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Effect of *Melaleuca Alternifolia* and Its Components on *Candida Albicans* and *Candida Tropicalis*

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Abstract: The essential oil of *Melaleuca alternifolia* is an herbal antifungal action and preventive scale pharmaceutical or cosmetic. The aim of this study was to evaluate the efficacy of *Melaleuca alternifolia* (tea tree oil—TTO) and its component (Terpinen-4-ol) on *Candida albicans* and *Candida tropicalis* in planktonic and biofilm form of dual species. MIC (Minimum Inhibitory Concentration) and MFC (Minimum Fungicidal Concentration) were determined of single species. Biofilms were quantified in CFU (colony forming unit per mL) and XTT {2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide} cell viability assay in single and multiple species. MIC was TTO (0.25%) and Terpinen-4-ol (0.11%) for both species. The MFC for *Candida albicans* showed TTO (0.25%) and Terpinen-4-ol (0.11%) were capable of inhibiting the growth and for *Candida tropicalis* was 0.50% and 0.24%, respectively. The results on biofilm for *Candida albicans* in single species and multiple species showed TTO (0.5%) and Terpinen-4-ol (0.24%) were effective. For *Candida tropicalis* in single species, treatments with TTO (0.5%) and Terpinen-4-ol (0.47%) were effective. For multiple species, the treatments with TTO (0.5%) and Terpinen-4-ol (0.24%) were also effective. In the XTT test, for *Candida albicans* in single species: TTO (0.25%) and Terpinen-4-ol (0.24%). For *Candida tropicalis*, the results were TTO (0.5%) and Terpinen-4-ol (0.11%), while for multiple species were TTO (0.25%) and Terpinen-4-ol (0.11%). Based on these factors, *Candida* spp. are susceptible to the antimicrobial agents TTO and Terpinen-4-ol, suggesting that these compounds might be beneficial as therapeutic.

Key words: *Melaleuca alternifolia* oil, biofilm, *Candida albicans, Candida tropicalis.*

1. Introduction

The interest in natural products is increasing because of their wide use and knowledge [1]. These products are being evaluated as treatment to fight infections due to resistance to existing drugs, ease of preparation, use and demand for lower costs [2].

These products are known as herbal remedies and have been shown to be potentially interesting with regard to their antimicrobial [3, 4] or antifungal activity [5]. Among them, the essential oil of *Melaleuca alternifolia*, also known as TTO (tea tree oil), stands out due to its broad spectrum of action [6]. In Australia, it is commonly used as a topical therapeutic agent and its medical use is primarily related to its anti-inflammatory [7, 8] and antimicrobial [9, 10] actions. The use as a topical analgesic agent is supported by an increasing clinical data indicating that TTO is effective in the treatment of infections or diseases such as herpes [11], acne [12], dandruff [13] and oral candidiasis [14-16].

Moreover, several studies have characterised the in vitro activity and mechanism of action of TTO oil against bacteria [9-17] and fungi [18-21]

The oil is composed of approximately 100 components [22], with the main ones being Terpinen-4-ol, γ-Terpinene, α-Terpineol, 1,8-Cineole and α-Pinene [23]. The antimicrobial activity of the oil is mainly attributed to Terpinen-4-ol which is present in the oil at a level of 30%-40% [1], and 1,8-Cienole acts to increase the permeability of the membrane which facilitates the penetration of other antimicrobial

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agents [24-25]. α-Terpineol is known for its antioxidant activity and has also demonstrated antimicrobial activity [26].

Based on the literature presented, TTO can be considered an effective herbal medicine and can work against various microorganisms. However, further studies are still needed in relation to their actions, interactions with other drugs and toxicity on mucosal epithelial cells. The antifungal effect of the oil should be analysed to determine the optimal concentration for use in fungal infections. Similarly, the soluble portions should be studied to determine their effectiveness and the concentration to be used on oil composition. Thus, with increasing knowledge of actions and reactions, the oil may have wide applicability in the clinical area.

Therefore, the objective of this study was to evaluate *in vitro* the antifungal effect of TTO and Terpinen-4-ol against *Candida* spp. planktonic cells and biofilm.

