



Schinus terebinthifolius (Brazilian Peppertree) extract used as antifungal to control *Candida* spp. in planktonic cultures and biofilms

Extrato de *Schinus terebinthifolius* (pimenta rosa) utilizado como antifúngico para controlar *Candida* spp. em culturas planctônicas e biofilmes

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ABSTRACT

Objective: The use of medicinal plants may be an alternative method for the control of *Candida* spp. responsible for human infections. This study evaluated the antifungal effect of *Schinus terebinthifolius* extract (Brazilian Peppertree) on *C. albicans*, *C. dubliniensis*, *C. glabrata*, and *C. krusei* planktonic cultures and biofilms. **Material and Methods:** Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of the plant extract were determined by the broth microdilution method. Biofilms formed in microplate wells were exposed to the extract for 5 min (50, 100 and 200 mg/mL) or 24 h (25, 50 and 100 mg/mL). After determination of colony-forming units per milliliter (CFU/mL), the data were analyzed by one-way ANOVA and Tukey's Test ($P \leq 0.05$). **Results:** Different MIC (mg/mL) were found, such as 0.39 (*C. dubliniensis*), 1.56 (*C. albicans*), and 3.13 (*C. glabrata* and *C. krusei*). Besides, MFC (mg/mL) of 0.78 (*C. dubliniensis*) and 3.13 (*C. albicans*, *C. glabrata* and *C. krusei*) were also observed. Regarding the biofilms, significant reductions (\log_{10}) were found after 5 min and 24 h exposure to the plant extract, compared to the control group. However, *C. dubliniensis* was significantly affected only in 24 h treatment. **Conclusion:** *S. terebinthifolius* extract presented a significant antifungal effect on *C. albicans*, *C. dubliniensis*, *C. glabrata*, and *C. Krusei* both in planktonic cultures and biofilms.

KEYWORDS

Biofilms; Brazilian peppertree; *Candida*; Planktonic cultures; *Schinus terebinthifolius*.

RESUMO

Objetivo: O uso de plantas medicinais pode ser um método alternativo para o controle de *Candida* spp. responsáveis por infecções humanas. Este estudo avaliou o efeito antifúngico do extrato de *Schinus terebinthifolius* (pimenta rosa) sobre culturas planctônicas e biofilmes de *C. albicans*, *C. dubliniensis*, *C. glabrata* e *C. krusei*. **Material e Métodos:** Concentração inibitória mínima (CIM) e concentração fungicida mínima (CFM) do extrato vegetal foram determinadas pelo método de microdiluição em caldo. Biofilmes formados em poços de microplacas foram expostos ao extrato por 5 min (50, 100 e 200 mg/mL) ou 24 h (25, 50 e 100 mg/mL). Após determinação de unidades formadoras de colônias por mililitro (UFC/mL), os dados foram analisados por one-way ANOVA e Teste de Tukey ($P \leq 0,05$). **Resultados:** Foram encontradas diferentes CIM (mg/mL), como 0,39 (*C. dubliniensis*), 1,56 (*C. albicans*) e 3,13 (*C. glabrata* e *C. krusei*). Além disso, CFM (mg/mL) de 0,78 (*C. dubliniensis*) e 3,13 (*C. albicans*, *C. glabrata* e *C. krusei*) também foram observadas. Em relação aos biofilmes, foram encontradas reduções significativas (\log_{10}) após 5 min e 24 h de exposição ao extrato vegetal, em comparação ao grupo controle. No entanto, *C. dubliniensis* foi significativamente afetada apenas no tratamento de 24 h. **Conclusão:** O extrato de *S. terebinthifolius* apresentou efeito antifúngico significativo sobre *C. albicans*, *C. dubliniensis*, *C. glabrata* e *C. Krusei*, tanto em culturas planctônicas quanto em biofilmes.

PALAVRAS-CHAVE

Biofilmes; *Candida*; Culturas planctônicas; Pimenta rosa; *Schinus terebinthifolius*.

