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Cinnamaldehyde and α-terpineol inhibit the growth of planktonic cultures of Candida albicans and non albicans

Cinamaldeído e α-terpineol inibem o crescimento de culturas planctônicas de Candida albicans e não albicans

El cinamaldehído y el α-terpineol inhiben el crecimiento de cultivos planctónicos de Candida albicans y no albicans

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Abstract
Agents based in natural products have been investigated for the treatment of oral candidiasis. This study aims to evaluate the antifungal effect of phytoconstituent cinnamaldehyde and α-terpineol in planktonic cultures of *Candida albicans*, *Candida glabrata*, *Candida krusei* and clinical isolates of *C. albicans*. Reference strains of *C. albicans* (ATCC 90028 and ATCC 60193), *C. glabrata* (ATCC 2001), *C. krusei* (ATCC 34135) and four clinical isolates were used. Nistatin 100,000UI was used as a positive control. After preparation of the inoculum (1×10^3 CFU / mL), serial microdilution technique was performed using RPMI 1640 medium. Results: in reference strains, the MIC for α-terpineol ranged from 312,5 μg / mL (*C. albicans* 90028) to 40 μg / mL (*C. krusei*); and the cinnamaldehyde ranged from 40 μg / mL (*C. albicans* 90028, *C. albicans* 60193 and *C. glabrata*) to 20 μg / mL (*C. krusei*). Whereas for clinical strains, the MIC for α-terpineol ranged from 156 μg / mL to 78 μg / mL and cinnamaldehyde ranged from 78 μg / mL to 40 μg / mL. Therefore, the cinnamaldehyde and α-terpineol present an inhibitory effect against planktonic cultures of *Candida albicans* and not albicans.
Keywords: *Candida albicans; Candida glabrata; Candida krusei*; Oral candidiasis; Phytotherap.

Resumo
Agentes à base de produtos naturais têm sido investigados para o tratamento da candidíase oral. Este estudo tem como objetivo avaliar o efeito antifúngico do fitoconstituinte cinamaldeído e α-terpineol em culturas planctônicas de *Candida albicans, Candida glabrata, Candida krusei* e isolados clínicos de *C. albicans*. Foram utilizadas cepas de referência de *C. albicans* (ATCC 90028 e ATCC 60193), *C. glabrata* (ATCC 2001), *C. krusei* (ATCC 34135) e quatro isolados clínicos. Nistatina 100.000UI foi usada como controle positivo. Após a preparação do inóculo (1 × 103 UFC / mL), foi realizada a técnica de microdiluição seriada em meio RPMI 1640. Nas cepas de referência, o MIC para α-terpineol variou de 312,5 μg / mL (C. albicans 90028) a 40 μg / mL (C. krusei); e o cinamaldeído variou de 40 μg / mL (C. albicans 90028, *C. albicans* 60193 e C. glabrata) a 20 μg / mL (C. krusei). Enquanto para as cepas clínicas, o MIC para α-terpineol variou de 156 μg / mL a 78 μg / mL e o cinamaldeído variou de 78 μg / mL a 40 μg / mL. Portanto, o cinamaldeído e o α-terpineol apresentam efeito inibitório contra culturas planctônicas de *Candida albicans* e não albicans.
Palavras-chave: *Candida albicans; Candida glabrata; Candida krusei;* Candidíase oral; Fitoterapia.

Resumen
Se han investigado agentes a base de productos naturales para el tratamiento de la candidiasis oral. Este estudio tiene como objetivo evaluar el efecto antifúngico del fitoconstituyente cinamaldehído y α-terpineol en cultivos planctónicos de *Candida albicans, Candida glabrata, Candida krusei* y aislados clínicos de *C. albicans*. Se utilizaron cepas de referencia de *C. albicans* (ATCC 90028 y ATCC 60193), *C. glabrata* (ATCC 2001), *C. krusei* (ATCC 34135) y cuatro aislados clínicos. Se utilizó nistatina 100.000 UI como control positivo. Después de preparar el inóculo (1 × 103 UFC / mL), se realizó la técnica de microdilución seriada en medio RPMI 1640. En las cepas de referencia, la CMI para α-terpineol osciló entre 312,5 µg / mL (*C. albicans* 90028) a 40 µg. / ml (*C. krusei*); y el cinamaldehído varió de 40 µg / mL (*C. albicans* 90028, *C. albicans* 60193 y *C. glabrata*) a 20 µg / mL (*C. krusei*). Mientras que para las cepas clínicas, la CMI para α-terpineol varió de 156 µg / mL a 78 µg / mL y el cinamaldehído varió de 78 µg / mL a 40 µg / mL. Por lo tanto, el cinamaldehído y el α-terpineol tienen un efecto inhibidor contra los cultivos planctónicos de *Candida albicans* y no albicans.
Palavras chave: *Candida albicans; Candida glabrata; Candida krusei*; Candidiasis oral; Fitoterapia.

