Original Research
Periodontics

Antibiofilm efficacy of tea tree oil and of its main component terpinen-4-ol against *Candida albicans*

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Abstract: Candida infection is an important cause of morbidity and mortality in immunocompromised patients. The increase in its incidence has been associated with resistance to antimicrobial therapy and biofilm formation. The aim of this study was to evaluate the efficacy of tea tree oil (TTO) and its main component – terpinen-4-ol – against resistant *Candida albicans* strains (genotypes A and B) identified by molecular typing and against *C. albicans* ATCC 90028 and SC 5314 reference strains in planktonic and biofilm cultures. The minimum inhibitory concentration, minimum fungicidal concentration, and rate of biofilm development were used to evaluate antifungal activity. Results were obtained from analysis of the biofilm using the cell proliferation assay 2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) and confocal laser scanning microscopy (CLSM). Terpinen-4-ol and TTO inhibited *C. albicans* growth. CLSM confirmed that 17.92 mg/mL of TTO and 8.86 mg/mL of terpinen-4-ol applied for 60 s (rinse simulation) interfered with biofilm formation. Hence, this *in vitro* study revealed that natural substances such as TTO and terpinen-4-ol present promising results for the treatment of oral candidiasis.

Keywords: Biofilms; *Candida albicans*; Mycoses; Phytotherapy.

Introduction

Candida albicans is among the most prevalent fungal species of the human microbiota. It is also an opportunistic pathogen that can cause severe, and often fatal, bloodstream infections.[1] However, among its different virulence factors, the initial adhesion, with biofilm formation on abiotic and biotic surfaces, plays a critical role in pathogenesis.[2] Drug resistance is becoming increasingly concerning, and the search for new agents may lead to the development of new antifungal agents that are effective against biofilms. Traditional medicinal plants and alternative therapies have been attractive sources for identifying new antimicrobial agents.[3]

Natural products are considered an important source for the development of new antifungal therapies. Several studies have shown antimicrobial activity of these products on oral pathogens, showing that these herbal agents can be vehicles for prevention and control of infectious oral

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diseases.[4, 5] *Melaleuca alternifolia*, of the botanical family Myrtaceae, is a plant native to Australia also known as tea tree. The steam distillation of its leaves produces an oil known as tea tree oil (TTO). TTO is a mixture of approximately 100 compounds, including terpinen-4-ol (at least 30% of the oil), 1,8-cineole, and terpinolene.[5] TTO demonstrates a broad spectrum of biological activities, including a wide variety of microorganisms that can be used to improve strategies for the treatment of chronic infections such as candidiasis, pharyngitis, vaginitis, and respiratory tract diseases. TTO acts within biological membranes, damaging their integrity and inhibiting the action of enzymes incorporated to increase membrane fluidity, with subsequent leakage of intracellular components.[6] According to Cox and Markham,[6] chemical constituents are characteristically hydrophobic and will accumulate in the lipid-rich environments of cell membrane structures and cause structural and functional damage.

The International Standard Organization[7] regulates the concentration ranges of the major TTO terpenes and related alcohols and ethers, including directives on the levels of these components: the minimum content of terpinen-4-ol should be at least 30% and the maximum content of 1,8-cineole should be less than 15% of the oil volume. Terpinen-4-ol is the main active compound within TTO and has gained attention because of its antimicrobial, antifungal, and anti-inflammatory properties.[8] Terpinen-4-ol is known to induce membrane loss, disrupting the integrity and physiology of microbial cells. Low concentrations of terpinen-4-ol are not toxic to fibroblasts and epithelial cells, allowing for topical use with reduced adverse effects.[9,10,11,12]

The aim of this study was to evaluate the efficacy of TTO and of its main component – terpinen-4-ol – against resistant clinical isolates of *Candida albicans* strains and *C. albicans* ATCC 90028 and SC 5314 reference strains in planktonic and biofilm cultures.