INTRODUCTION

Yeasts, such as *Candida*, have been continuously responsible for the majority of fungal infections [1]. Species such as *C. albicans*, *C. dubliniensis*, *C. glabrata* and *C. krusei* are considered commensal, belonging to the resident microbiota of oral, genital, urinary and gastrointestinal mucosa [2]. However, these species are opportunistic and can cause local and systemic infections with potentially high mortality rates, usually in patients hospitalised due to candidemia. These fungal infections may be related to the host's health condition as there is a direct relationship with the use of immunosuppressants or antibiotics, disruption of mucosal barrier, radiotherapy and chemotherapy [3-5].

Candida pathogenicity is associated with its capacity to form hyphae, which can favour the invasion of the host's tissues, formation of biofilm on biotic and abiotic surfaces and production of hydrolytic enzymes such as proteases and phospholipases [4].

There are several antifungal agents commercially available for treatment of candidiasis, such as nystatin, amphotericin B, clotrimazole, miconazole, itraconazole, fluconazole and ketoconazole. However, these drugs may produce adverse effects such as bitter taste, allergic reactions and resistant cell selection [6].

S. terebinthifolius (Brazilian Peppertree), Anacardiaceae family, is a native species from the South and Central America, but it can also be found in tropical and subtropical regions of the United States and Africa. In Brazil, this plant species is distributed throughout the country's eastern coast, from northern to southern region [7]. Antimicrobial, anti-inflammatory and antifungal activities of this plant have been reported [7,8].

The search for therapeutic applications

using medicinal plants and their derivatives has been increasing around the world. The use of products obtained from these plants such as extracts, dyes, essential oils and phytochemicals may be an important alternative for control of strains resistant to the antimicrobial drugs available, as well as for treatment of infections caused by *Candida* spp. Therefore, this study evaluated the antifungal effect of *S. terebinthifolius* extract on *C. albicans*, *C. dubliniensis*, *C. glabrata* and *C. krusei* both in planktonic cultures and biofilms.

MATERIAL AND METHODS

Plant extract

S. terebinthifolius glycolic extract was commercially obtained at 200 mg/mL, in propylene glycol (All Chemistry, São Paulo, Brazil).

Fungal strains

Reference strains (ATCC - American Type Culture Collection) of *C. albicans* (ATCC 18804), *C. dubliniensis* (ATCC MYA646), *C. glabrata* (ATCC 9030) and *C. krusei* (ATCC 6258) were used. The strains were acquired from Laboratory of Microbiology and Immunology, UNESP/ICT, and stored in yeast extract peptone dextrose broth (YPD – Himedia, Mumbai, India) containing 16% glycerol at -80°C.

Antifungal activity on planktonic cultures

For determination of MIC, the broth microdilution method was used according to Clinical and Laboratory Standards Institute (CLSI) [9,10]. Fungal suspensions were prepared in sterile saline solution (0.9% NaCl) from a culture incubated at 37°C for 24 h and standardised to 10⁶ CFU/mL in a spectrophotometer (Micronal, São Paulo, Brazil). From this suspension were performed two dilutions, one of 1:50 and other of 1:20, in order to obtain a concentration of approximately

5×10^2 to 2.5×10^3 CFU/mL. The assay was performed in microplates with addition of $100 \mu\text{L}$ /well of culture medium and $100 \mu\text{L}$ of extract only in the first well from where 10 serial dilutions were obtained, then $100 \mu\text{L}$ /well of standardised yeast suspension were added. Negative control composed by inoculum and culture medium and positive control constituted only by culture medium were added. The culture medium used was RPMI 1640 (Himedia) with glutamine, without bicarbonate and phenol red indicator, buffered to $\text{pH } 7,0 \pm 0,1$ with MOPS [3-(N-morpholino) propanesulfonic acid] (Sigma-Aldrich, St. Louis, USA). After 24 h incubation, MIC was determined in the first well that showed no turbidity. For determination of MFC, $100 \mu\text{L}$ of MIC and adjacent concentrations were seeded in Sabouraud dextrose (SD – Himedia) agar. After 48 h incubation at 37°C the MFC was determined on the plaque with no colonial growth.

