk): 22 mm	Germacrene D (18.5%), epi-zonarene (8.2%), cis-calamenene (8.2%), terpinen-4-ol (6.3%), linalool (6%), umbellulone (6%), di-epi-α-cedrene (4.9%), α-curcumene (4.1%), limonene (2.3%), α-amorphene (2%), β-caryophyllene (1.5%), linalyl acétate (1.2%), cedrol (1.2%), α-muurolene (1.1).	[25]
			C. glabrata (IP 35)	100%, 10%, 1% (*v/v*): respectively, 6 mm, 6 mm, 6 mm	-	Nystatin (10 μg/disk): 23 mm		
			C. krusei (ATCC 6258)	100%, 10%, 1% (*v/v*): respectively, 10 mm, 6 mm, 6 mm	-	Nystatin (10 μg/disk): 18 mm		

			C. lusitaniae (ATCC 200950)	100%, 10%, 1% (*v*/*v*): respectively, 13 mm, 10 mm, 6 mm	-	Nystatin (10 µg/disk): 33 mm		
			C. parapsilosis (ATCC 22019)	100%, 10%, 1% (*v*/*v*): respectively, 7 mm, 6mm, 6 mm	-	Nystatin (10 µg/disk): 24 mm		
			C. tropicalis (ATCC 750)	100%, 10%, 1% (*v*/*v*): respectively, 14 mm, 7 mm, 6 mm	-	Nystatin (10 µg/disk): 23 mm		
C. lusitanica	Leaves non-flowering	Macrowell dilution	*C. albicans* (ATCC 9002)	0.16% (*v*/*v*)	0.16% (*v*/*v*)	Nystatin (MIC): 2 µg/mL; (MFC): 2 µg/mL	Germacrene D (18.5%), epi-zonarene (8.2%), cis-calamenene (8.2%), terpinen-4-ol (6.3%), linalool (6%), umbellulone (6%), di-epi-α-cedrene (4.9%), α-curcumene (4.1%), limonene (2.3%), α-amorphene (2%), β-caryophyllene (1.5%), linalyl acétate (1.2%), cedrol (1.2%), α-muurolene (1.1%).	
			C. glabrata (IP 35)	1.25% (*v*/*v*)	1.25% (*v*/*v*)	Nystatin (MIC): 16 µg/mL; (MFC); 16 µg/mL		
			C. krusei (ATCC 6258)	1.25% (*v*/*v*)	1.25% (*v*/*v*)	Nystatin (MIC): 4 µg/mL; (MFC): 4 µg/mL		
			C. lusitaniae (ATCC 200950)	0.62% (*v*/*v*)	0.62% (*v*/*v*)	Nystatin (MIC): 2 µg/mL; (MFC): >2 µg/mL		
			C. parapsilosis (ATCC 22019)	1.25% (*v*/*v*v)	1.25% (*v*/*v*)	Nystatin (MIC): 16µg/mL; (MFC): 16µg/mL		
			C. tropicalis (ATCC 750)	1.25% (*v*/*v*)	1.25% (*v*/*v*)	Nystatin (MIC): 8 µg/mL; (MFC): 8 µg/mL		
C. arizonica var. *glabra*	Aerial parts	Broth mcrodilution	*C. albicans* (ATCC 18804)	5×10^{-2} (0.05 µL/mL))	-	-	α-Pinene (26.53%), umbellulone (15.05%), β -cubebene (6.71%), cal-	[26]