1. Introduction

Oral candidiasis is an opportunistic fungal infection caused by poor oral hygiene conditions, and consequently, the presence of biofilm (Hellstein et al., 2019). This disease is prevalent in patients with immunosuppression (Suryana et al., 2020), diabetic mellitus (Tretin et al., 2017) and denture wearers (Radovic et al., 2014). Although *Candida albicans* is the prevalent fungi in this infection (Hellstein et al., 2019), other *Candida* spp are related with this disease, as *Candida glabrata* and *Candida krusei* (Hu et al, 2019). These species form communities of microorganisms embedded within an extracellular matrix (Silva et al., 2012), which facilitates the epithelial invasion, protects microbial cells from host immune responses, promotes protection to biofilm by limiting the penetration of substances through the matrix. As a consequence, these microorganisms establish the disease and conferring significant resistance to antifungal therapy (Gulati et al., 2016).

Generally, the oral candidiasis treatment requires use of topical or systemic antifungal agents, such as Nystatin, Miconazole and Fluconazole (Lyu et al., 2016; Zhang et al., 2016; Quindóes et al., 2019). The selection of the agent should consider the site of the infection, oral or oropharyngeal, as well as the patient's systemic condition (Hellstein eet al., 2019). Patients with living with HIV or with immunological deficits, must be treated as fast as possible, because this infection could develop a nosocomial infection, which may lead to death (Kabwe et al., 2016).

Despite those antifungal agents are widely used, it has been reported that intensive application of Nystatin, Miconazole and Fluconazole could promoting antifungal resistant fungi (Gulati et al., 2016; Perlin et al., 2017), causing a risk to human health. Hence, resistance to these drugs clearly challenges treatment due to the limited therapeutic options. Due this effect, natural products have been investigated as alternative for oral candidiasis treatment (Sardi et al., 2013). Assorted essential oils and hydroalcoholic extracts had demonstrated antifungal activity (Ferreira et al., 2015). However, these substances had nonspecific effects due the presence of several molecules in your composition. In recent years, the investigations focus on bioactive molecules isolated from these products, as are known as phytoconstituent, because these molecules could reach specific biological effects, as the control of *Candida* spp biofilm formation.

Molecules such as cinnamaldehyde and α-terpineol had antimicrobial effect against *C. albicans* by inhibiting the adhesion, morphological transition and biofilm formation (Trinh et al., 2011; Taguchi et al., 2013). The cinnamaldehyde is an aldehydic component extracted from cinnamon bark (Wu et al., 2018). While the α-terpineol is a monoterpenoid compound existing in plants and it was often used as perfume and repellent in the cosmetic industry (Zhang et al., 2019). Although these molecules had been previously investigated, still not totally explored the antifungal activity against non albicans pathogens and clinical isolates of *C. albicans*. Therefore, this study aimed to evaluate the antifungal effect of cinnamaldehyde and α-terpineol in planktonic cultures of *C. albicans*, *C. glabrata*, *C. krusei* and clinical isolates of *C. albicans*.

2. Methodology

Microbial strains and growth conditions

Fungal strains used were as follows: *C. albicans* (ATCC 90028), *C. albicans* (ATCC 60193), *C. glabrata* (ATCC 2001), *C. krusei* (ATCC 34135). Collection of four clinical strains of *C. albicans* were in accordance with the Declaration of Helsinki, approved by the Ethics Committee of Federal University of Paraíba (CAAE 55844316.9.0000.5188). The clinical strains were supplied by the Micology Laboratory of Federal University of Paraíba and were isolated from the palatal region of denture stomatitis subjects, who provided written informed consent. The clinical isolates were identified by CHROMagar *Candida*, each strain used the designation S1 to S4. The reference strains were cultivated aerobically on Sabouraud Dextrose (SD) agar (Difco, Detroit, USA) at 37°C, and suspensions were grown in RPMI 1640 broth (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C for 24 h. For preparation of cells for experiments, cultures were grown for 16 h at 37°C, centrifuged (5000 *g* for 5 min), the cell pellets were suspended and washed twice with NaCl 0,9%, and suspended in RPMI medium at an optical density at 600 nm (OD_{600}) of 1.0. Subsequently, this initial inoculum was diluted 1000×, at a 1×10^3 CFU /mL concentration, which in accordance with planktonic cultures.