Antifungal activity on biofilms

Monomicrobial biofilms were formed in 96-well plates (TPP, Trasadingen, Switzerland). Initially, the yeasts were cultured in SD agar and then in yeast nitrogen base (YNB – Himedia) supplemented with 100 mM of glucose at 37°C for 24 h each one. Posteriorly, the fungal suspension was centrifuged ($358 \text{ g}/10 \text{ min}$) and the supernatant disregarded and the pellet suspended in sterile saline solution. After another centrifugation, the suspension was standardised to 10^7 CFU/mL in spectrophotometer (Micronal, São Paulo, Brazil). After, $200 \mu\text{L}$ /well of this suspension was added in the microplate and followed to incubation (37°C) under shaking (75 rpm) for 90 min, for adherence of the biofilms. The supernatant was discarded and YNB broth was added. After 48 h, the biofilms were exposure to the extract for 5 min at 50,

100 and 200 mg/mL; or, for 24 h at 25, 50 and 100 mg/mL. Saline solution (0.9% NaCl) and culture medium were used as controls on the treatments of 5 min and 24 h, respectively. The biofilm was washed three times with sterile saline solution before being disaggregated by ultrasonic homogenizer (Sonopuls HD 2200 – Bandelin Eletronic, Berlin, Germany) with power of 25% for 30 s. The suspension was serially diluted and $20 \mu\text{L}$ were added to SD agar in single drops in triplicate. After 48 h incubation, the concentration of CFU/mL was determined by calculating the mean CFU of each drop multiplied by 50 and dilution factor used and the values were converted in \log^{10} . Three assays were performed on an independent basis, with four repetitions each, totalising 12 for each experimental group.

Statistical analysis

Results were analysed by ANOVA and Tukey's Test ($P \leq 0.05$), using GraphPad Prism software version 5.0.

RESULTS

In planktonic cultures, MIC (mg/mL) of 1.56 (*C. albicans*), 0.39 (*C. dubliniensis*) and 3.13 (*C. glabrata* and *C. krusei*) and MFC of 0.78 (*C. dubliniensis*) and 3.13 (*C. albicans*, *C. glabrata* and *C. krusei*) were observed.

Regarding the biofilms, this plant extract at 50, 100, and 200 mg/mL produced significant reductions of CFU/mL after 5 min exposure (Figure 1) in relation to the control group, except *C. dubliniensis*, when applied the concentrations of 100 and 50 mg/mL. After 24 h exposure to the extract at 25, 50 and 100 mg/mL, significant reductions of all fungal species were observed (Figure 2).