			C. dublinenensis (CIPO 82)	1×10^{-2} (0.01 µL/mL)	-	-	manene (4.5%), limonene (4.12%), terpinen-4-ol (4.08%), 14-norcadin-5-en-4-one (2.78%), 6-terpinene (2.06%), β -sesquiphellandrene (2.01%), camphor (1.83%), sabinene (1.7%), aromadendrene (1.65%), β -fenchol (1.48%), þ-cymene (1.47%), cedrol (1.36%), ionole (1.26%), 6-3-carene (1.02%).	
			C. glabrata (8D)	5×10^{-2} (0.05 µL/mL)	-	-		
			C. parapsilosis (28 B)	5×10^{-2} (0.05 µL/mL))	-	-		
			C. tropicalis (IGC 3097)	1×10^{-3} (0.001 µL/mL)	-	-		
C. arizonica var. *arizonica*	Aerial parts	Broth mcrodilution	*C. albicans* (ATCC 18804)	5×10^{-2} (0.05 µL/mL))	-	-	α-Pinene (29.76%), umbellulone (11.86%), terpinen-4-ol (5.72%), α-cedrene (4.12%), limonene (4.09%), camphene hydrate (3.82%), β -sesquiphellandrene (3.11%), 6-Terpinene (2.86%), camphor (2.68%), sabinene (2.51%), 6-3-Carene (1.72%), þ -cymene (1.56%), Labd-(13E)-8,15-diol (1.55%), β -fenchol (1.38%), α-terpinene (1.07%), delta-cadinene (1.04%), α-terpinolene (1.03%), cedrol (1.01%).	
			C. dublinenensis (CIPO 82)	1×10^{-2} (0.01 µL/mL)	-	-		
			C. glabrata (8D)	1×10^{-2} (0.01 µL/mL)	-	-		
			C. parapsilosis (28 B)	1×10^{-2} (0.01 µL/mL)	-	-		
			C. tropicalis (IGC 3097)	1×10^{-2} (0.01 µL/mL)	-	-		
C. semper-virens	Aerial parts - flowering period	Broth microdilution	*C. albicans* (ATCC 10231)	0.42 ± 0.027 µL/mL	1.319 ± 0.066 µL/mL	Nystatin (MIC_{90}): 0.84 ± 0.062; (MFC): 1 ± 0.062	(+)-Cedrol (74.03%), α-pinene (4.6%), thymol (4.25%), δ-3-carene (3.8%), (-)-caryophyllene oxide (3.31%), (-)-borneol (2.33%), α-cadinol (2.19%).	[27]
			C. glabrata	<64 µL/mL	-	Nystatin (MIC_{90}): 0.84 ± 0.032; (MFC): 1 ± 0.045		
			C. krusei	<64 µL/mL	-	Nystatin (MIC_{90}): 1.148 ± 0.065; (MFC): 2 ± 0.034		

			C. parapsilosis	0.757 ± 0.067 µL/mL	1.64 ± 0.058 µL/mL	Nystatin (MIC_{90}/MFC): 0.84 ± 0.076 /1 ± 0.041		
C. semper-vires	Leaves	Broth microdilution	*C. albicans* (SC 5314)	250 µg/mL	-	-	Sabinene (20.3%), citral (20%), terpinen-4-ol (15.4%), α-pinene (8%), myrcene (6%), þ -cymene (5%), neral (5%), δ-2-carene (4%), γ-terpinene (4%), limonene (3.9%), δ-cadinene (3%), α-terpineol (2.4%), cedrol (2.1%).	[16]
			C. glabrata (ATCC 2001)	31.25 µg/mL	-	-		
			C. krusei (ATCC 62580)	62.5 µg/mL	-	-		
			C. orthopsilosis (ATCC 93141)	31.25 µg/mL	-	-		
			C. parapsilosis (ATCC 22019)	62.5 µg/mL	-	-		
			C. tropicalis (ATCC 13803)	250 µg/mL	-	-		
C. semper-virens	-	Broth microdilution	*C. albicans* (ATCC 18804)	625 µg/mL	-	-	α-Pinene (49.7%), δ-3-carene (27.0%).	[5]
C. macro-carpa	Leaves	Broth microdilution	*C. albicans* (ATCC 10231)	1 mg/mL (1000 µg/mL)	40% of all MFCs were no longer than a higher dilution.	Amphotericin B (MIC): 4 mg/mL (4000 µg/mL) Nystatin (MIC): 1,5 mg/mL (1500 µg/mL)	α -Pinene (63.2%), cedrol (7.21%), β -myrcene (4.2%), β -pinene (4.1%), γ -terpinene (3.1%), germacrene (1.9%), sabinene (1.56%).	[18]
			C. albicans	2 mg/mL (2000 µg/mL)		Nystatin (MIC): 4 mg/mL (4000 µg/mL)		
			C. tropicalis (ATCC 750)	2 mg/mL (2000 µg/mL)		Amphotericin B (MIC): 4 mg/mL (4000 µg/mL) Nystatin (MIC): 2 mg/mL (2000 µg/mL)		

