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Review

Recent Advances in the Application of Essential Oils as Potential Therapeutic Candidates for Candida-Related Infections

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Abstract: Candidiasis (oral, vulvovaginal, or systemic bloodstream infections) are important human fungal infections associated with a high global prevalence in otherwise healthy adults but are also opportunistic infections in immunocompromised patients. With the recent discovery of the multidrug resistant—and often difficult to treat—*Candida auris*, as well as the rising costs associated with hospitalisations and the treatment of infections caused by *Candida* species, there is an urgent need to develop effective therapeutics against these pathogenic yeasts. Essential oils have been documented for many years as treatments for different ailments and are widely known and utilised in alternative and complementary therapies, including treating microbial infections. This review highlights knowledge from research on the effects of medicinal plants, and in particular, essential oils, as potential treatments against different *Candida* species. Studies have been evaluated that describe the experimental approaches used in investigating the anticandidal effects of essential oils (in vivo and in vitro), the established mode of action of the different compounds against different *Candida* species, the effect of a combination of essential oils with other compounds as potential therapies, and the evidence from clinical trial studies.

Keywords: *Candida* infections; antifungal activity; essential oils; mode of actions; combinative therapies; in vitro; in vivo; clinical trials

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1. Introduction

Candidiasis is a multifaceted fungal infection caused by commensal fungi *Candida* spp., which reside on the skin, mucosa, and gastrointestinal tract of 30–50% of healthy adults at any given time—with everyone colonised at some point in their lifetime [1]. Under normal host conditions, *Candida* spp. is not usually pathogenic and normally resides in genital and gastrointestinal tracts in healthy humans; however, Candidiasis infection appears when the balance between the fungus, mucosa, and host defence mechanisms is interrupted [2]. *Candida* spp. are known to cause superficial infections (mucosal and cutaneous) and systemic infections. Superficial infections caused by *Candida* spp. vary and present in the form of oral Candidiasis, vaginal Candidiasis, oropharyngeal Candidiasis, onychomycosis, etc., with a relatively high prevalence worldwide (20–25%) [3]. Systemic Candidiasis infections on the other hand are associated with high morbidity and mortality rates as well as increased hospitalisation [4].

Oral Candidiasis is one of the most common human fungal infections, especially in elderly people, human immune deficiency virus (HIV)-positive individuals, or patients receiving radiotherapy treatments [5]. It is reported that the oral carriage rates of *Candida*

spp. range from 20–75% in the general population, and up to 95% in HIV patients [6]. Candidiasis that develops in the vagina is commonly called vulvovaginal Candidiasis. In fact, approximately 75% of women suffer from vulvovaginal Candidiasis at least once in their lifetime, and up to 8% of them have recurrent infections [7]. In immunocompromised patients, pathogenic *Candida* spp. can spread through the bloodstream and affect internal organs like the upper gastrointestinal tract, kidney, heart, or brain, leading to systemic Candidiasis with significant morbidity and mortality [6]. According to Fraser et al. [8], systemic Candidiasis carries a mortality rate of 71–79%. Cavayas et al. [9] reported that *Candida* spp. are responsible for the third-highest incidence of isolates from bloodstream infections in neutropenic or immunocompromised hospitalized patients from intensive care units (ICUs). Moreover, the morbidity and mortality rate associated with Candidiasis is gradually rising because of the increase in the number of high-risk patients and the emergence of new *Candida* spp. and drug resistant strains [10].

Pfaller and Diekema [11] estimated that approximately 62% of all invasive candida infections are caused by *C. albicans*. *C. glabrata* was ranked as the second most common pathogen of candida bloodstream infections in the US with reported prevalence rates of 20–24%. This may be of pertinence as *C. glabrata* is a leading pathogen associated with echinocandin resistance, which is of great concern as echinocandin drugs are the front-line therapeutics for invasive Candidiasis [12]. Some other common *Candida* spp. are *C. tropicalis*, *C. parapsilosis*, and *C. krusei.* Notably, *C. auris* is a recently emerged *Candida* spp. that was first isolated from a Japanese patient in 2009 and is continuously spreading worldwide [13]. This newly described fungal pathogen can resist several antifungal agents, with some multiple drug resistant (MDR) strains exhibiting resistance to three classes of antifungals (azoles, polyenes, and echinocandins) [14]. *C. auris* can be transmitted person-to-person and it has a high mortality rate (30–60%) in patients who suffered from *C. auris* bloodstream infections [15,16]. Essential oils (EOs) are complex liquid mixtures of volatile and low molecular weight substances that can be extracted from the whole plant or plant parts, such as the leaf, bark, fruits, and flowers of aromatic plants [17,18]. Currently, approximately 3000 EOs have been discovered and about 300 EOs are known to be commercially important [19]. A wide range of effects of EOs from different plant species and botanical families have been reported, including immunomodulatory, psychotropic, acaricide, expectorant, antidiabetic, cancer suppressive, and antibacterial effects [20–22]. In addition to these, other effects, such as antifungal properties against various plant and human pathogenic fungi including yeasts, have been reported from numerous EOs [18,23].