Methodology

Melaleuca alternifolia Cheel (tea tree) oil and its components

TTO composition was analyzed by gas chromatography in compliance with ISO 4730.[7] To prepare the samples, 1 µL of TTO was dissolved in 1 µL of ethanol and injected into an HP35 capillary column with carrier gas at a flow rate of 0.8 mL/min. The temperature of the injector and detector was 250ºC. The temperature regimen was as follows: initial temperature of 80ºC for 2 min, increase by 10ºC/min to 250ºC, and constant temperature for the final 10 min.[13] The concentrations were determined at the time of fractionation. Gas chromatography showed TTO complied with ISO 4730:2017[7] and the relative concentrations of the components showed terpinen-4-ol was present in 47.66%. Terpinen-4-ol was purchased from Sigma-Aldrich.

Tea tree oil and terpinen-4-ol: stock solutions

The following stock solutions of TTO were prepared: TTO 2.24 mg/mL – 17.92 mg/mL and terpinen-4-ol 1.02 mg/mL – 8.86 mg/mL (Sigma-Aldrich, St. Louis, MO, USA) were diluted in Roswell Park Memorial Institute (RPMI 1640) culture medium (Sigma-Aldrich) at pH 7.0 with 0.165 M of 3-(N-morpholino) propanesulfonic acid (MOPS) and 0.4% of dimethyl sulfoxide (DMSO) as a solubilizing agent.[8] The standard powder used to prepare nystatin was dissolved in DMSO. The nystatin stock solution (500 µg/mL) was kept at -70°C until use.

Candida albicans strains

This study included two *C. albicans* clinical strains previously identified by molecular typing as genotypes A and B, isolated from diabetic patients with chronic periodontitis (Ethics Committee process no. 062/2008).[14] *C. albicans* ATCC 90028 and SC 5314 reference strains were also used.

Preparation of the initial *Candida albicans* suspension

Prior to each experiment, the yeast strains were cultured in RPMI at 37ºC for 18 h. After incubation, the cells were harvested, washed with phosphate-buffered saline (PBS) at pH 7.2, and resuspended in RPMI. All *C. albicans* suspensions (10^7 CFU mL^{-1}) were prepared by adjusting the optical density (OD) at 600 nm (Multiskan Ascent 354, EC Labsystems, Les Ulis, France).

Determination of minimum inhibitory concentration and minimum fungicidal concentration values for TTO and terpinen-4-ol

The activity of TTO and terpinen-4-ol against the *C. albicans* strains tested was determined by the broth microdilution method (M27-A, 2008).[15] TTO and terpinen-4-ol were serially diluted in RPMI 1640 with 0.4% DMSO. The same volume of the *C. albicans* suspension was added to each well to obtain a final density of 1.0×10^3 CFU mL^{-1}. RPMI medium with DMSO and *C. albicans* and nystatin (without TTO and terpinen-4-ol) were used as control groups. After 24 h of incubation at 37 °C, cell growth was assessed visually and by absorbance at 590 nm.

The MIC was defined as the lowest concentration of TTO and terpinen-4-ol that led to no visible growth of the cell strains tested. The MFC value was defined as the lowest concentration of TTO and terpinen-4-ol that showed no growth of *C. albicans* colonies. To determine the MFC, the samples were seeded onto Sabouraud dextrose agar (SDA) plates and maintained thereon at 37°C for 48 h. All assays were performed in triplicate as independent experiments.

Metabolic activity of *Candida albicans* biofilm

XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) (Sigma) assay was used to determine the metabolic activity of the biofilm.[16] Biofilms were allowed to form on microtiter plates and after 24 h of exposure to TTO, terpinen-4-ol, and nystatin. The solutions and non-adherent cells were removed by washing twice with sterile PBS. The biofilms were then treated with 158 µL of PBS containing 200 mM of glucose, 40 µL of XTT, and 2 µL of diluted menadione. After incubation with the XTT solution, absorbance was recorded at 490 nm.