Note: Diameter of inhibition zones includes diameter of disks (6 mm). * MIC: Minimum inhibitory concentration. ** MFC: Minimum fungicidal concentration. ATCC: American Type Culture Collection .

Table S4. Anti-*Candida* activity of *Litsea* spp. essential oils and their main components.

Litsea Species	Part of the Plant	Antifungal Activity					Major Components (>1%)	Reference
		Method of Antifungal Susceptibility	Species of *Candida*	MIC *	MFC **	Comparison Drug Results		
L. viridis	Leaf	Broth microdilution	*C. albicans* (ATCC 10231)	128 μg/mL	-	Nystatin (MIC): 8 μg/mL	Bicyclogermacrene (25.5%), decanal (14.4%), α-pinene (11.1%), β-pinene (8.3%), aromadendrene (3%), dodecanal (2%), β-elemene (1.9%), limonene (1.8%), methyl (E)-cinnamate (1.5%), germacrene B (1.3%), β-selinene (1.2%), (E)-nerolidol (1.1%), γ-elemene (1%), germacrene D (1%), (E)-γ-bisabolene (1%).	[28]
L. cubeba	Fruits	Broth microdilution	*C. albicans* (SC 5314)	500 μg/mL	-	-	Limonene (37%), neral (31.4%), citral (12%), linalool (4%), α-terpineol (2.3%), α-pinene (2%), β-pinene (2%), β-caryophyllene (1.7%), sabinene (1.3%), myrcene (1.3%), geraniol (1.2%).	[16]
			C. tropicalis (ATCC 13803)	1000 μg/mL	-	-		
			C. krusei (ATCC 62580)	62.5 μg/mL	-	Amphotericin B (MIC): 1.00 μg/mL		
			C. glabrata (ATCC 2001)	250 μg/mL	-	-		
			C. parapsilosis (ATCC 22019)	500 μg/mL	-	Amphotericin B (MIC): 0.25 μg/mL		
			C. parapsilosis (ATCC 96141)	250 μg/mL	-	-		

Note: * MIC: Minimum inhibitory concentration. ** MFC: Minimum fungicidal concentration.

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CAPÍTULO 1

O ÓLEO ESSENCIAL DE *Citrus limon* COMO ALTERNATIVA PARA O TRATAMENTO DE CANDIDÍASE

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RESUMO: O uso crescente de produtos naturais para fins terapêuticos tem sido uma alternativa segura e de baixo custo para as formulações sintéticas no tratamento de infecções. As espécies de fungos do gênero *Candida* são agentes oportunistas de infecções em humanos, causando infecções superficiais e disseminadas. As plantas e seus derivados apresentam uma diversidade de aplicações, cujos metabólitos podem ser utilizados para fins terapêuticos, como os óleos essenciais. O óleo essencial de *Citrus limon* têm mostrado eficácia na inibição antifúngica e na redução da formação de biofilme *in vitro* de espécies de *Candida*. Este estudo de revisão relata as informações atualizadas sobre ação de *C. limon* sobre espécies de *Candida*. A capacidade *in vitro* do óleo essencial de *C. limon* em inibir o crescimento de *Candida* spp. demonstra o potencial para o desenvolvimento de formulações farmacêuticas para o tratamento de candidíases, especialmente aquelas superficiais e mucocutâneas, como candidíases vulvovaginal e bucal.