Natural compounds found in medicinal plants could be considered as one of the greatest sources for the development of innovative modern medicine [24]. The isolation and characterization of active compounds in EOs is more of a growing science due to the development of new technologies, and detection and characterisation systems such as gas/liquid chromatography and mass spectrometry (GC/LC-MS). Additionally, various available in vitro tests have been utilised to verify the antimicrobial activities of EOs. A significant number of studies have been conducted to evaluate natural compounds of plant origin that are effective but less toxic than those in drugs already in use [25,26].

This review critically evaluates the antifungal activity of EOs against *Candida* spp., including *C. auris*, a newly emerged *Candida* spp. The authors discuss the methods currently used in the screening of EO antifungal activity, the mode of action of the compounds, and the findings from clinical trials investigating therapeutic formulations that contain various EOs.

2. Mode of Action of EOs

Depending on the species, EOs have been shown to comprise a mixture of tens to hundreds of different compounds [27]. Due to this reason, a single EO can possess more than one mechanism of action against a microorganism. Of note, given that every single EO seems to have a wide range of cellular targets (Table 1), the probability of the generating new, resistant strains to the EOs as fungicidal agents are low [28].

Table 1. Monoterpenoids and their mode of action upon *Candida* species.

Antifungal Compound	EOs	Mode of Action on *Candida* Species	References
Aldehydes		C=O	
Cinnamaldehyde	Camphor, Cassia, Cinnamon.	ATPase inhibition Induces apoptosis Induction of oxidative stress Reduction of ergosterol biosynthesis	[29–31]
Citral	Lemon, Lime, Orange.	Induction of oxidative stress Inhibition of pseudohyphae formation	[32,33]
Cyclic Terpenes		C_6 ring	
α-pinene	Frankincense, Juniper, Pine, Rosemary.	Disruption of cellular membranes Reduced biofilm formation	[34]
β-pinene	Cannabis, Lavender, Mint, Pine.	Disruption of cellular membranes Reduced biofilm formation	[34]
Limonene	Lemon, Lemongrass, Lime, Orange.	Disruption of cellular membranes Induces apoptosis	[35]
p-cymene	Anise, Basil, Camphor, Cumin, Eucalyptus, Oregano, Thyme.	Disruption of cellular membranes Inhibition of germ tube formation	[36]
Phenols		-OH	
Carvacrol	Oregano, Thyme, Wild Bergamot	Binds to sterol components of membranes	[37,38]
Eugenol	Basil, Cinnamon, Clove, Nutmeg.	Altered protein functionality Increases membrane fluidity and permeability Inhibits ergosterol biosynthesis Inhibits proton efflux	[39]
Linalool	Basil, Lavender, Rose, Sage.	Altered protein functionality Increases membrane fluidity and permeability Inhibits proton efflux	[39]
Menthol	Geranium, Mint, Sunflower, Tarragon.	Inhibition of ergosterol biosynthesis	[40]
Thymol	Citrus, Coriander, Oregano, Thyme, Wild Bergamot.	Altered protein functionality Inhibition of ergosterol biosynthesis	[41]

2.1. Phenolic Terpenes

Of the many reactive compounds found in EOs of interest for antifungal agents, monoterpenoids are a diverse class of low molecular weight, isoprene-derived molecules that can be further divided into groups depending upon their functional activities. Phenolic terpenes (e.g., carvacrol, eugenol, and thymol) possess an −OH moiety that is able to be transferred to protein structures, thereby altering their integrity and functional capacity [41]. This may be of critical importance when considering the protein content of the cell wall in *Candida* spp., which remains a key factor for adhesion and virulence [42]. However, the most favoured and well-researched target for many phenolic terpenes is the cell membrane structural and regulatory sterol, ergosterol.

Analogous to cholesterol in animal and plant cells, ergosterol has been identified as a primary target for phenolic terpene interactions. As the cardinal sterol component of the plasma membrane of fungal species [18], ergosterol is responsible for the maintenance of cell membrane structure and integrity. Inhibition of ergosterol biosynthesis by numerous monoterpenes, including carvacrol, eugenol, menthol, and thymol, have been demonstrated to affect the fluidity, integrity, and permeability of fungal membranes [29,42]. Here,

inhibition of the lanosterol 14-α demethylase enzyme negatively regulates transmethylation processes at position C24 of the sterol sidechain, an effect that has been demonstrated to affect the protein–protein binding functionality of this essential fungal sterol [40].

In addition to the effect phenolic moieties can impose on the structural stability of fungal cells, monoterpenoid alcohols have also been described as having an inhibitory effect on efflux pumps (e.g., carvacrol, thymol) in *Candida* spp. [43,44]. Inhibition of these essential fungal defences could indicate that EO extracts may be viable as an adjunctive therapy, working synergistically alongside current antifungal treatments (e.g., clotrimazole and fluconazole). Similarly, eugenol and linalool have been suggested to inhibit proton efflux channels, which are responsible for regulating cellular pH, and perhaps more importantly, are associated with the electrochemical gradient required for ATP production [39]. Carvacrol, a common component of many EOs, has also been demonstrated to induce temporal changes in both cytosolic and vacuolar pH, leading to a dose-dependent increase in cellular Ca+ and subsequent activation of the target of rapamycin (TOR) stress response pathways [36], which leads to apoptosis [38]. With such multifarious actions being attributed to the phenolic class of terpenoids, it is seemingly the combination of molecules within various EOs that provide continued fungicidal activity.