2. Methods

2.1 Strains

Reference *C. albicans* (American Type Cell Culture-ATCC 90028) and *C. tropicalis* (ATCC 4563) strains were selected for this study.

2.2 Preparation of Yeast Strains Suspension

Prior to each experiment, the yeasts strains were cultured aerobically at 37 ℃ for 21 h in Tryptic Soy Broth (TSB, Acumedia, Lasing, MI, EUA). After incubation, the cells were harvested, washed twice with phosphate buffered saline (PBS, pH 7.2) and resuspended in TSB. Reference *Candida* suspensions were prepared to a concentration of 10^7 cells/mL by adjusting the OD (optical density) using a spectrophotometer (Eppendorf AG, 22331 Hamburg, Germany) at the absorbance of 600 nm.

2.3 TTO and Components

The composition of TTO (Sigma, MO, USA) used in this study was analysed by gas chromatography at the Institute of Chemistry of São Carlos—USP for quality control of the oil according to ISO 4730. The portions of soluble TTO used were Terpinen-4-ol (Sigma, MO, USA). For the use in the experiments, dilutions of TTO were made to concentrations of 2%, 1%, 0.5%, 0.25% and 0.12% in TSB. The concentrations of Terpinen-4-ol were 0.95%, 0.47%, 0.24%, 0.11%. Dimethyl sulphoxide (DMSO, Sigma, MO, USA) at a final concentration of 0.4%, was added in order to increase the solubility [18]. Nystatin (Medley, Campinas, Brazil), the gold standard for treating fungal infections, was used for comparison of the efficacy of TTO and its soluble portions. The solution was prepared with 1 mL of Nystatin to 1 mL of TSB.

2.4 Minimum Inhibitory Concentration Determination

The MIC was determined using the method according to microdilution broth method M27-A2 determined by the CLSI (Clinical and Laboratory Standards Institute). This test was performed using a 96 well cell culture plate. For each experiment, nine wells (n = 9) and control groups were used to determine the MIC. Each well was filled with 200 μl of TSB broth containing oil solutions and 2 μl of the inoculated broth with reference strains; the plate was incubated at 37 ℃ for 24 h. Wells without a solution of oils were used as controls, but these contained the microorganism to be evaluated. Wells without fungus were used as negative controls. Thereafter, the wells were visually examined to confirm the presence of turbidity.

2.5 Minimum Fungicidal Concentration Determination

For wells which showed no visible growth (OD 600nm ≤ 0.05), 10 μl of the sample was removed and plated in culture medium Saboraude Dextrose Agar (SDA, Acumedia, Lansing, MI, USA); this was maintained at 37 ℃ for 48 h. The MFC was defined as the lowest concentration able to reduce the initial inoculum ≥ 99.9%.

2.6 Biofilm Development

Biofilm formation was performed as described by Thein, Smaranayake and Smaranayake [27]. The biofilm formation was performed with single species of microorganism (SSB: single-species biofilms), *C. albicans* or *C. tropicalis*, and two species biofilm (DSB: dual-species biofilms), *C. albicans* and *C. tropicalis*, in association. Cultures were reactivated as mentioned above. For each experimental group and control, eighteen wells (n = 18) of the 96-well cell culture plate were used for the formation of biofilms. For the formation of DSB, aliquots of 50 µl of standard cell suspensions of yeast *C. albicans* and *C. tropicalis* were transferred to the same well, and for the SSB, aliquots of 100 µl of each yeast were transferred for the wells and incubated for 1.5 h (adhesion phase) at 37 ℃ at 75 rpm (rounds per minute) in an orbital shaker. After the adhesion phase, the cell suspensions were gently aspirated and each well was washed twice with PBS to remove any remaining planktonic cells, taking care not to disturb the adhered cells. In order to allow the growth of the biofilm (biofilm phase), 100 µl of TSB was added to each well. The plates were incubated for 48h at 37 ℃ at 75 rpm in an orbital shaker. After incubation, the medium was aspirated and biofilms were washed twice with PBS. Aliquots of 100 µl of TTO and its components were added to the wells and incubated for 24 hours at 37 ℃ at 75 rpm. Biofilms were quantified using the CFU and XTT reduction assay.