PALAVRAS-CHAVE: Óleos voláteis. *Candida*. Testes de sensibilidade antimicrobiana. Substâncias bioativas.

THE ESSENTIAL OIL OF *Citrus limon* AS AN ALTERNATIVE FOR THE TREATMENT OF CANDIDIASIS

ABSTRACT: The increasing use of natural products for therapeutic purposes has been a safe and cost-effective alternative to synthetic formulations in the treatment of infections. Fungal species of the genus Candida are opportunistic agents of infections in humans, causing superficial and disseminated infections. Plants and their derivatives have a diversity of applications, whose metabolites can be used for therapeutic purposes, such as essential oils. Citrus limon essential oil has shown efficacy in antifungal inhibition and reduction of in vitro biofilm formation of Candida species. This review study reports the updated information on action of C. limon on Candida species. The in vitro ability of C. limon essential oil to inhibit the growth of Candida spp. demonstrates the potential for the development of pharmaceutical formulations for the treatment of candidiasis, especially those that are superficial and mucocutaneous, such as

vulvovaginal and oral candidiasis.
KEYWORDS: Volatile oils. *Candida*. Antimicrobial susceptibility testing. Bioactive substances.

1 I INTRODUÇÃO

A microbiota humana é constituída por diversos microrganismos comensais que, por alterações estruturais ou de defesa natural do hospedeiro, se tornam patogênicos. Espécies de fungos do gênero *Candida* são encontradas na forma de leveduras em diversos sítios anatômicos, habitando pele e mucosas, uretra, trato gastrointestinal e microbiota vaginal (ANTINORI *et al,* 2016).

Espécies de *Candida* têm se destacado nas últimas duas décadas pelo crescente registro como agentes causadores de infecções em humanos (MATTA; SOUZA; COLOMBO, 2017). Dentre as cerca de 200 espécies que compõem o gênero, *C. albicans* é a principal relacionada a doenças (DIGNANI, SOLOMKIN, ANAISSIE, 2003). As demais espécies, comumente denominadas *Candida* não-*Candida albicans* (NCA), são reconhecidas como outros agentes causadores de candidíase, tanto superficiais como invasivas, sistêmicas e oportunistas (MATTA; SOUZA; COLOMBO, 2017). Estudo realizado no Brasil aponta que NCA representam 65,7% das leveduras isoladas de infecções, sendo que as principais espécies relatadas são *C. parapsilosis* (24,1%), *C. tropicalis* (15,3%), *C. glabrata* (10,2%), *C. krusei* (1,5%), *C. lusitaniae* (0.7%), *C. famata* (0.7%) e *C. guilliermondii* (0.7%) (DOI *et al.,* 2016).

Além do favorecimento oportuno obtido pelo imunocomprometimento do hospedeiro, espécies de *Candida* apresentam mecanismos ativos de agressão direcionados à adesão tecidual e à invasão, como produção de enzimas hidrolíticas (proteinases, fosfolipases, hemolisinas) (SILVA *et al.,* 2017). As manifestações clínicas do hospedeiro dependem do sítio acometido, condições imunes do hospedeiro e virulência do agente, variando em infecções envolvendo pele e anexos, mucosa oral e vaginal, e em quadros mais graves, caracterizados por infecção sistêmica invasiva (DIGNANI, SOLOMKIN, ANAISSIE, 2003).

As infecções causadas por espécies de *Candida*, na grande maioria das vezes, são infecções oportunistas, o que implica dizer que para a infecção ocorrer, deve haver um fator predisponente ou fator de risco. No caso das infecções invasivas, o imunocomprometimento desenvolvido, ou pregresso, à internação, juntamente com outros fatores, como utilização de dispositivos invasivos, uso de antibacterianos de amplo espectro, síndrome de imunodeficiência adquirida, infecções bacterianas concomitantes são diretamente envolvidas no desenvolvimento de candidíases invasivas (ENOCH *et al*., 2017).