2.2. Cyclic Terpenes

Cyclic terpenes contain a hydrophobic six-carbon ring that is known to penetrate, disrupt, and increase the fluidity of the cytoplasmic membrane of *Candida* and other fungal species [45]. This particular class of terpenes include α-pinene, β-pinene, limonene, and p-cymene, among others, which are prominent in many EOs. An in silico study of *C. albicans*, conducted by Pinto et al. [36], suggests p-cymene may act primarily as an antagonist to fungal membranes, promoting cellular permeability, the leakage of cytosolic content, and cessation of activity. An alternative mode of action, however, is displayed by limonene, a major constituent of citrus derived EOs, which are noted to induce the apoptotic pathway in *C. albicans* [35].

In addition to the membrane disruption modality displayed by the majority of cyclic terpenes, α-pinene and β-pinene isomers, when examined by Rivas et al. [34], showed a notable reduction in *C. albicans* biofilm formation. However, whether this effect is due to quorum sensing interference [46], interruption of fundamental cellular processes [47], or by other means is still unclear. The diverse mechanisms of fungicidal activity exhibited by this class of phytochemicals could explain the continued susceptibility of *Candida* spp. to EOs and their active components, although much research will be required if the intricacies and efficacies of such biochemical processes are to be fully understood.

2.3. Aldehyde Terpenes

Aldehyde compounds such as cinnamaldehyde and citral are another class of antifungal phytochemicals that are found extensively in the EOs of cinnamon bark and citrus rinds, respectively. Although these molecules have analogous functional moieties, their modes of fungicidal action appear to be diacritic. To illustrate, cinnamaldehyde contains an aromatic ring structure, and in common with other terpenes, exhibits antifungal properties that include dissolution into the hydrophobic domain of cellular membranes, reduction in ergosterol biosynthesis, and inhibition of ATPase and proteinaceous activities [30,31]. Furthermore, the addition of cinnamon-derived EOs to in vitro colonies of *C. albicans* and *C. auris* reveals the inhibition of haemolysin production and reduced hyphae formation [29]. Interestingly, cell wall perturbations and interference with ergosterol processes and production were ruled out as targets for citral. Instead, it has been posited that the inhibition of pseudohyphae and chlamydoconidium may be responsible for the fungicidal effects seen in *C. albicans* [32,33].

As each individual EO can contain varying amounts of active compounds, and each compound may have a similar or unique mode of action against *Candida* cells, future

research may involve comparative analysis that assesses the financial and sustainable viability of EOs as modern therapeutics.

3. Activity of EOs against Drug-Resistant *Candida* spp.

Invasive Candida infections pose major health concerns, especially in hospitalised, immunocompromised, or critically ill patients [48,49]. However, there are only four major classes of antifungals in clinical use, which include azoles, polyenes, echinocandins, and pyrimidine analogs [50,51]. For this reason, an intense search for new alternative antifungal compounds is very urgent and necessary.

Previous research has explored the effect of 21 plant essential oils against multidrug resistant *Candida* spp., where it was discovered that *Cymbopogon martini* (lemongrass, LEO), citral, and cinnamaldehyde exhibited great inhibitory activities with MIC ranging from 90–100 μg/mL [52]. Furthermore, these EOs were more effective than fluconazole and amphotericin B. Therefore, the enhanced tolerance to antifungal drugs among *Candida* spp. and the role of biofilm in disease development has necessitated research for new antifungal treatment strategies [52].

A recent study by Jafri and Ahmad [53], which examined the effect of the *Thymus vulgaris* EO (Thyme, TEO) and thymol—its major active compound—on *C. tropicalis* resulted in the discovery that thymol at 0.78–25 μg /mL and TEO used at the same concentration contributed to the significant reduction of biofilm formation by *C. tropicalis*. Furthermore, the same research showed that, when treated with thymol, the biofilm cells of *C. albicans* showed disaggregation and had deformed shapes. Additionally, there was reduced hyphae formation in *C. tropicalis* biofilms.

As a result of the globally emerging threats of multidrug resistant *C. auris* [54], further research by Hamdy et al. [55] led to the development of novel antifungal drugs that are effective against not only *C. albicans*, but also *C. auris* through the use of cuminaldehyde isolated from the *Calligonum comosum* plant, which has demonstrated broad-spectrum antifungal activities. In this research, new compounds were designed and developed with the incorporation of azoles, whereby the new compounds developed showed significant anti-Candida activities against both *C. auris* and *C. albicans*. This resulted in the formulation of polymeric nanoparticles that possess significantly enhanced activities against *C. albicans* and *C. auris* while maintaining prolonged action and no toxicity at lower concentrations [55].