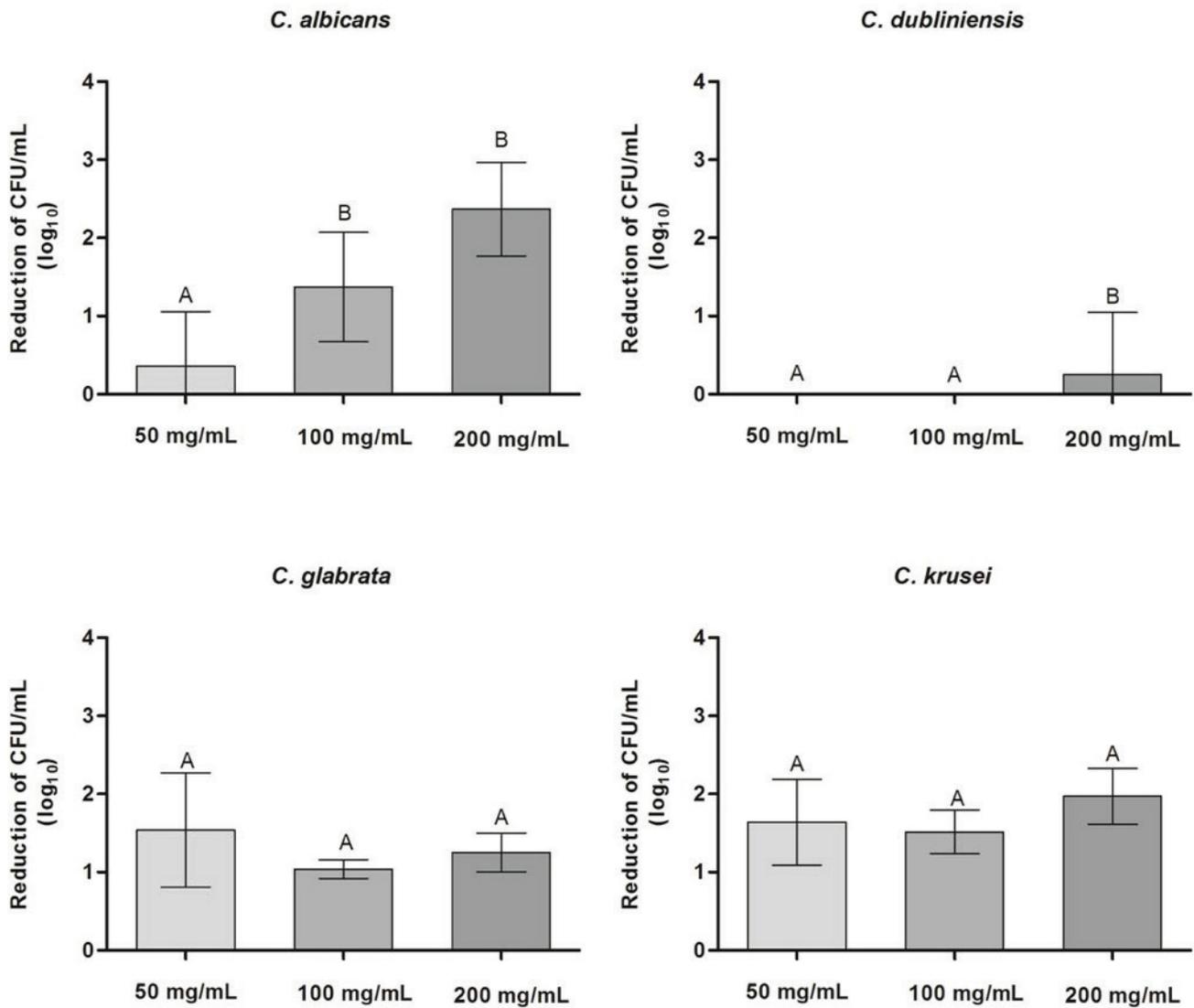


Figure 1 - Mean values (\pm standard deviation) of reductions (CFU/mL – log₁₀) of *C. albicans*, *C. dubliniensis*, *C. glabrata* and *C. krusei* biofilms obtained after 5 min exposure at 50, 100 and 200 mg/mL of *S. terebinthifolius* extract, regarding to the control group. Different letters indicate significant statistical difference (n = 12; one-way ANOVA and Tukey's test, P ≤ 0.05).