Biofilm growth inhibition

Biofilm was allowed to grow in microtiter plates.[8] The biofilms were treated with a range of different concentrations of TTO, terpinen-4-ol, and RPMI 1640 containing 0.4% DMSO (control group). *C. albicans* biofilms were prepared by adding 100 µL of the isolated suspension containing 1×10^5 CFU mL^{-1} into the wells, which were kept at 37°C for 1.5 h. Thereafter, the cell suspension was replaced with 100 µL of RPMI 1640 medium and the plates were incubated at 37°C for 24 h in a shaker at 75 rpm. Metabolic activity was quantified by the XTT assay after 48 h of incubation.[16] All experiments were performed on three independent occasions.

Biofilm formation on denture base resin specimens and mouthwash simulation

Denture base acrylic resin was chosen for this study (Vipi Colorless Wave). The final dimensions of the disc-shaped acrylic resin patterns were 10 mm in diameter and 2 mm in thickness. The surface roughness of specimens was measured (0.2 *µm)* (Mitutoyo Surftest SJ-401, Mitutoyo Corporation, Japan). Distribution between experimental and control groups was determined by randomization and restriction. Prior to the experiment, the specimens were sterilized with ethylene oxide.

The assays for adhesion and biofilm formation were performed according to the literature.[17] Each denture base acrylic resin specimen was placed in sterile 24-well flat-bottomed microtiter plates. Then, 1 mL of human saliva was added, incubated for 1 h at 37°C, and shaken at 75 rpm to allow salivary pellicle formation on the specimens.[18] Human unstimulated whole saliva (Certificate of Presentation for Ethical Consideration - CAAE 06687412.5.0000.5416) was collected and clarified by centrifugation at 3,800 g for 10 min at 4°C. The supernatant was sterilized by filtration (22 µm) and stored at -70° C until use.

After incubation, *C. albicans* was dispensed into each well and the plates were incubated at 37°C for 90 min (adhesion assay) at 75 rpm and for 72 h. The culture medium was renewed every 24 h during biofilm formation. After biofilm formation, each specimen was treated with 1 mL of the solutions for 60 s, simulating the clinical application of mouthwash: 17.92 mg/mL of TTO, 8.86 mg/mL of terpinen-4-ol, 256 µg/mL of nystatin and phosphate-buffered saline. Subsequently, residual biofilms were disrupted and adhered microorganisms were removed from the specimens by sonication (7 W for 20 s)[19] and then washed with PBS. The cell viability of the biofilm was evaluated by XTT assay and by confocal laser scanning microscopy.

Confocal laser scanning microscopy

Biofilms formed on the denture base acrylic resin surfaces were stained using the Live/Dead BacLight Viability kit, comprising SYTO-9 (Invitrogen). Images of the stained biofilms were captured using a CLSM system (Leica TCS SPE, Leica Microsystems, Wetzlar, Germany).

Statistical analyses

Statistical analysis was performed using Statistical Package for the Social Sciences – SPSS version 17.0 (Chicago, USA). All data were analyzed for the presence of outliers, assumptions of normality (Shapiro-Wilk test), and homogeneity of variance (Levene's test). One-way ANOVA, followed by Tukey's test, was also used. The significance level for all statistical tests was set at 5% ($p < 0.05$).

Results

Antifungal activity

The concentrations of TTO, terpinen-4-ol, and nystatin able to inhibit growth (MIC) in planktonic cultures of *C. albicans* clinical strains (A, B) were 8.96 mg/mL, 4.53 mg/mL, and 8 μg/mL, respectively. Regarding the MFC, lower concentrations were observed for TTO (17.92 mg/mL), terpinen-4-ol (8.86 mg/mL), and nystatin (16 μg/mL). In general, the clinical samples from genotypes A and B showed greater resistance. The MIC and MFC values were similar for *C. albicans* reference strains.