Treatment substances

Cinnamaldehyde (Sigma-Aldrich, St. Louis, MO, USA) and α-terpineol (Sigma-Aldrich, St. Louis, MO, USA) were used in the present study. The chemical specifications of the substances were shown in Figure 1. The phytoconstituent solutions were prepared for the assay using RPMI 1640 medium supplemented with 0.1% Tween 80 (Dinamica, São Paulo, Brazil), which was used as vehicle, followed by vigorous agitation. A 40.000 µg/ mL working solution was formulated for each phytoconstituent, with the concentrations of both substances ranging from 10.000 µg/ mL to 5 µg/ mL. As a positive control, 100.000UI solution of Nystatin (Neo Química, São Paulo, Brazil) was used.

Table 1. Chemical specifications of the substances selected for the microdilution assay

Fhytoconstituents	Molecular Fórmula	Chemical classification	Molecular weight	Density
Cinnamaldehyde	C9H8O	Aldehyde	132.16 g/mol	1050 mg/mL
α-Terpineol	C9H18O	Alcohol	154.25 g/mol	950 mg/mL

Source: Authors (2021).

Inhibitory Minimum Concentration (MIC): evaluation in planktonic culture

Minimum Inhibitory Concentration (MIC) was performed in compliance with Clinical Laboratory and Standards Institute (CLSI) M27-A3 standards (CLSI., 2008). Polystyrene 96-well plates with a conical bottom were used to determine the MIC of the substances. Initial serial dilutions were performed in 1 : 2 (v / v). 100 µL of the inoculum were inserted in the 96-well and incubated at 37 °C for 48 hours. Subsequently, 100 µl of RPMI 1640 medium were dispensed into each well. Then, 100 µL of the initial dilution of cinnamaldehyde, α-terpineol and Nystatin was inserted and the serial dilutions were performed. RPMI 1640 was used as sterility control. The plates were aerobically incubated at 37°C for 20 h. The experiments were performed in triplicates. The growth within the wells was determined visually (CLSI 2008), therefore, the Minimum Inhibitory Concentration was defined as the lowest concentration of the test substance inhibiting microbial growth.

Statistical analysis

Data were analyzed descriptively, being the mode measure to determine the MIC value of each test substance against each microorganism.

3. Results

Concerning the reference strains, the minimum inhibitory concentration (MIC) of cinnamaldehyde was found to range from 40 µg / ml to 20 µg / ml depending on the strain evaluated. Whereas for α-terpineol was found to 312,5 µg / ml to 40 µg / ml (Table 1). *C. krusei* presents a challenge for oral candidiasis treatment due to intrinsic resistance character, especially to commercially available antimicrobials. Surprisingly, this strain was the less resistant to the effects of cinnamaldehyde and α-terpineol.

Table 2. Minimum Inhibitory Concentration (MIC) of α-Terpineol and cinnamaldehyde against the microorganisms.

Microorganisms	**MIC**	**MIC**
	α-Terpineol	**Cinnamaldehyde**
C.albicans (ATCC 90028)	312,5 µg/mL	40 µg/mL
C.albicans (ATCC 60193)	156 µg/mL	40 µg/mL
C.glabrata (ATCC 2001)	312,5 µg/mL	40 µg/mL
C.krusei (ATCC 34135)	40 µg/mL	20 µg/mL
C.albicans S1	156 µg/mL	78 µg/mL
C.albicans S2	156 µg/mL	40 µg/mL
C.albicans S3	156 µg/mL	40 µg/mL
C.albicans S4	78 µg/mL	40 µg/mL

Source: Authors (2021).