2.7 Quantification of the Biofilm

The viable fungal cell counting method was used to evaluate the effect of oil on biofilm formation. The biofilms were washed twice with PBS. After, 100 µl of PBS were transferred for the wells and the biofilm was scraped carefully and the suspensions obtained gently sonicated for 1 min to destroy aggregates. The suspensions were diluted and plated on CHROMagar (CHROMagar *Candida*, Difco, Sparks, MD, USA) when the solution contained two microorganisms and plated on SDA when the solution contained one microorganism. The plates were incubated for 48 h at 37 ℃. The results were quantified by CFU/mL.

2.8 Analysis of Biofilm Viability (XTT Test)

XTT reduction assay was performed as previously described [28]. XTT salt (Sigma, MO, USA) was dissolved in PBS at a final concentration of 1 mg/mL. This solution was filter-sterilized and stored frozen at -80 ℃ until use. Menadione solution (Sigma, MO, USA) was prepared in acetone at a concentration of 0.4 mM prior the experiment. The biofilms were washed twice with 100 µl of PBS. Next, 200µl of the XTT solution (with 158 µl of PBS with 200 mM glucose, 40 µl of XTT and 2 µl of diluted Menadione) was added. The plates were incubated in dark at 37 ℃ for 3 h. The colorimetric changes were measured at 492 nm using a spectrophotometer (ELX 800-Universal Microplate Reader; Bio-Tek instrument, Inc., VT, EUA).

2.9 Effect of Nystatin on SSB and DSB

To analyse the effect of Nystatin in the formation of SSB and DSB of *C. albicans* and *C. tropicalis* within 48h of formation, the culture medium was replaced with 100 mL of solution of Nystatin, previously prepared with TSB. 1mL of Nystatin was used for 1mL of TSB. Wells without Nystatin solution were used as negative control. The plates were incubated at 37 ℃ for 24 h, after which the effect of biofilm on the Nystatin was analysed by quantification and analysis of cell viability, was performed using the XTT method. This test was performed to compare the results of TTO with a substance that is often used in the treatment of *Candida* spp. infection.

2.10 Statistical Analysis

To assess the significance of the effect of TTO and it component (Terpinen 4-ol) in different concentrations on biofilm of *Candida albicans* and *Candida tropicalis*, a factor ANOVA (one way) was carried out followed by a post-hoc Tukey test. Thus, the data obtained by

CFU/mL after processing in Log (CFU + 1/mL), and values obtained by XTT test were analysed according to the conditions of one-way ANOVA. For this, the outliers were removed and the normality and homogeneity of variances were tested with the Shapiro-Wilkes and the Levene test, respectively. The statistical analysis was carried out using the software PASW Statistics (v. 17, SPSS Inc., Chicago, IL) with the significance level at $\alpha = 0.05$.

3. Results

3.1 MIC (Minimum Inhibitory Concentration Determination) and MFC (Minimum Fungicidal Concentration) Determination

The concentrations of TTO and Terpinen-4-ol were equal for *Candida albicans* and *Candida tropicalis*. The results were: TTO 0.25% and Terpinen-4-ol 0.11%. TTO and Terpinen-4-ol demonstrated fungicidal activity against both species. The results of MFC for *Candida albicans* were TTO 0.25% and Terpinen-4-ol 0.11%, and for *Candida tropicalis* were TTO 0.50% and Terpinen-4-ol 0.24%.

3.2 Quantification of Biofim (CFU/mL)

The actions of Nystatin, TTO and Terpinen-4-ol upon biofilm growth are presented in Fig. 1. As can be seen, biofilm exposure to components resulted in significant fungal death. Treatment with TTO and Terpinen-4-ol gave better results than treatment with Nystatin. Considering the results obtained for *C. albicans* as a single species, the lower concentration that differed from the control were TTO (0.5%) and Terpinen-4-ol (0.24%). Nystatin differed from the control. For *C. albicans* in multiple species, the results were TTO (0.5%) and Terpinen-4-ol (0.24%). For *C. tropicalis* in a single species, the effective concentrations were TTO 0.5% and Terpinen-4-ol 0.47%. For multiple species, TTO (0.5%) and terpinen-4-ol (0.24%) were different from the control. Nystatin showed antimicrobial activity, but this was lower than that of TTO and Terpinen-4-ol.