Cell viability assay

Figure 1 shows the higher effect of 17.92 mg/mL of TTO and 8.86 mg/mL of terpinen-4-ol ($p < 0.05$) on all *C. albicans* biofilms formed in polystyrene plates. No significant difference was found between 17.92 mg/mL of TTO and nystatin on *C. albicans* biofilms A and B. Thus, we considered that 8.86 mg/mL of terpinen-4-ol had a significantly higher effect against cell viability of all isolates.

Figure 2 shows the XTT results expressed by OD at 492 nm, where 17.92 mg/mL of TTO and 8.86 mg/mL of terpinen-4-ol were effective against the biofilm formed on the specimens when compared to the control for all strains tested, excepted for strain A, where TTO and terpinen-4-ol were not statistically different from all groups ($p < 0.05$).

Confocal laser scanning microscopy on acrylic resin specimens

A confocal microscope was used to examine images of the biofilm species in terms of structure and interactions between *C. albicans* biofilms exposed to TTO and to terpinen-4-ol, which grew on the denture base acrylic resin specimens (Figure 3).

Representative CLSM images of the control group (A1, B1, C1, D1) showed a contiguous layer of cells of *C. albicans*, which initially presented formation of hyphae and then evolved into a mature biofilm, producing a thick layer of co-aggregated cells with a homogeneous mass of viable microorganisms and some dead cells.

The activity of nystatin, TTO, and terpinen-4-ol showed a non-contiguous layer of cells, with preferential association to the hyphal elements of *C. albicans* and, to a lesser extent, round yeast cells and reddish yellow coloration, which means non-viable cells. Furthermore, in most samples, the biofilm presented adhesion with a lower quantity of uniformly distributed cells.

The results from antimicrobial activity assessments and analysis of the CLSM images showed that TTO, terpinen-4-ol, and nystatin obtained similar results, except for genotype A, which was the most resistant to the action of the test components and nystatin (C1–C4).

Discussion

TTO and its components are the most commonly used essential oil because of their antibacterial and antifungal properties.[8,12,20] In this study, we evaluated the efficacy of TTO and its main bioactive component – terpinen-4-ol – against *C. albicans* clinical strains (genotypes A and B), and ATCC 90028 and SC 5314 *C. albicans* reference strains in planktonic and biofilm cultures.

The ability of *C. albicans* to adhere and form biofilm is essential to the development of candidiasis. The antifungal activity of TTO and terpinen-4-ol was able to control biofilm proliferation *in vitro*. MIC and MFC results proved to be highly active against

