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In Vitro Antifungal Activity of Melaleuca alternifolia Essential Oil against Clinical Isolates of Candida Species from the Oral Mucosa: Current Status and Challenges

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Abbreviations: CLSI: Clinical Laboratory Standards Institute; EUCAST: European Committee on Antimicrobial Susceptibility Testing; MIC: Minimum Inhibitory Concentration; DMSO: Dimethyl Sulf-oxide; MFC: Minimum Fungicidal Concentration.

Introduction

Among the microorganisms present in the oral cavity, fungi can be highlighted, not only as opportunistic pathogens but also as members of the oral microbiota [1,2]. In a detailed characterization by Ghannoum MA, et al. [3] it was observed that about 100 species of fungi are present in the oral cavity of healthy people, ranging from 9-23 species per individual, and Candida was the most frequent occurring genus [2].

However, certain predisposing factors can make *Candida* pathogenic, leading to several diseases. Among these diseases, oral candidiasis (OC) displays different clinical manifestations, such as pseudomembranous candidiasis, hyperplastic candidiasis, acute atrophic candidiasis, and chronic atrophic candidiasis [3]. In addition, *Candida* can also be associated with prosthetic stomatitis, a chronic disease that affects the palatal mucosa in people using complete or partial dentures. In these cases, *Candida albicans* is one of the main etiologic agents, although other *Candida* species, such as *Candida glabrata* or *Candida tropicalis*, may be involved as well [4].

For oral candidiasis, therapy usually involves the use of topical antifungals such as nystatin or miconazole, which are useful for the treatment of initial episodes. Nevertheless, most patients suffer from recurrences or new episodes of candidiasis, making the use of systemic antifungals such as fluconazole or itraconazole, necessary [4]. This recurrence may be related to intrinsic or acquired resistance from some *Candida species* [5].

Therapeutic management of mucocutaneous infections by *Candida* is mostly performed with allopathic antifungals, which are scarce, compared to the development and availability of synthetic antibiotics [6]. On the contrary, despite the scarcity of antifungals, a substantial increase in infections by *Candida* spp. that are resistant to commonly used antifungals, has been observed as well [7]. Therefore, new drugs, based mainly on natural products, are being studied as potential antifungal agents [6,8]. Medicinal plants and their derivatives are the sources of more than 50% of the medicines currently under use [9,10]. Yet, it is estimated that about 80% of the population use plants or plant-based preparations in primary health care [9].

Melaleuca alternifolia (tea tree) is a plant native to Australia and has been used in traditional medicine for centuries. Tea tree essential oil is a common ingredient in formulations used to treat cutaneous infections and is widely available over-the-counter in Australia, Europe, and North America, marketed as a remedy for several diseases [11]. Tea tree oil is well known for its applicability against viruses, bacteria, and fungi [11]. Taking into account the shortage of drugs for the treatment of mucocutaneous candidiasis and resistance to compounds commonly used

for this purpose, the search for new therapeutic options is of utmost importance.

In vitro Assessment of Antifungal Activity

A variety of laboratory methods can be used to evaluate the *in vitro* antifungal activity of tea tree oil, like agar diffusion, disk diffusion, and poisoned food and broth micro dilution, making it difficult to compare results by different research teams. Therefore, this study only discusses investigations that used the broth micro dilution method, standardized by two distinct organizations: Clinical Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST). It is worth mentioning that these documents were made available in 2002 and have been through some adaptations in the last decade [12]. Furthermore, it must be noted that the broth micro dilution method is the gold standard for *in vitro* antifungal susceptibility testing.

Essential oils are volatile, hydrophobic, viscous, and composed of dozens of naturally occurring organic compounds from different classes. One of the first challenges scientists face while executing the broth micro dilution test, to verify tea tree antifungal activity, is that it's highly hydrophobic and won't mix with the growth medium or water unless a third substance is used to make the solution homogeneous. Because of that, different solvents or surfactants are also used to form the emulsion between the water and the essential oil during the susceptibility tests [13]. The standardized solvents mentioned in the CLSI and EUCAST documents, for diluting antifungal drugs, are water and Dimethyl sulfoxide (DMSO). Thus, one of the alternatives to solubilize tea tree essential oil would be DMSO, but prior literature shows that common solubilizing agents also include Tween 20 and Tween 80. Therefore, it is necessary to choose a solubilizing agent that allows the minimum inhibitory concentration (MIC) or minimum fungicidal concentration (MFC) to be determined more effectively, forming a homogeneous emulsion with the tested compounds. This is crucial, especially regarding the broth microdilution method, since through serial dilutions, a heterogeneous initial solution may lead to indeterminate oil concentrations in the subsequent dilutions [13].

Investigating prior literature, some studies show that the use of solvents and their final concentration interfere with inhibitory and fungicidal values since these compounds can interact with the components of the oil or even with the microorganisms, altering its susceptibility [14-16]. That is, if, on the one hand, the use of solvent or surfactant causes the oil to be soluble in the culture medium, ensuring dispersion and emulsification, on the other hand, two variables that can interfere with the inhibitor values are also gained: the agent used as a surfactant, and the concentration of that agent [17].

Another important variable is the chemical profile of the oil. Because of that, chromatographic analysis is important to detect substances that characterize the adulteration of the oil [18]. Examples of common adulterations are mixtures of synthetic substances or by-products of other cheaper essential oils, such as eucalyptus oil (*Eucalyptus globulus*), pine tree oil (*Pinus spp.*), and camphor oil (*Cinnamomum camphora*). This lowers oil production costs and generates a higher percentage of profit for the company [19]. However, this can reduce the antifungal activity of tea tree oil.