The enhanced ability of some *Candida* species to form biofilms that promote yeast survival upon exposure to drugs contributes to the acquisition of resistance [56]. According to research by Khan and Ahmad [57], pre-formed biofilms of *C. albicans* showed $\geq$1024 times increased resistance to antifungal drugs. However, at a concentration of 50–180 μg/mL, oils of *Cymbopogon citratus*, commonly known as west Indian lemongrass, and *Syzygium aromaticum* (clove) inhibited biofilm formation. Here, in the presence of a *C. citratus* EO, the three-dimensional structures of the biofilms produced by both *Candida* species showed deformation. In addition to the drug resistance described earlier, the phase in which the EOs are administered can also determine the type of effect they have on *Candida* species. An example of this was reported by Santomauro et al. [58], whereby the vapour and liquid phases of the *Artemisia annua* EO were analysed against several strains of *Candida* spp. The authors describe that the antifungal activity of *A. annua* is influenced by the type of method adopted, as the inhibitory action of this EO was, in fact, greater in the vapour phase than liquid. This research determined that the average MIC in the liquid phase was 11.88 μL/mL, while the vapour phase was 2.13 μL/mL. However, it was discovered that a strain of *C. glabrata* was more susceptible to the liquid phase than vapour phase. It is also interesting to note that *C. albicans* and *C. dubliniensis* were the most susceptible to vapourised *A. annua*, while *C. parapsilosis* was the least susceptible strain.

4. Combinative Therapies against Candidiasis Infections

Due to the growing resistance of pathogens against antimicrobials, patients may be prescribed a combinative therapy of broad-spectrum drugs to eradicate the infection.

This combination may be with conventional drugs, although studies have shown that conventional drugs in combination with natural products may have a more positive effect [25,52,59]. This approach has been shown to decrease the side effects and toxicity observed in recovering patients, but also may be able to overcome the resistance against conventional antimicrobials [52].

The use of TEO and its major component, thymol, in combination with conventional antifungal drugs demonstrated synergistic effects against *Candida* spp. In a study conducted by Jafri and Ahmad [53], the antifungal properties of fluconazole increased when combined with thymol and TEO as opposed to being used alone against sessile cells of the fungus. The thymol and fluconazole combination exhibited greater synergy against sessile *Candida* spp., where sessile MIC was reduced by up to 16-fold.

In a similar investigation by Essid et al. [60], the combination of a fluconazole and *Cinnamomum verum* (cinnamon) EO demonstrated synergistic effects against *C. albicans*. A *Pelargonium graveolens* EO (Rose geranium, REO) in combination with fluconazole also displayed synergistic action against fluconazole-resistant strains of *Candida* spp. The combination of two different compounds, each exhibiting their own mode of action, essentially prevents the yeast from recovering. This metaphorical double-edged sword may also be a factor in overcoming antimicrobial resistance [52].

As well as the combination of conventional drugs and EOs, the combination of multiple EOs have demonstrated synergistic activity against pathogens. A screening of commercial EO combinations against fungal pathogens by Orchard et al. [61] showed an overall decrease of the MIC of essential oils used in combination when compared to them being used alone. The combination of a *Santalum austrocaledonicum* EO (sandalwood, SEO) and REO presented one of the strongest synergistic actions against *C. albicans*; overall, MIC values decreased with the use of essential oils in combination with SEO.

Angiolella [62] investigated the combination of REO and LEO against *Candida* spp. The EOs were also combined with fluconazole separately and showed that the combination of REO and fluconazole displayed synergistic activity, as well as the combination of REO and LEO. On the other hand, the combination of LEO and fluconazole displayed additive activity. It is also worth noting that although not every EO combination has a synergistic effect, not many studies have reported antagonistic effects of essential oil combinations. In a study conducted by Giordani et al. [63], low concentrations of TEO and amphotericin B showed antagonistic activity. This also has been displayed when amphotericin B was combined with imidazole [64], amphotericin B, and miconazole [65]. Therefore, it cannot be assumed that all EOs will complement each other and/or conventional drug treatments, and further empirical studies will be required if the synergistic effects of EOs are to be fully understood.

5. Approaches to Investigate Anti-Candida Activity of EOs

5.1. In Vitro Methods

Clearly, there has been a growing interest in researching the antimicrobial properties of EOs in the scientific community. As a result, several screening and evaluation methods have been developed to determine such antimicrobial activity of EOs. Owing to their simplicity and low cost, many bioassays, such as agar disk-diffusion, vapour phase diffusion, broth dilution, and direct bioautography, are well known and commonly used in testing the antifungal activity of EOs [66]. Other techniques, such as electron microscopy, bioluminescence, or flow cytofluorometric methods, are not widely used as they require specified equipment or further evaluation for reproducibility and standardization. Below, Figure 1 shows an overview of some commonly used in vitro tests to evaluate the anti-Candida activity of EOs.