Regarding clinical isolates of *C. albicans*, the MIC of cinnamaldehyde was to range from 78 µg / ml to 40 µg / ml; and for α-terpineol was found from 156 µg / ml to 78 µg / ml (Table 1).

The MIC values found in this study are lower those indicated for the clinical efficacy of an antimicrobial (> 400 µg / mL).

4. Discussion

Long-term administration of Nystatin, Miconazole and Fluconazole caused antifungal resistance (Gulati et al., 2016; Perlin et al.,2017). This fact demand inquest and challenge for clinicians and researchers in oral candidiasis treatment. As a result, the development of a product that reduces the risk and severity of oral candidiasis infections would be ideal (Sardi et al., 2013). Natural products, as bioactive molecules, may be taken in the treatment of infection without potential resistance and side-effects. Our results shown that cinnamaldehyde and α-terpineol demonstrated antifungal effect in cultures of *C. albicans*, *C. glabrata*, *C. krusei* and clinical isolates of *C. albicans*. Therefore, these findings shown that cinnamaldehyde and α-terpineol exhibit comparable efficacy to a commercially available antifungal.

Our results are consistent with newly published findings that showed that these molecules were effective at antimicrobial activity against *C. albicans* and *C. Glabrata* (Trinh et al., 2011; Dogan et al., 2017; Bakhtiari 2019). However, here we highlight the effectiveness cinnamaldehyde and α-terpineol on inhibiting growth of *C. krusei*. This fungal can produce extracellular ammonia (NH_3), which contribute to increase the pH levels and synthesis of ATPase within cells (Jorgensen et al., 2017). Hence, these processes provide an intrinsic resistance character in the fungi, especially in commercially available antimicrobials, which difficult the treatment (Dias et al., 2018). α-terpineol and cinnamaldehyde shows the strongest inhibition of *C. krusei* in lower concentrations, suggesting that the mechanisms of action of these molecules are different from azole components. This effect could be explained due to their chemical composition, which perhaps inhibiting factors that modulate resistance.

The cinnamaldehyde is the most important phytoconstituent present in the oil extracted from the bark of the species *Cinnamomum cassia* (Taguchi et al., 2013). This molecule is described as a suppressor of bacterial cell division and had the capacity of interfere the permeability of the cytoplasmic membrane of fungal, thus, compromising the cellular integrity (Taguchi et al., 2013). On the other hand, α-terpineol is a monoterpene alcohol, extracted from several species such as *Eucalyptus cinerea* (Franco et al., 2015), *Salvia libanotica* (Hassan et al., 2010) and *Melaleuca alternifolia* (Nogueira et al., 2014). The α-terpineol activity also is via disrupting cell walls and cytoplasm, resulting in abnormal hyphae. In this process, the molecule can downregulate metabolic pathways and further energy metabolisms, which weakening the fungal (Kong et al., 2019). Thereby, the chemical composition of cinnamaldehyde and α-terpineol can promote the fungal death.

As investigations now point to the antimicrobial effect of cinnamaldehyde and α-terpineol in several reference strains (Dogan et al., 2017., Pootong et al., 2017; Bakhtiari et al., 2019), we also investigated antifungal effects in clinical isolated of *C. albicans*. Although reference strains are widely used in microbiology reports, the evaluation against clinical isolates strains demonstrate a challenge to antifungal drugs due to phenotypic modifications in the cells, which could interfere in the action these drugs (Akers et al., 2015). Our findings shown that the use of cinnamaldehyde and α-terpineol in clinical isolates reach similar effects, if not slightly more efficacious, when compared with reference strains of *C. albicans*. Thus, as clinical benefit, these results indicated that these substances may provide alternate mechanisms to prevent and treat oral candidiasis.

Despite the minimum inhibitory concentration found in this study are lower those indicated for the clinical efficacy of an antimicrobial (> 400 µg / mL)[30], it is reported that concentrations lower than 100 µg / mL are considered excellent to antimicrobial activity *in vitro* studies (Holetz et al., 2002). Thus, according with this, the results found in this study suggested that cinnamaldehyde could be clinically effective against all strains evaluated.