As infecções superficiais e cutâneas geralmente são menos graves, mas não menos importantes, pois em muitos casos, apresentam importância para a saúde pública. Para essas, os fatores predisponentes podem ser diabetes mellitus, uso de medicações imunossupressoras, imunocomprometimento geral, alterações hormonais, dentre outros

fatores, locais ou sistêmicos (COLOMBO *et al.,* 2013). As infecções podem ocorrer em unhas, cavidade bucal, regiões genitais de homens e mulheres, e outros. A candidíase vulvovaginal, por exemplo, é um transtorno que leva muitas mulheres ao ginecologista a cada ano (FELIX *et al*., 2018).

Estima-se que até 75% das mulheres tenham algum evento relacionado à infecção fúngica vaginal em algum período da vida, principalmente durante a idade reprodutiva (SILVA et al., 2017). *C. albicans* é a espécie prevalente em cerca de 95% dos casos de candidíase vulvovaginal, seguida da *C. glabrata* (14.5%) (COLOMBO *et al.,* 2013). *Candida tropicalis e C. parapsilosis* também são relatadas como espécies de NCA causadores de infecção vaginais (FELIX *et al*., 2018).

A indicação de terapia medicamentosa antifúngica é baseada na espécie de fungo, gravidade da infecção, local acometido, condição clínica e imunológica do indivíduo (RAI; 2017). Classes de medicamentos eficazes atualmente são representadas pelos azóis, poliênicos, alilaminas e equinocandinas, que são as opções terapêuticas para o tratamento de diversas infecções de etiologia fúngica, incluindo aquelas causadas por espécies de *Candida* (COLOMBO *et al*., 2013).

Os derivados azólicos representam a primeira escolha pelo espectro de ação, segurança e disponibilidade, em formulações orais, tópicas e intravenosas, a depender da gravidade do caso, e do agente infeccioso (EGGIMANN, GARBINO, PITTET; 2003; COLOMBO *et al*., 2013; SILVA *et al.,* 2017). Em casos de candidíases superficiais, os azóis, poliênicos e alilaminas (terbinafina) de uso tópico podem ser combinados com os de uso oral para aumentar a eficácia do tratamento (RAI, 2017). Imidazóis e nistatina tópicos, combinados com triazóis orais são eficazes na candidíase vulvovaginal não complicada, sendo contraindicado durante a gravidez (COLOMBO *et al*., 2013). Triazóis orais, como fluconazol e itraconazol, são alternativas eficientes em casos de moderada gravidade, em indivíduos imunocomprometidos e no caso de candidíase vulvovaginal complicada, geralmente combinado ao uso de imidazóis (RAI, 2017; FELIX *et al*., 2018).

Em infecções invasivas, formulações fungicidas contendo anfotericina B (lipossomal e complexo lipídico) e equinocandinas (anidulafungina, caspofungina e micafungina) constituem a primeira escolha em pacientes infectados por isolados com resistência demonstrada, após exposição prévia a azóis e com candidemia (SILVA *et al.,* 2017). A anfotericina B apresenta considerável efeito de nefrotoxicidade, altos custos, e baixa tolerabilidade em decorrência de diversos efeitos adversos decorrentes de seu uso (COLOMBO *et al,* 2013).

O amplo emprego de azóis, diferenças na duração e o uso como profilático, principalmente após a década de 80, podem explicar a redução da incidência de infecções por *C. albicans*, e o aumento do percentual relativo de infecções causadas por espécies NCA (EGGIMANN, GARBINO, PITTET; 2003). A partir daí, tornou-se necessário maior atenção em relação à identificação da espécie, e em casos de infecções invasivas, muitas

vezes a necessidade de realização de testes de sensibilidade *in vitro* aos antifúngicos, similarmente com o que ocorre com as bactérias (COLOMBO *et al,* 2013; MATTA; SOUZA; COLOMBO, 2017).