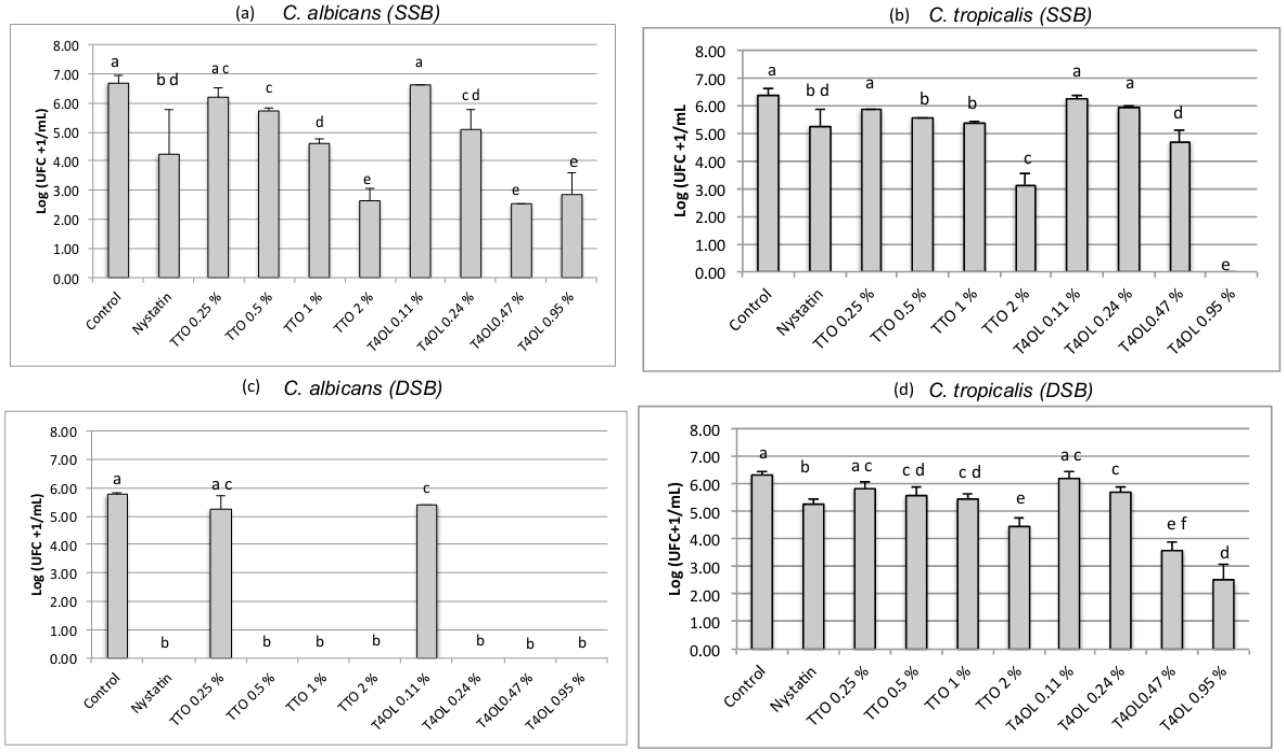

Fig. 1 Anti-Candida activity (CFU/mL) of TTO, Terpinen-4-ol and Nystatin on biofilm of *Candida albicans* (SSB) (a), *Candida tropicalis* (SSB) (b), *Candida tropicalis* (DSB) (c), *Candida tropicalis* (DSB) (d). *Different letters denote statistically different results.