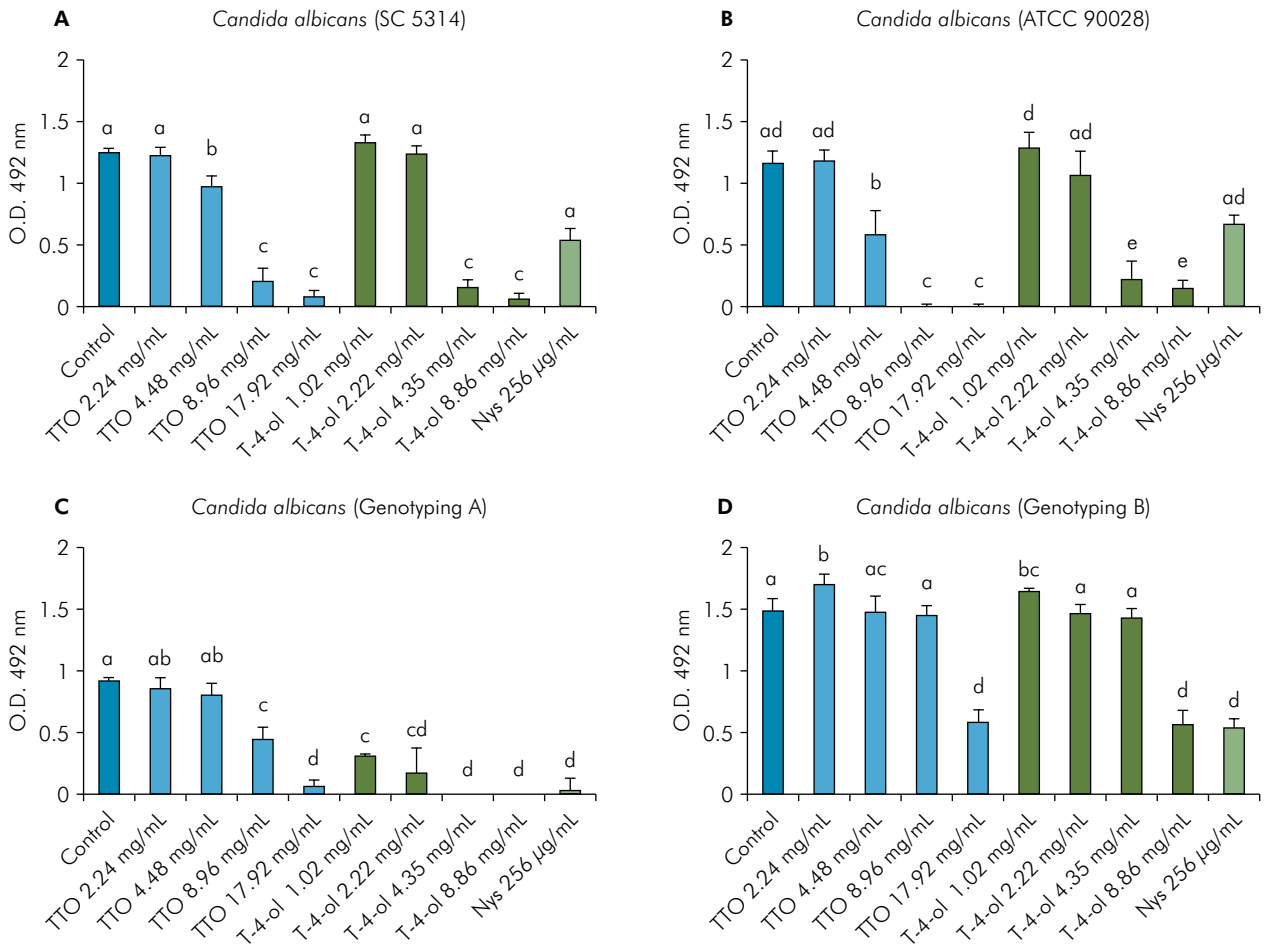

Figure 1. Analysis of cell viability (XTT) of TTO, terpinen-4-ol, and nystatin on biofilm of *Candida albicans* SC 5314 (A), ATCC 90028 (B), Genotyping A (C), Genotyping B (D), formed in polystyrene (sterile 96-well flat-bottomed microtiter plates). Statistical differences are indicated by different letters.

planktonic cells of *C. albicans*, corroborating the findings of previous studies.[9,20,21,22] Mondello et al.[23] found that terpinen-4-ol was as effective as TTO in accelerating vaginal clearance of all the examined *Candida*-resistant strains. The present study also showed that terpinen-4-ol has been investigated as an antifungal in resistant *C. albicans* strains, since resistance to antimicrobial drugs is the major obstacle to the treatment of candidiasis. In this study, the increasing concentrations of TTO and terpinen-4-ol were significantly able to eliminate biofilm from ATCC 90028 and SC 5314 reference strains. However, the activity of TTO and terpinen-4-ol was lower on the biofilms of *C. albicans* strains (genotypes A and B), but not effective enough to eliminate *C. albicans* strains completely. This could be occurring because the strains isolated from diabetic patients might have been subjected to antifungal activity and become highly prone to environmental stress. However, both compounds showed a lower effect on the eradication of *C. albicans* (genotype B) biofilm when compared to the control group (nystatin) (Figure 1). The highest concentrations could be explained by the fact that the biofilm becomes more resistant than in planktonic culture because of intraspecies and interspecies metabolic changes.[19] The effectiveness of TTO and its main component has been observed previously.[9]