However, the minimum and maximum values of the compounds present in tea tree oil are regulated as per ISO 4730: 2017. According to this regulatory standard, the highest concentrations of compounds present must include terpinem-4-ol, at 35% to 48% concentration, followed by γ-terpinene, at 14% to 28% concentration. In addition to these, other components also have their range regulated. It is known that variation within the minimum and maximum values established by ISO 4730 is common since the constituents of the oil vary according to the external factors the tree was exposed to during its cultivation, such as weather, temperature, availability of water, and soil cultivation [20]. Therefore, the composition of the oil is another variable that can change the minimum inhibitory concentrations noted by investigators [21].

Even though the MIC and CFM values vary according to factors previously discussed and the species, tea tree oil shows promising fungi static and fungicidal activity against isolated *Candida* strains. That tea tree oil was fungi static against all the 91 *Candida* strains tested, with an MFC90 of 10%. Tea tree oil was also found to be effective against *Candida* strains that showed fluconazole resistance, with a MIC range of 0.07-0.15%. In a study conducted by Ninomiya K, et al. [21], the MIC of tea tree oil against a clinically isolated strain of *Candida* was 20 mg/mL, and that against an azole-resistant strain, was 5 mg/ml. Both were *C. albicans* strains isolated from patients with AIDS.

Nikolić MM, et al. [22] also studied the antifungal activity of tea trees against *C. albicans*, *Candida krusei, and C. glabrata*, but found it to have a lower antifungal activity as compared to its antibacterial properties. The MIC values were 2250-9000μg/mL and the MFC values were 4500-18000μg/mL.

Francisconi RS, et al. [23] investigated the antimicrobial activity of tea tree oil against both planktonic cultures and biofilms of *C. albicans* [24]. The concentration of tea tree oil that inhibited growth in planktonic cultures (MIC) of clinical strains of *C. albicans* was 1%. In the same study, *Candida* biofilms grown on denture base acrylic resin specimens were exposed to 2% tea tree oil for 60 seconds, simulating the clinical application of mouthwash. A confocal microscope

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was used to examine the biofilm species, showing a non-contiguous layer of cells and reddish-yellow coloration, indicating the presence of non-viable cells. A similar result was obtained on biofilms that were exposed to nystatin.

Nonetheless, it is impossible to compare the inhibitory values obtained by various studies, since different methodologies were applied by each. Francisconi RS, et al. [23] used similar methodological designs, evaluating a total of 95 clinical isolates from the oral cavity. The second study determined the MIC and CFM values of 4 isolates of *C. albicans* by broth microdilution method in RPMI medium with 0.4% DMSO for oil solubilization also determined MIC and MFC values through the broth microdilution method in RPMI but did not specify the DMSO concentration used. The results of these studies show that even almost identical methodologies can produce different MIC values, probably due to the oil used, the clinical isolate tested, or the concentration of the solvent present.

Different species also have different susceptibilities, so it's crucial to understand that tea tree oil may not have the same antifungal effect on every isolate. Studies have shown that *C. glabrata* exhibited reduced sensitivity to tea tree oil, as compared to *C. albicans, C. krusei, Candida tropicalis, Candida dubliniensis, and Candida parapsilosis* [23-25]. Nevertheless, these are still initial data as the number of clinical strains of *Candida* apart from the *C. albicans* species in these studies is low. This is because *C. albicans* is still the most frequent species isolated from the oral mucosa, and hence the results concerning its MIC when exposed to tea tree oil are also more accurate.

Mechanism of Action of Melaleuca Alternifolia

Cox SD, et al. [24] evaluated the membrane permeability of *C. albicans* against tea tree oil. The study found that the membrane permeability of C. *albicans* was disrupted when it was exposed to 0.25% concentration of tea tree oil for 30 minutes. The exposure to tea tree oil allowed propidium iodide staining of *C. albicans* cells, hardly seen in the control group, which didn't receive any tea tree oil treatment. Cellular respiration of the fungus was also inhibited by the oil in a concentration of 0.125%, which was further determined using an oxygen electrode. The study, therefore, suggests that tea tree oil directly inhibits a specific respiratory enzyme or a metabolic process in *Candida*.

Hammer KA, et al. [25] obtained similar results while investigating possible changes in the permeability and fluidity of the *C. albicans* membrane when treated with tea tree oil. The study concluded that tea tree oil and its components affected the membrane properties and its integrity, and was able to inhibit the acidification of the medium as well. It is important to emphasize that two isolated components of the oil, 1,8-cineole, and terpinolene, caused major changes in the membrane fluidity but did not increase the permeability of Methylene Blue dye, which was used as a marker in the *in vitro* assay. On the other hand, 0.25% terpinen-4-ol caused a significant increase in the permeability of Methylene Blue into the fungal cell but did not change the membrane fluidity. These discrepancies cannot be completely elucidated yet, but different components of tea tree oil are considered to vary in their modes of action, causing the oil to have multiple antifungal mechanisms.

Conclusion

The antifungal activity of *Melaleuca alternifolia* essential oil has been investigated by several researchers, however, most studies are still in vitro. It has been observed that almost all isolated strains from the oral mucosa were susceptible to tea tree oil, even at low concentrations. However, there is no standardized test to measure the antimicrobial activity of essential oils, which prevents homogeneity in methodological analysis, and consequently, prevents direct comparison of results from studies that used different methodologies. It is also evident that the solvent and its concentration, as well as the chromatographic profile of the oil, result in different inhibitory values. In this context, we concur that more clinical and laboratory studies are needed, using standardized susceptibility methods, for the complete elucidation of the antifungal potential of tea tree and other essential oils.

Competing Interests

The author declared no competing of interest.

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