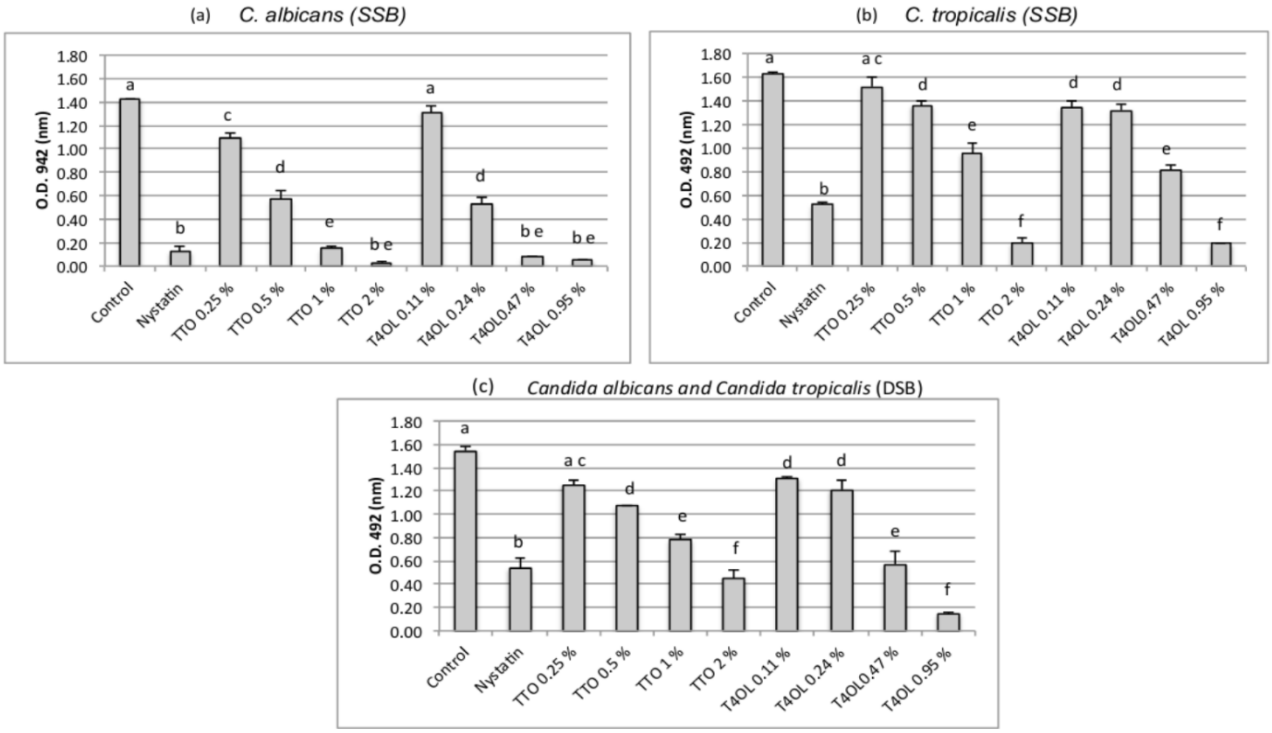

Fig. 2 Anti-Candida activity (XTT) of TTO, Terpinen-4-ol and Nystatin on biofilm of *Candida albicans* (SSB) (a), *Candida tropicalis* (SSB) (b), *Candida albicans* and *Candida tropicalis* (DSB) (c). *Different letters denote statistically different results.

3.3 Analysis of Biofilm Viability (XTT Test)

Treatment with essential oils showed a decrease in the viability of microrganisms. The results are shown in Fig. 2. When *Candida albicans* was treated with TTO (0.25%) and Terpinen-4-ol (0.24%), a decrease in viability was observed. Against *Candida tropicalis*, the best results were for TTO (0.5%) and Terpinen-4-ol (0.11%). For double biofilm, the effective treatments were found to be with TTO 0.25% and Terpinen-4-ol 0.11%. *Candida* spp. were all sensitive to treatment with nystatin.

4. Discussion

Oral candidosis is a common opportunistic infection among patients with immunological problems or prosthesis users; therefore, it is important to treat this effectively [29]. For the treatment of fungal infections, there are several drugs on the market, such as nystatin and fluconazole; however, with candidosis recurrence in these patients and the frequent use of these drugs, populations of resistant fungi are occurring.