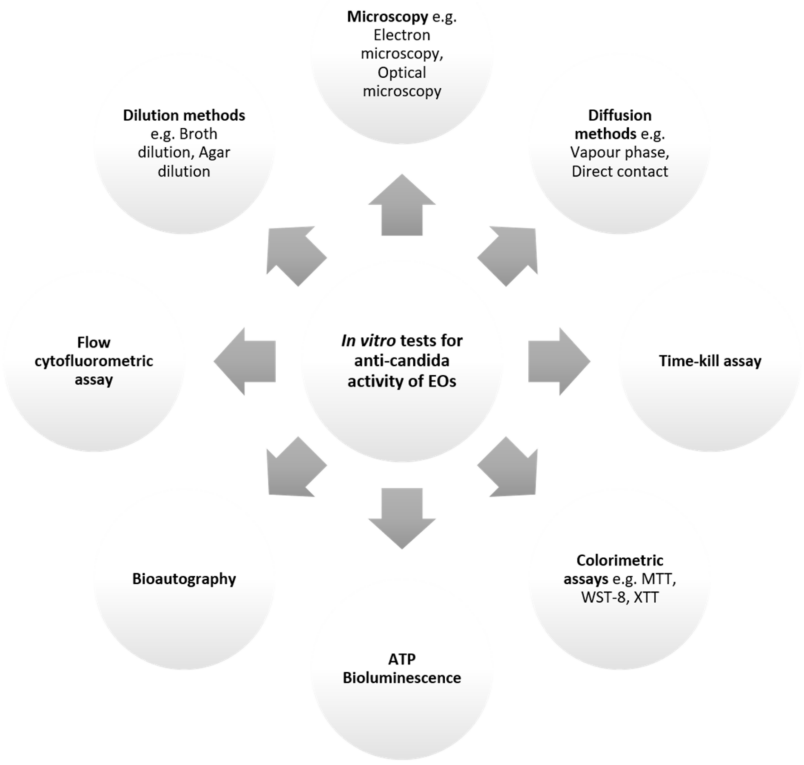

Figure 1. In vitro methods for investigating antifungal activities of essential oils against *Candida* species.

5.1.1. Diffusion Assays

Diffusion tests are widely considered as a primary screening assay because of the simplicity, low cost, capacity to conduct a huge number of tests and investigate many compounds at a time for their activity against different microorganisms, and, finally, the ease to interpret the data provided. However, this method cannot distinguish fungicidal and fungistatic effects [66]. There are two diffusion methods that are commonly in use: direct contact disc diffusion and vapour phase diffusion. In the direct contact disc diffusion test, EOs diffuse into the agar and the assessment of antifungal activity is the inhibition of germination and growth of the test microorganism. The antifungal activity can be estimated from the size of the inhibition zone. One problem associated with the use of the agar diffusion technique is that the results from this test sometimes show little antimicrobial activity, but the same EO is observed to have high activity when using other tests. This phenomenon could be explained by the diffusion coefficient and water solubility of EOs [67]. On the other hand, the vapour phase diffusion test is a well-known technique to study the importance of volatile compounds on the biological activity of EOs [68,69]. Volatile compounds in EOs will evaporate, diffuse into the agar, and inhibit the growth of the test microorganism. Research into the vapour phase antifungal activity of EOs is growing in interest as it represents potentially different routes of administration to be used when treating against fungal infection (e.g., in clinical settings to improve air quality by means of decontamination). It is important with the vapour phase diffusion test to differentiate the direct from the indirect effects of EOs on microorganisms. Nevertheless, it is important to point out that this methodology also depends on a good vapour density of the active compounds [31–70].

5.1.2. Dilution Assays

Dilution assays are the most appropriate techniques used for quantitatively measuring the effect of EOs against microorganisms [71]. The methodologies for dilution assays are simple and cost-effective. However, the inoculum used for dilution tests must follow a

standard, as any variability in the inoculum size, turbidity, incubation time, and preparation method can affect the MIC results [72–74]. The most recognised standards are provided by the Clinical & Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST). There are two common dilution techniques: broth dilution and agar dilution. Broth dilution tests are conducted by preparing dilutions of the EOs in a liquid growth medium. This is noteworthy because EOs are not water soluble and need to be dissolved in solvent (e.g., dimethyl sulfoxide) or mixed with an emulsifying agent (e.g., Tween20) before being added to the liquid medium with yeast inoculum. The prepared tubes or 96-well microtitration plates are then incubated under suitable conditions, followed by an optical density (OD) measurement at a suitable wavelength. In an agar dilution test, EOs with varying desired concentrations will be incorporated into agar medium. A standardised inoculum of the test microorganism is spread onto agar plates and incubated under suitable conditions. The MIC values can be determined as the lowest concentration of EOs, which completely inhibits the visible growth of yeast colony forming units (CFU). The agar dilution assay offers the possibility to test several different strains/species against a single EO on the same plate if they have similar growing conditions. It presents a good correlation with other methods such as disk diffusion and broth dilution assays [75]. However, these tests require a large quantity of medium, Petri dishes, and laborious handling. Therefore, although agar dilution is an appropriate method for studying the antimicrobial activity of EOs, it is not frequently used.

5.1.3. Time-Kill Assays

The time-kill assay is the most appropriate method to evaluate the dynamic interaction between EOs and fungal cells. Hence, a time-dependent or a concentration-dependent fungicidal effect of EOs will be determined [76]. In this test, the fungal suspension should be well standardised by following the McFarland standard. After incubation with EOs, the cell viability will be calculated relative to the growth control by using spectrophotometry at a suitable wavelength [77,78]. EO–EO or EO–antifungal drug synergism can also be evaluated by this method [11].