Em termos de resistência, *C. krusei* apresenta resistência intrínseca aos triazóis. *C. glabrata*, por outro lado, pode necessitar de dosagens elevadas para o sucesso terapêutico (EGGIMANN, GARBINO, PITTET; 2003). A resistência a anfotericina B e equinocandinas é incomum, porém *C. krusei* e *C. glabrata* podem desenvolver resistência, conforme relatam alguns estudos (COLOMBO *et al.,* 2017; MATTA; SOUZA; COLOMBO, 2017).

Os mecanismos de resistência de fungos aos antifúngicos relacionam-se com os mecanismos de ação das drogas, em nível molecular (EGGIMANN, GARBINO, PITTET; 2003). A classe dos azóis tem seu mecanismo de ação relacionado à inibição da via enzimática da C14-α-lanosterol desmetilase, sintetizada a partir da expressão do gene ERG11 (CAMPOY, ADRIO, 2017). Em alguns casos de resistência do fungo à droga azólica, este gene sofre mutação, podendo levar à expressão de forma aumentada dos níveis da enzima, ou ainda, impedir a ligação da droga à proteína (CARMONA, LIMPER, 2017). Além disso, a resistência aos antifúngicos têm sido associadas a alterações gênicas, expressão de enzimas e a presença de bombas de efluxo. Dessa forma, os mecanismos de resistência estão associados à regulação positiva de transporte de medicamentos, alterações em alvos de drogas, assim como, sua superexpressão (PERLIN; RAUTEMAA-RICHARDSON; ALASTRUEY-IZQUIERDO, 2017). Contudo, as bombas de efluxo são o principal tipo de mecanismo, sendo estas associadas a genes de cassete de ligação ao ATP ou a superfamília principal facilitadora. Os fenômenos de resistência aos polienos, têm sido associados a redução da produção de ergosterol, o que dificulta a interação dos polienos com os esteróis e a formação dos poros (CAMPOY, ADRIO, 2017).

Muitos isolados de diferentes espécies de *Candida* formam biofilme, o que contribui para a resistência antifúngica (DOI *et al*., 2016). O biofilme é resultante da multiplicação das células que se unem formando agrupamentos firmemente aderidos em multicamadas, que se conectam por matriz de exopolissacarídeos auto-secretadas, concedendo vantagens ecológicas estruturais e coordenação de funções voltadas à nutrição, metabolismo e proteção mútuas contra agressores externos, quando comparado com a forma planctônica dos microrganismos constituintes (DONLAN, COSTERTON, 2002; SILVA *et al.,* 2017). A baixa penetração das drogas antifúngicas entre as camadas constituintes do biofilme constitui um mecanismo de defesa que protege as células do contato e ação do antifúngico (SILVA *et al.,* 2017).

2 | O USO DE PLANTAS MEDICINAIS, ÓLEOS ESSENCIAIS E ATIVIDADE ANTIFÚNGICA

Os conhecimentos e o uso de plantas e suas preparações acompanham a

humanidade desde os primórdios como recurso à saúde (REAUT; KARUPPAYIL, 2014). No mundo, a incorporação de práticas tradicionais ao contexto da atenção primária foi uma das recomendações estabelecidas pela declaração de Alma Ata (1979), incluindo o uso de plantas medicinais (WHO, 1979). Assim, a relevância desse recurso pode ser expressa pela ação dos compostos ativos presentes nos vegetais para a elaboração de novos medicamentos (WHO, 2003).