Nystatin binds to steroids within the cell membranes of susceptible fungi, disrupting the permeability of the cell membrane and causing the cytoplasmic content to leak from the cell; however, it has several side effects in humans, including

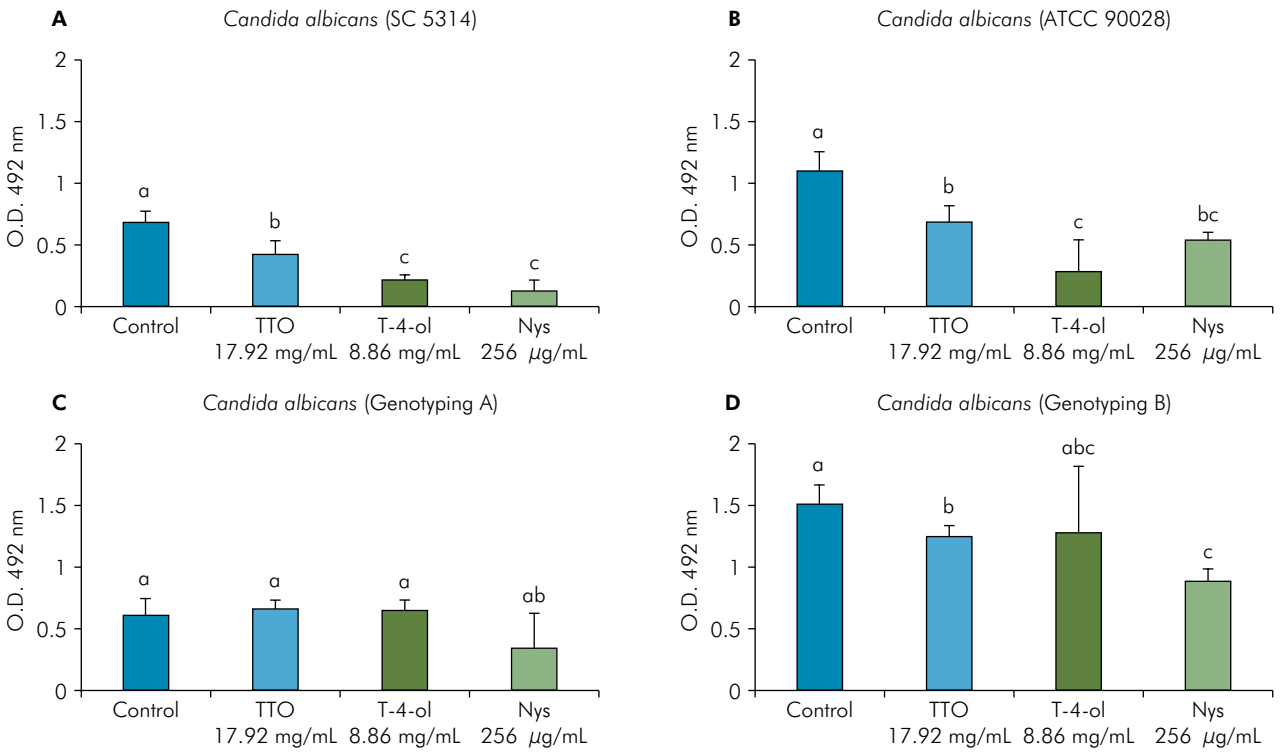

Figure 2. Analysis of cell viability (XTT) of TTO, terpinen-4-ol, and nystatin on biofilm of *Candida albicans* SC 5314 (A), ATCC 90028 (B), Genotyping A (C), Genotyping B (D), grown on denture base resin specimens. Statistical differences are indicated by different letters.