5.1.4. Colorimetric Methods

The colorimetric assays allow for the quantitative measuring of the effects EOs have against microorganisms by using colour indicators. Therefore, these techniques offer a possibility to determine MIC endpoint or cytotoxicity testing. Several dyes have been developed to record changes in cell viability. These dye reagents work on the basis of being converted to a coloured formazan in the presence of metabolic activity [79]. For example, the MTT assay, based on the reduction of a yellow tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to its respective purple formazan, is often used for testing the fungicidal activity of EOs and has been extensively reported [80–82]. Other dyes, including 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT), and [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphonyl)-2H-tetrazolium] (WST-8), are also widely employed as assays of yeast viability due to their ease of use [83].

5.1.5. ATP Bioluminescence Assay

Similar to the aforementioned colorimetric assay, the ATP bioluminescence assay can quantitatively measure the number of living cells in a given sample. This test is based on the capacity to detect the amount of adenosine triphosphate (ATP) produced by living fungal cells. The ATP bioluminescence assay processes a large range of applications, such as determining the MIC endpoint [84], cytotoxicity test [85], or evaluating the Candida biofilm development [86]. Compared to other colorimetric tests, such as the MTT assay, the ATP bioluminescence assay provides better reproducibility and sensitivity when cells were grown in microtiter plates over several days [87]. Therefore, this assay is particularly useful for the measurement of viability with low cell numbers. However, the widespread use of

this assay currently appears unlikely due to the inaccessibility of the required luminometer equipment in various laboratories.

5.1.6. Direct Bioautography

The thin-layer chromatography (TLC) direct bioautography is a high-throughput analysis method that is used for detecting antifungal substances in complex mixtures like EOs [66]. In this test, a TLC plate that contains the testing agent is dipped into or sprayed with a microbially contaminated liquid. Following incubation under suitable conditions and the use of tetrazolium salts, zones of inhibition on the bioautogram are uncovered. These salts are transformed into their corresponding intensely-coloured formazan by the dehydrogenase enzymes of viable cells. As a result, the zones of inhibition can be observed and measured as such zones are colourless and easily visualised [88].

5.1.7. Flow Cytofluorometric Method

The flow cytofluorometric method uses flow cytometry to discriminate dead, viable, and injured cells based on the difference in the fluorescent marker of these cells. The detection of damaged yeast cells by this technique depends on the use of appropriate dying agents [89]. Propidium iodide, a fluorescent and intercalating agent, is widely used as a DNA staining agent in this technique [90]. This method offers the possibility to estimate the impact of the molecule of interest on the viability and cell damage of the tested microorganism. The number of injured cells in this test exhibits cellular component damage with subsequent impairment of reproductive growth [89].

5.1.8. Microscopy Assays

Microscopy assays are the most appropriate techniques for determining changes in the morphology of Candida cells before and after treatment with EOs. Hence, these methods are often used for evaluating the ability to form hyphae of the testing Candida strains. The effects of EOs on the integrity of the Candida cell membrane and Candida biofilm, or the possible cytological damages, are caused by the synergism of EOs [59,76,91].

Optical microscopy, also referred to as conventional light microscopy, is the most common imaging technique and uses a system of lenses and visible light to generate magnified images of small objects. This technique is very simple; therefore, it is usually used as the first-line method to evaluate the change in morphology of fungal cells, especially the germination ability before and after treatment with EOs. Germination could be considered as one of the major virulence factors known to contribute to Candida pathogenesis. In *C. albicans*, virulence gene expression is linked to its ability to transition from yeast-form to hyphal-form [92]. The yeast-to-hyphal transition in *C. albicans* is known to promote virulence because hyphae can exert a mechanical force to breach and damage endothelial cells [93]. Understanding the changes in hyphae formation could contribute to the evaluation of the anti-Candida activity of EOs. When compared with electron microscopy, light microscopy is more popular because of its low cost and simplicity.

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) are two imaging techniques that use a beam of electrons as a source of illumination to provide bi- and tri-dimensional views, respectively. SEM images the surface of a sample, therefore it offers a possibility to evaluate the integrity of the fungal membrane and biofilm under treatment with EOs. Hence, the mechanisms of action of testing EOs could be predicted [94]. TEM, on the other hand, possesses a higher magnification and resolution than can be achieved with a SEM. This technique not only offers the possibility to observe the morphological changes, but also the intracellular damages in exposed cells [95].

5.2. In Vivo Methods

Several in vivo models have been used in evaluating the effect of essential oils against Candidiasis infections. Some advantages and disadvantages of using in vivo models for Candidiasis research will be discussed in this section.

5.2.1. Mammalian Models

The most utilised species of mice used in Candida research are the Swiss white mouse or the species from the Institute of Cancer Research (ICR mice). Unlike in vitro tests, the induction of Candida infections in mice can be used to simulate Candidiasis in more susceptible human populations, due in part to the ability to replicate various human diseases, such as immunopathies or diabetes, for example. Candidiasis can also be induced through a variety of routes, with intravenous inoculation being the most common [96].

The mouse model is advantageous when researching the immunogenic response to Candidiasis and acquired immunity to *Candida* spp., thus creating the opportunity (or increasing the prospect) to develop a suitable vaccine. The pathogenic mechanism of Candidiasis can be analysed and compared between species, which is paramount when developing new anti-Candida drugs—particularly concerning pharmacokinetic parameters or characteristics. The availability of this information from the mouse model can be further used to establish preventative measures against Candida infection.