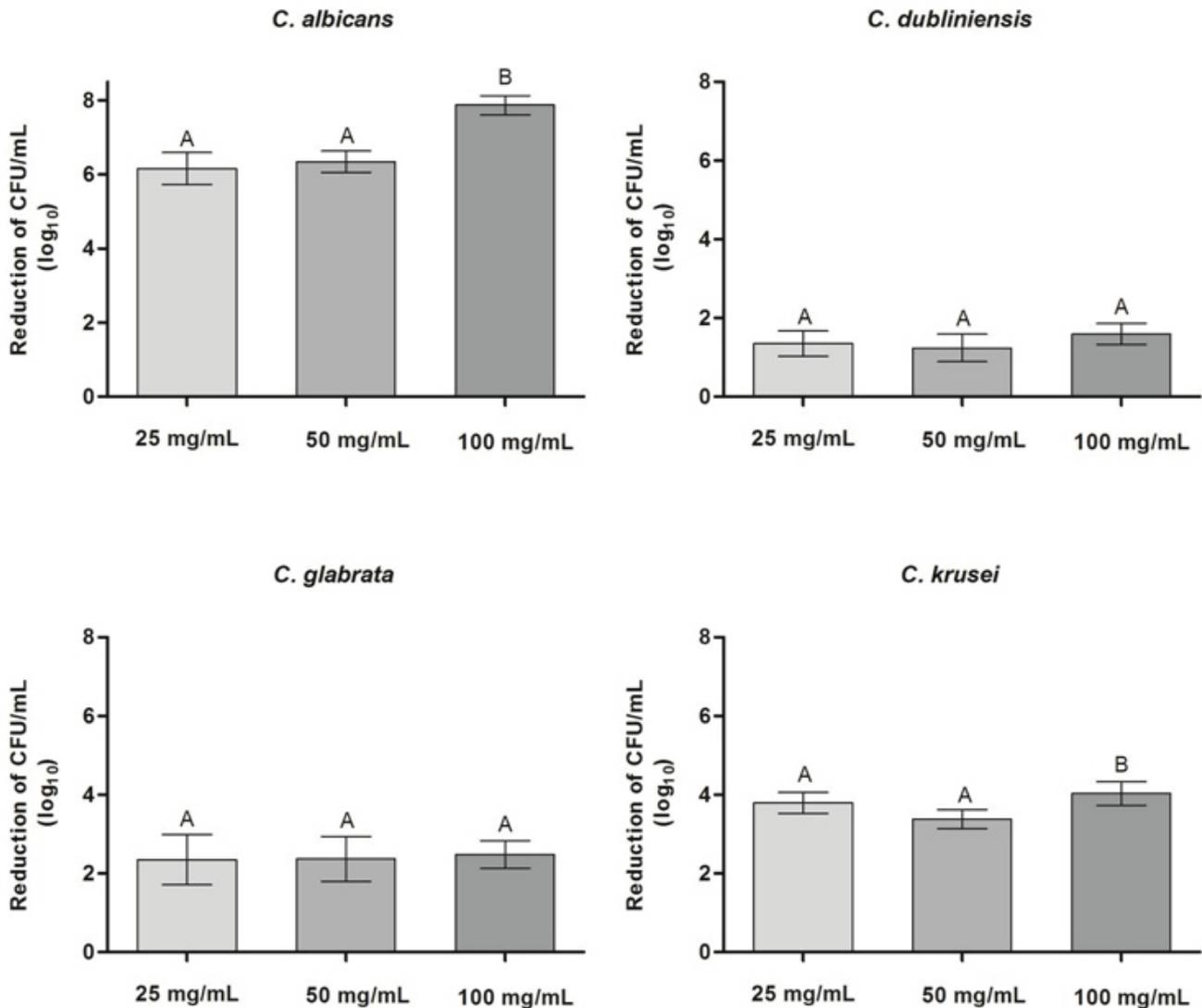


Figure 2 - Mean values (\pm standard deviation) of reductions (CFU/mL – \log_{10}) of *C. albicans*, *C. dubliniensis*, *C. glabrata* and *C. krusei* biofilms obtained after 24 h exposure at 25, 50 and 100 mg/mL of *S. terebinthifolius* extract, regarding to the control group. Different letters indicate significant statistical difference ($n = 12$; one-way ANOVA and Tukey's test, $P \leq 0.05$).

DISCUSSION

Antifungal effect of *S. terebinthifolius* extract on *C. albicans*, *C. dubliniensis*, *C. glabrata* and *C. krusei* both in planktonic and biofilm forms was demonstrated in the present study. *C. dubliniensis* presented the lowest MIC (0.39 mg/mL) and MFC (0.78 mg/mL) values compared to the species analysed like *C. albicans* (MIC = 1.56 mg/mL; MFC = 3.13 mg/mL) and *C. glabrata* and *C. krusei* (MIC/MFC = 3.13 mg/mL).