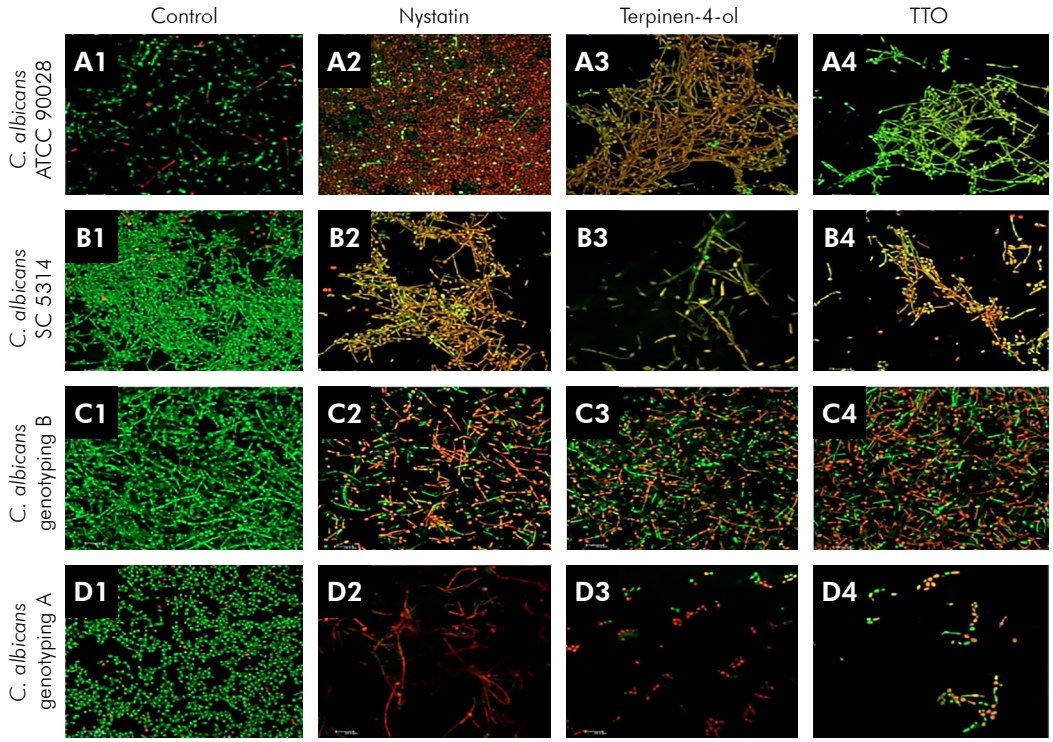

Figure 3. Representative CLSM images of *Candida albicans* ATCC 90028 (A), SC 5314 (B) genotyping A (C), and genotyping B (D) biofilms formed on the specimens and exposed to PBS (control group) (1), nystatin (2), terpinen-4-ol (3), and TTO (4).

gastrointestinal disorders and an unpleasant taste.[24] The topical use of nystatin is limited in terms of the period of localization, mainly as a result of the addition of flavoring agents, which increase the volume of saliva in the region, thus reducing its residence time at the injured site, hindering the effectiveness of treatment.[25,26] By contrast, terpinen-4-ol disrupts the fungal cell membrane and interferes with the pathogen's cell integrity and physiology. The present results are in line with those of Ramage et al.[9] and Tonon et al.[27] who showed strong antimicrobial properties of terpinen-4-ol against planktonic cultures and fungal biofilms, indicating that it may be suitable for the prophylaxis and treatment of candidiasis.