The most commonly used non-mammalian model is the *Drosophila melanogaster* [96]. Its short life cycle, ease to keep in large numbers, and affordability makes this a favourable non-mammalian model. Drosophila can be bred with a variety of defects from the few thousand genes available. Additionally, when results obtained from *D. melanogaster* have been compared to those of mice models, many similarities were found [97]. Thus, *D. melanogaster* could be considered as an appropriate model for studying Candida infections.

Caenorhabditis elegans is a transparent nematode of 1 mm in length. It is a transparent eukaryotic, multicellular organism that allows for observations to be made with ease. They are affordable and easy to grow and can remain intact after freezing for long periods. *C. elegans*, however, only have an innate immune system and are unable to grow at 37 °C; researchers, therefore, may find it difficult to generalise results gained from using *C. elegans*.

Galleria mellonella, a moth larva, has many functional and structural similarities to the innate mammal immune response. Its benefits include affordability, being commercially available, and being handled easily. However, unlike the mouse model, morbidity cannot be assessed. However, there are questions as to whether this model produces similar results to that of the mammalian mouse model. Amorim-Vaz et al. [98] reported a significant difference in the phenotype of *C. albicans* from the *G. mellonella* model and mice model, which is contradictory to Hirakawa et al. [99] and Brennan et al. [100], who all reported similar findings from both models. These discrepancies should be considered when drawing conclusions from results obtained from *G. mellonella*, which cannot be investigated to the same extent as mammalian models.

Clearly, most results obtained from in vivo models concur with those from mammalian models. However, some did not correlate with the mammalian model, and thus researchers should be cautious of this. The main advantages that non-mammalian models have over mammalian models lie in the affordability, ease of use, and economy of human resources required to maintain and handle such organisms. A significant disadvantage of non-mammalian models is that they are not suitable for studies involving microbial vaccination as they have no adaptive immune system, and some do not function at 37 °C—the mammalian body temperature. This greatly reduces the validity of results when generalising to the human population.

Findings from In Vivo Studies Investigating Anti-Candida Activity of EOs

The antifungal activities of EOs make them promising alternatives to treat superficial Candidiasis. The study by Pedroso et al. [101] investigated the fungicidal actions of EOs extracted from *Citrus limon* and *Cupressus sempervirens* by using the *C. elegans*–Candida model. The results showed that the *C. limon* and *C. sempervirens* EOs exhibited fungicidal activity after treatment with these agents for 48 h. In addition, the *C. sempervirens* EO was not toxic and increased the survival of *C. elegans* worms infected with *C. glabrata* or *C. orthopsilosis*. Rasteiro et al. [102] reported that *Melaleuca alternifolia* oil (tea tree oil, TTO)

at 12.5% (*v/v*) effectively reduced yeasts of *C. albicans* in an experimental model of oral Candidiasis in immunosuppressed mice.

In order to investigate the therapeutic actions of EOs in vulvovaginal Candidiasis, many in vivo studies have been conducted using female mice models. Pietrella et al. [85] focused on the antifungal activity of the *Mentha suaveolens* (applemint) EO against the infection of vaginal Candidiasis. Here, cell suspensions of *C. albicans* were administered from a mechanical pipette into the vaginal lumen of female CD1 mice. The results of both photon emission and CFU measurements demonstrated the accelerated clearance of *C. albicans* during vaginal Candidiasis in EO treated mice. Further, when oophorectomized female Wistar rats were infected with fluconazole-susceptible and fluconazole-resistant *C. albicans* strains, TTO was administered intravaginally for Candidiasis treatment. TTO caused a rapid clearance of the *C. albicans* (both fluconazole-susceptible and fluconazole-resistant strains). With all dose regiments (5%, 2.5%, and 1%, *v/v*), the infection was cleared within 3 weeks, whereas the untreated control rats remained infected.

Similarly, Wang et al. [103] studied the anti-Candidiasis activity of EO extracted from the seed of *Anethum graveolens* L. (umbelliferae) on female BALB/c mice and found that umbelliferae EO at low concentration (2%, *v/v*) was highly efficacious in accelerating *C. albicans* clearance from experimentally infected mice vaginas through both prophylaxis and therapeutic treatments. Toledo et al. [104] also researched the in vivo antifungal effects of EO from the leaves of *Cymbopogon nardus* (citronella) against *C. albicans* in female mice C57BL/6 models. All mice treated with citronella EO in microemulsion (a nanostructured drug delivery system that increase drug solubilization and absorption) were cured on the third day of treatment. The results showed that treatment with the mixture of citronella EO in microemulsion was more effective than that of free EO or commercial cream used in clinical therapy (amphotericin B and tetracycline).