Several authors have studied the action of diverse components on microrganisms of the oral cavity [30-32]. There is, therefore, a need to find new approaches to treat infections in an era of emerging infections and the spread of antimicrobial resistance; as a result, alternative products for these treatments are sought [33]. This line of research found that TTO is one of the natural products used for treatment of fungi, especially *C. albicans* [6].

In the literature, there are several reports of tea tree oil acting as an antifungal agent in tests with planktonic microorganisms. However, there are few reports on the effect of oil on biofilm. Therefore, in the present study, the essential oil TTO and its components (Terpinen-4-ol), with previously known antimicrobial activity, were evaluated against planktonic cells and biofilm of *Candida* spp..

The results of MIC and MFC of TTO in planktonic cultures of *C. albicans* and *C. tropicalis* demonstrate that TTO is effective against the fungi in suspension. The concentrations found are similar to those described in the literature [19-22].

The composition of TTO includes terpenes such as monoterpene alcohols. Its function has been attributed to their interactions with cellular membranes. Using appropriate methods, Hammer et al. [22] identified that most components of TTO are active against *C. albicans* and other fungi. This contradicts several earlier reports [23, 24] and challenges beliefs on various attributes and properties of the components of Melaleuca. TTO contains a single active component, Terpinen-4-ol; many of the other components still need to be better exploited.

Hammer et al. [22] tested TTO and quantified the results on *C. albicans* biofilms. The results show that at concentrations of 0.5% there was a decrease in the amount of microorganisms present; however, at the concentration of 1%, it was the most effective. In our study, the best concentration was 2% for all species biofilms.

One important finding of this study and of other published papers [6] is the effectiveness of Terpinen-4-ol against *Candida* spp. in planktonic form and biofilms. Mondello et al. [6] reported that the oil is composed of various substances, with terpinen-4-ol and 1,8-cineole being the main components. In their studies, they demonstrated in vitro antifungal activity to *C. albicans* and *C. tropicalis* using TTO, terpinen-4-ol and 1,8-cineole. To analyse the effect of TTO, MIC and MFC was performed resulting in 0.25% to four different strains ATCC of *C. albicans* and 0.06% to *C. tropicalis*. To analyse the terpinen-4-ol and 1,8-cineole, MIC were determined by the broth microdilution method. We should emphasise the effectiveness of terpinen-4-ol, which in the result of the MIC for *C. albicans* was 0.045% on average and *C. tropicalis* was 0.03%; the CFM was 0.108% to 0.125% on average for *C. albicans* and *C. tropicalis*, respectively. The study suggests that terpinen-4-ol is a mediator of TTO activity.

Moreover, the action of TTO on biofilm was analysed using XTT. In this analysis, where the purpose was to verify the viability of the biofilm on various oil concentrations, the results show that with increasing concentrations, the viability of the biofilm decreased, and with a concentration of 2% viability was almost zero. The concentration of 1% TTO and 0.47% terpinen-4-ol resulted in a significant decrease in biofilm. The treatments were as good as Nystatin, which is the antifungal that is considered the gold standard for treating diseases caused by fungi.

Further studies are needed before this oil may be used to treat fungal infections; for example, cytotoxicity tests and drug interactions. Hammer et al. [35] analysed contact allergy and systemic problems of TTO and stated that although TTO is not considered a toxic product, it can generate changes if used incorrectly, such as via high local concentrations and improper applications.

Studies have shown that the oil is effective for the treatment of infections [9, 10] but it is unclear whether TTO is able to interfere in the mechanisms of adhesion of the fungus during the biofilm development. Hammer et al. [22] suggest that the oral cavity has exogenous proteins that can interfere with the effect of TTO, which may play a role.

The results of this in vitro susceptibility study suggest that TTO and its components should be considered potential therapeutic agents against fungal infections.

5. Conclusion

Although TTO is still widely used as an alternative treatment, there are many studies in vitro and in vivo that are proving the effectiveness of oil for the treatment of fungal infections. The results presented here demonstrated the potential of the antifungal activity of this essential oil and its main component (Terpinen-4-ol), arousing interest for drug development.

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