Studies demonstrated that TTO decreases biofilm formation and attachment of different *C. albicans* strains to biotic and abiotic surfaces. However, TTO has some problems associated with its physical properties, such as low water miscibility and high volatilization rates, resulting in low stability.[28,29]

Biofilms demonstrate wettability (ability of a liquid to maintain contact with a solid surface), as a result of the great number of intermolecular interactions. This explains some factors that affect the penetration of antifungal agents into different cell walls and into the polymeric matrix of the biofilm.[30,31]

Candida species are microorganisms that live commensally in the oral cavity of healthy individuals, but their incidence and virulence seem to be increased in those who are immunocompromised. This explains why *C. albicans* clinical strains previously identified as genotypes A and B, isolated from diabetic patients with chronic periodontitis, present physiological changes in the host, causing oral candidiasis or invasive systemic infections. This growing trend has been associated with resistance to the antimicrobial therapy and with the ability of the microorganism to form biofilms. The increasing microbial resistance rates may also result from long-term drug exposure or from the selection of strains with intrinsic resistance mechanisms.[14]

Another property of *Candida* spp. is their ability to grow either aerobically or anaerobically as they have developed adaptive mechanisms to survive in both situations. Oxygen can generate reactive products during an infection and induce an oxidative stress response. Treatment of *C. albicans* with low concentrations of superoxide-generating agents, such as hydrogen peroxide, induces a redox potential with the activation of antioxidant enzymes, protecting cells from the lethal effects of a subsequent challenge with higher concentrations of these oxidants. Another problem is posed by anaerobic environments, such as root canal systems and periodontal pockets, which lead to polymicrobial infections.[32]

This study also assessed biofilm viability in acrylic resin specimens. Denture materials themselves can contribute to denture stomatitis, as the roughness and hydrophobicity of denture surfaces can promote microbial adhesion and biofilm formation. Surface roughness was also evaluated. It has been reported that surface roughness is the main factor that affects microbial adhesion and biofilm formation on denture base surfaces.[17] Therefore, we measured the surface roughness of each specimen to avoid the effect of an irregular surface on *C. albicans* adhesion and biofilm formation. An essential event in the onset of denture stomatitis is the adhesion of *C. albicans* to the denture base. After adhesion occurs, mature biofilm forms on the surface of dentures, resulting in denture stomatitis in the presence of a suitable environment. Therefore, controlling *C. albicans* adhesion and biofilm formation on the denture base is vital for preventing denture stomatitis.[33]

The results show that the images obtained by CLSM confirmed that 17.92 mg/mL of TTO and 8.86 mg/mL of terpinen-4-ol applied for 60 s (rinse simulation) are effective and comparable to treatment with nystatin and interfere with biofilm formation. These results were confirmed by the XTT assay and our *in vitro* data confirm that TTO and terpinen-4-ol have the same antimicrobial activity. Results showing a preventive effect were observed using 2.24 mg/mL of TTO to inhibit biofilm formation on various *C. albicans* samples.[28] Under these conditions, low concentrations of TTO can be used to prevent infection and to treat existing fungal infections.[20]

The results of the present study indicate that TTO and terpinen-4-ol have antifungal activity against *Candida* strains. The mechanism of action of TTO on cells has not been elucidated, but it is believed to

have a lipophilic character as TTO interacts with the cell membrane and then disrupts its normal activity.[11]

In summary, studies suggest that TTO penetrates through the cell wall and cytoplasmic membrane of fungal strains, causing damage to these structures, with subsequent loss of cytoplasmic material. Essential oils can penetrate the cytoplasmic membrane because of their lipophilicity.[22,34] Li et al.[22] showed that TTO and terpinen-4-ol might penetrate fungal organelle membranes and induce organelle damage. Finally, these irreversible TTO-mediated changes lead to cell death and showed antifungal activities against *C. albicans* strains. This activity was mainly attributed to the presence of terpinen-4-ol, which showed equivalent antimicrobial activity against these fungal strains.

Conclusions

The experimental data strengthen the contribution of TTO and terpinen-4-ol activity *in vitro*, suggesting that they can be used for both prevention and treatment of fungal infections.

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