6. Clinical Trials of Therapeutic Formulations with EOs

EOs have been used in many in vitro studies and, to date, have shown remarkable antifungal effects, especially against *Candida* spp. These findings have been supported with similar results from clinical trials, further establishing EOs as an alternative therapy against many fungal diseases. In a randomised, double-blind, and controlled ketoconazole study [105], the effectiveness and tolerability of a *Solanum chrysotrichum* herbal medicinal product was assessed in 101 women (aged 17–54 years) clinically diagnosed with vulvo-vaginal Candidiasis. The trial formulated an experimental treatment with 125 mg of the dry S. chrysotrichum extract against a control treatment of 400 mg ketoconazole. Clinical assessment and mycological studies were carried out at (i) 3–4 days of treatment initiation, (ii) 7 days after treatment, and (iii) 3 weeks after concluding treatment. The study results showed similar levels of therapeutic clinical effectiveness between the EO based product and ketoconazole with 100% tolerability; however, a higher percentage of fungus eradication was observed in the *S. chrysotrichum* treatment after 7 days, suggesting a residual antifungal effect of the EO treatment. Interestingly, Ellah et al. [106] have developed a cumin seed EO (CSEO)-containing vaginal suppositories and have clinically examined its efficacy in the treatment of vulvovaginal Candidiasis. In this pilot study, a total of 32 women (aged 18–49 years) with symptoms and signs of vulvovaginal Candidiasis were included. CSEO suppositories were inserted in patients' vaginas once at night for six consecutive days. After treatment, there was a statistically significant lower rate of itching, discharge, and dyspareunia. Moreover, the results showed that 70% of patients' cultures were negative for *Candida* spp. after treatment with CSEO suppositories.

Another randomised control clinical trial investigated the in vivo activity of TTO mixed with tissue conditioner (Coe-Comfort) on *C. albicans* in the treatment of Denture Stomatitis (DS) type II patients [107]. In this trial, 27 patients (26 women and 1 man, aged 50–77 years) who exhibited clinical evidence of Denture Stomatitis type II were randomly divided into three treatment groups. The treatment groups were as follows: (i) 1 mL TTO with conditioner, (ii) 2 mL Nyastin (a conventional antifungal medication) with

conditioner (positive control group), and (iii) pure conditioner mixture (negative control group). Clinical evaluations were assessed in three sessions (2 h after treatment, 4 days after treatment, and 8 days after treatment) and the results showed that both the TTO and Nyastin mixtures were effective in producing a clinical remission of DS. Furthermore, statistical data suggested a faster decrease in *C. albicans* with TTO than that with Nyastin, suggesting that TTO could be used as an alternative therapy for DS that is resistant to traditional therapies. Similarly, another randomised, open-label study by Vazquez and Zawawi [108] also confirms the antifungal effects of TTO against *C. albicans*. In this single-site study, 27 patients (men and women, aged 18–65 years) with AIDS and fluconazole-refractory oropharyngeal Candidiasis were recruited and tested over a 4-week period to investigate the efficacy of alcohol-based and alcohol-free TTO oral solution on clinical lesions of oral Candidiasis. Patients were categorised into two cohorts, with cohort 1 being treated with 15 mL of an alcohol-based TTO oral solution and cohort 2 being treated with 5 mL of an alcohol-free TTO oral solution 4 times daily. The results showed that both oral solutions of TTO demonstrated good and equal antifungal efficacy rates of over 60% in the patients. However, the alcohol-free TTO solution had fewer side effects (such as a burning sensation) than the alcohol-based TTO solution. A *Cinnamomum zeylanicum* (cinnamon) EO is also a promising agent for oral Candidiasis treatment. A randomised, controlled, and blinded clinical trial conducted by Araujo et al. [109] also reported that *C. zeylanicum* EO exhibited clinical efficacy in treating DS and in reducing *Candida* spp.

On the contrary, some clinical trials have reported that some EOs did not demonstrate superior antifungal effects to the positive controls used in the studies [110,111]. In the randomised, single blind, controlled study by Chalhoub et al. [111], which lasted 45 days, 25 elderly participants (aged 65 or over) with a significant loss of autonomy were recruited and divided into two treatment groups to investigate the effectiveness of an alcohol-free essential oil mouthwash (AF-EOMW) (Listerine Zero; Johnson & Johnson) in reducing plaque accumulation and oral levels of pathogens, including *C. albicans*. For the duration of the trial period, treatment group 1 rinsed their mouth with the AF-EOMW whilst group 2 rinsed their mouth with a water control. While no significant difference was observed between the two groups, the trial highlighted various limitations, such as the small sample size, several sources of bias, an undisclosed EO used, and the withdrawal of participants during the study. Additionally, as the study stated its actual primary objective was to evaluate the feasibility of conducting the trial, it is necessary for a well-designed, controlled, large sample study on AF-EOMWs to be conducted in order to draw firmer conclusions on its effectiveness in inhibiting oral pathogens such as *C. albicans*.

7. Conclusions

In summary, there is an increasing demand for natural therapies and rising need for clinical research on various EOs, as empirical evidence suggests these natural compounds could be highly efficacious as antifungal agents. This is in part due to the high therapeutic efficiency and low toxicity in the treatment of different fungal infections caused by *Candida* spp. Of particular note are current studies that demonstrate that EOs seem to have a wide range of cellular targets. Therefore, numerous EOs should be considered as a potential anti-Candida agent, especially for the treatment of drug-resistant Candida strains. In addition, EOs could be used in combination with conventional drugs to increase the therapeutic efficacy and decrease the side effects and toxicity that may be observed in recovering patients. Many clinical studies have reported that the usage of EOs is effective in vulvovaginal Candidiasis and oral Candidiasis treatment. However, more in vivo studies are required to validate the potential clinical use of EOs as an alternative antifungal therapy.

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