Diversas substâncias encontradas em produtos derivados de plantas apresentam mecanismos de ação com valor bioativo individual que, combinadas, tornam a ação mais efetiva contra bactérias e fungos de interesse à saúde, como nos casos dos óleos essenciais (REAUT; KARUPPAYIL, 2014; MERTAS *et al*., 2015), que são compostos em que estão associadas muitas substâncias diferentes, que em conjunto são responsáveis pelas suas propriedades aromáticas e terapêuticas.

Espécies de plantas encontradas em diversas famílias, como Myrtaceae, Cupressaceae, Lauraceae, Rutaceae, Apiaceae demonstram considerações quanto à aplicabilidade na composição de produtos para fins medicinais, em específico, pelo rendimento e diversidade de compostos característicos dos OE obtidos de diferentes partes de cada planta (REAUT; KARUPPAYIL, 2014; SWAMY, AKHTAR, SINNIAH, 2016).

Os OE resultam da combinação de substâncias lipofílicas complexas de baixo peso molecular extraídos de partes especializadas de plantas (BAKKALI *et al*., 2008). Geralmente, os OE apresentam derivados terpenóides como substâncias bioativas predominantes, (MERTAS *et al*., 2015); destes, os monoterpenos são responsáveis por cerca de 90% das estruturas identificadas em OE de diversas espécies de plantas (SWAMY; AKHTAR; SINNIAH, 2016). Metodologias *in vitro* trazem evidências da ação isolada do OE de *C. limon* contra diversas espécies do gênero *Candida,* podendo ser ampliado de maneira significativa pela ação sinérgica de outros óleos essenciais combinados em diferentes concentrações (SWAMY; AKHTAR; SINNIAH, 2016; NIKOLIC *et al*. 2017; ORCHAND *et al.,* 2019).

3 I ÓLEO ESSENCIAL DE *CITRUS LIMON* E SUA APLICAÇÃO ANTICANDIDA

Citrus limon (Rutaceae) é uma das 40 espécies do gênero, naturalizada nas partes do mundo com clima quente e ameno; é de reconhecida importância econômica, produzindo anualmente cerca de 100 milhões de toneladas de frutos (MARIN et al., 2002, MABBERLEY, 2004). O OE é conhecido pela característica aromática, sendo empregado como insumo na indústria de alimentos, farmoquímica e na formulação de cosméticos (MARIN et al., 2002, DOSOKY, SETZER, 2018).

As condições que influenciam a constituição do OE podem modificar a quantidade e a sua composição, e assim sua atividade biológica, como o cultivo e o desenvolvimento da planta (SPADARO et al., 2012; GONZÁLEZ-MAS *et al*., 2019). Embora presente nos

OE das folhas, flores e frutos em concentrações variáveis, é zzna casca de *C. limon* que os hidrocarbonetos monoterpênicos são abundantes, geralmente majoritários na caracterização química da espécie. O limoneno, substância característica do gênero, pode representar até 90% da constituição do OE extraído da casca da espécie. Além disso, o grupo dos monoterpenos oxigenados, subdivisão que inclui os aldeídos terpênicos e os álcoois monoterpênicos, são classes representativas quanto o valor biológico atribuído ao OE da espécie (SPADARO *et al*., 2012; NIKOLIC *et al.,* 2017; GONZÁLEZ-MAS *et al*.; 2019).

O OE de *C. limon* têm chamado atenção pela ação inseticida, antiviral, estimulante do sistema nervoso central, analgésica, imunomodulatória, antioxidante, anti-inflamatória, anticarcinogênese e no tratamento de infecções microbianas (DOSOKY, SETZER, 2018; NIKOLIC *et al.,* 2017; LAMINE *et al*., 2019; SINGH *et al*., 2020).