The effect of cinnamaldehyde on a clinical isolate of *C. albicans* was evaluated, according with the medium temperature and time of exposition. The results shown that at 37 °C the IC50 after 60 minutes ranged from 128 µg / mL to 320 µg / mL (Taguchi et al., 2012). Interestingly our results demonstrated that minimum inhibitory concentration of cinnamaldehyde was 40 µg / mL against clinical isolates of *C. albicans*. This divergence could be explained due to different grown conditions. Taguchi et al. (2012) evaluated the effect of cinnamaldehyde on Candida isolates with initial adhesion of 3 hours, therefore, higher cell density. This grown condition could have influenced the results because higher amounts of cells difficult the action of antimicrobials. Thus, future investigations should evaluate the effect of cinnamaldehyde against clinical isolates using a biofilm model, which represents a complex of microorganisms and matrix, offering similar conditions in relation to the mouth.

Although *C. albicans* is the mainly microorganism in the oral candidiasis (Hellstein et al., 2019) the presence of bacteria increases the virulence of the fungi, and thus, the capacity to cause the disease (Cavalcanti et al., 2015). Thereby, studies have been demonstrated the efficacy of cinnamaldehyde against bacterial cultures, such as *E. faecalis* and *S. aureus* (Ferro et al. 2016). The MIC of cinnamaldehyde ranged from 0.5 to 0.25 µg / mL for *E. faecalis* and *S. aureus*, as well as

clinical isolates of these species (Ferro et al., 2016). This finding could be explained due to higher virulence and pathogenicity of these bacteria or the presence of extracellular matrix, which require higher concentrations of the substance to promote an antimicrobial effect against bacterial species. Thus, could be suggested that cinnamaldehyde effect is better in fungal cultures than bacterial species. These effects, as a fungistatic agent, also would be related with the farnesol production, which is a *quorum sensing* molecule that regulates virulence and morphogenesis in *C. Albicans* (Polke et al., 2017). However, this relation remains unclear.

Regarding the α-terpineol, the minimum inhibitory concentration of was ranging from 312.5 μg / mL to 40 μg / mL. The lowest concentration was found in *C. krusei*, followed by *C. albicans* and *C. glabrata*. The antibiofilm effect of tea tree oil compounds (TTO) was determinate, evaluating the effect of α-terpineol on 100 strains of *C. albicans*, including clinical and reference strains (Ramage et al. 2012). These results shown that 5 mg / mL of concentration was enough to determine 90% cell death in biofilms of *C. Albicans* (Ramage et al., 2012). However, the cell concentration used was 10^6 cells / mL, which could be a challenge for α-terpineol disrupt the fungi cells.

Concerning the α-terpineol effect on bacterial proliferation, evidence demonstrate the MIC for *Streptococcus mutans* and *Streptococcus sobrinus* was ranged from 0.8 to 1.6 mg / ml (Park et al., 2012). These microorganisms are related with the cariogenic biofilm, which is able to live in a lower pH environment (Chu et al., 2016). Both microorganisms can establish synergistic relationships with *C. albicans*, and then, increase the fungal virulence and its capacity to antimicrobial resistance (Cavalcanti et al., 2016). However, remains not totally explored about the effect of α-terpineol in co-cultures of fungal and bacteria.

Furthermore, α-terpineol has been evaluated in relation to its immunomodulatory potential. This phytoconstituent modulate the NF-K B and ERK MAPK activation, in macrophage culture, in low concentrations as 28.8 and 13.9 μg / mL (Nogueira et al., 2014). However, it is noted that the effects on IL-1B and IL-8 cytokines production may be dependent on the concentration used. It is suggested that the α-terpineol antimicrobial activity is by disrupting the cell membrane (Nogueira et al., 2014). In addition, the anti-inflammatory effect of α-terpineol occur on the regulation of NFK-β in tumor cell lines, such as CCRF-CEM (leukemia), U937-GTB (lymphoma) and NCI-H69 (lung cancer cells) (Hassan et al., 2010). Although the activity of cinnamaldehyde and α-terpineol have been investigated, the cytotoxic effects of these molecules, in human gingival fibroblasts cells or *in vitro* tree-dimensional epithelium model, and their capacity of promoting cellular biostimulator should be evaluate.

5. Conclusion

Our findings shown that cinnamaldehyde and α-terpineol are effective against *C. albicans*, *C. glabrata*, *C. krusei* and clinical isolates of *C. albicans*. Therefore, these preliminary results could guide clinicians and researchers for explore the effect of these molecules in complex models of biofilm and cells, as a possible drug treatment to oral candidiasis.

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