O potencial dos OE extraídos da casca, fruto e folhas de *C. limon* tem sido avaliado extensivamente para diversas espécies do gênero, principalmente quanto a inibição *in vitro* de *C. albicans*, e demais espécies NCA, incluindo *C. glabrata*, *C. tropicalis, C. lusitaniae*, *C. guillermondii*, *C. parapsilosis* e *C. orthopsilosis* (SPADARO *et al*., 2012; HAMDAN *et al.,* 2013; EL ASBAHANI *et al*., 2014; NIKOLIC *et al.,* 2017; LAMINE *et al*., 2019; PEDROSO *et al*., 2019; USACH *et al*., 2020; MEZZOMO *et al.,* 2021). Além disso, o OE de *C. limon* foi capaz de inibir etapas do desenvolvimento do biofilme de formando por *C. albicans, C. glabrata, C. tropicalis, C. krusei, C. parapsilosis* e *C. orthopsilosis* (PEDROSO *et al.,* 2019; SAHAL *et al.,* 2020).

Portanto, nos últimos anos têm sido crescente o desenvolvimento de novas abordagens estratégicas e metodológicas com o objetivo de aumentar a eficiência da atividade de OE contra microrganismos, como no visto para o OE de *C. limon* frente a *Candida* spp. A atividade sinérgica de *C. limon* em combinação ao OE de *Thymus vulgaris* apresentou melhores resultados de inibição em proporções próximas (1:1) de ambos os óleos essenciais contra *C. albicans* (ORCHARD, VAN VUUREN, VILJOEN, 2019). O sinergismo também foi mostrado quando associado com *Melaleuca alternifolia* para a mesma espécie de *Candida* (NIKOLIC *et al.,* 2017).

Nesse sentido, a abordagem envolvendo o uso de OE é bastante promissora, envolvendo aplicações além da aromaterapia. Assim o uso de metodologias e tecnologias farmacêuticas como a dispersão de compostos biocompatíveis por meio de técnicas de encapsulamento e do potencial bioativo do OE na fase de vapor (FEYAERTS *et al.,* 2018; PINNA *et al.,* 2019 USACH *et al.,* 2020), poderão ampliar o espectro de ação do OE de *C. limon* contra espécies de *Candida*.

4 I CONSIDERAÇÕES FINAIS

A elevação progressiva das taxas de infecções, unida a condições predisponentes

dos pacientes, e a crescente resistência dos microrganismos causadores à terapia habitual, tornam o assunto uma questão preocupante no contexto da saúde pública no presente e nos próximos anos. Espécies de *Candida* são responsáveis por acometimentos infecciosos variáveis à saúde de pacientes, preferencialmente em condições para o desenvolvimento de infecções oportunistas. Os óleos essenciais são alternativas na medicina tradicional, e foco de estudos sobre sua bioatividade nos últimos anos.

O óleo de *C. limon* é uma importante fonte de fitoquímicos empregados pelo aroma e sabor em alimentos e bebidas, e em cosméticos, na aromaterapia e em produtos para usos medicinais. A ação efetiva desse OE contra microrganismos envolve múltiplos fatores, seja para o OE isolado ou quando combinado a outros. Os efeitos sobre o espectro de ação da combinação de óleos essenciais não podem ser restritos à capacidade de determinados metabólitos ativos isoladamente, mas à diversidade e à concentração encontrada na constituição de forma global. O uso de tecnologias para a liberação apropriada dos princípios ativos foram capazes de ampliar a eficácia, contornando hidrofobicidade e a volatilidade características, direcionando o seu uso de acordo com a especificidade físico-química com a finalidade pretendida, quando incorporado em formulações farmacêuticas.

Em conclusão, os resultados relatados nos estudos demonstram que o OE de *C. limon,* ou combinado a outros óleos essenciais, é uma possibilidade viável para o desenvolvimento de produtos voltados ao tratamento de infecções superficiais, a exemplo da candidíase seja vulvovaginal e outras mucocutâneas causadas por *Candida* spp. No entanto, são necessários mais estudos, para avaliar a eficácia, a segurança e a possível toxicidade dos produtos voltados à saúde humana.

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