The anti-*Candida* activity of *S. terebinthifolius* has also been reported by some studies using other types of products from this plant. Alves et al. [11] used dye extracted from *S. terebinthifolius* and found antifungal activity on *C. albicans* (MIC = 0.312 mg/mL; MFC = 2.5 mg/mL). Moura-Costa et al. [12] analysed the aqueous extract of this plant and verified antifungal activity on *C. albicans* (MIC = 0.49 μ g/mL), *C. parapsilosis* (MIC = 3.9 μ g/mL) and *C. tropicalis* (MIC = 15.6 μ g/mL). These same authors also reported antifungal effect

of the hydroalcoholic extract on *C. albicans* (MIC = 0.49 $\mu\text{g}/\text{mL}$), *C. parapsilosis* (MIC = 62.5 $\mu\text{g}/\text{mL}$) and *C. tropicalis* (MIC = 62.5 $\mu\text{g}/\text{mL}$), whereas Alves et al. [13] demonstrated that the dye extracted from *S. terebinthifolius* presented effective action against *C. tropicalis*, with MIC and MFC values of 625 $\mu\text{g}/\text{mL}$. Martínez et al. [14] conducted a study with different concentrations of *S. terebinthifolius* alcoholic extract and found no microbial growth inhibition in Gram-positive and Gram-negative bacteria and *C. albicans* at the lowest concentration used (10%), but an inhibitory effect was observed at higher concentrations (50% and 100%). In addition, Gomes et al. [15] reported that lectin, a compound extracted from *S. terebinthifolius*, had an effect on *C. albicans* (MIC = 6.5 $\mu\text{g}/\text{mL}$; MCF = 26 $\mu\text{g}/\text{mL}$). Johann et al. [16] demonstrated that *S. terebinthifolius* leaf extract contains saponins, flavonoids, triterpenes, steroids and tannins, all capable of affecting the development of fungal specimens. Studies on plant compounds are necessary to find out bioactive molecules and elucidate their action mechanisms, thus allowing their addition to new drugs.

In this study was also shown the effect of *S. terebinthifolius* extract on *Candida* spp. biofilms. Nevertheless, higher concentrations were needed to achieve an effective control of biofilms compared to those used on planktonic forms. In fact, it was reported that biofilms can be more resistant than planktonic cells [17]. *C. albicans* biofilm exposed to *S. terebinthifolius* extract presented significant reductions of CFU/mL. However, it was found that concentrations of 100 and 200 mg/mL were more effective than 50 mg/mL in 5 min exposure. Similar reductions of these biofilms were observed after 24 h exposure to the extract at concentrations of 25, 50 and 100 mg/mL. Alves et al. [11] also reported that the dye extracted from *S. terebinthifolius* showed antibiofilm effect on *C. albicans* demonstrating that the MIC values (1x, 2x and 4x) of this product affected significantly this fungal species, causing significant reductions of CFU/mL after 60 min exposure; however they were similar to controls after 120 and

180 min exposures. Barbieri et al. [8] reported inhibitory effect on the initial formation of *C. albicans* biofilm. According to the authors, this effect can be measured by the presence of some compounds of the plant such as alkaloids, phenols and terpenes. Therefore, it can suggest that this product may be potentially used for both prevention and treatment of infections associated with *Candida* biofilm formation.

Non-*albicans Candida* biofilms were also significantly affected such as *C. dubliniensis*, *C. glabrata* and *C. krusei* after 24 h exposure to different concentrations of *S. terebinthifolius*. There were also significant reductions in CFU/mL after 5 min exposure, except with *C. dubliniensis* at 50 and 100 mg/mL. These results are important because non-*albicans Candida* are present in different candidoses such as oropharyngeal candidiasis in HIV-infected individuals [18], invasive candidiasis in intensive care units [19] and vulvovaginal candidiasis [20], including strains resistant to most conventional antifungal like fluconazole and itraconazole.

Similarly to *C. albicans*, *C. dubliniensis* can be considered as human commensal yeast and thus can cause opportunistic infections [21]. However, some differences regarding adherence to oral epithelium [22] and filamentation of biofilm cells [23] were related. *C. krusei* was cited as a pathogen involved in cases of systemic infections, mainly in patients with acquired immune deficiency syndrome [24]. This specie can also inhibit the development of *C. albicans* in an interspecific association [25].

CONCLUSION

The antifungal potential of the *S. terebinthifolius* extract suggests opportunities to study this plant more closely so that other possible biological effects (i.e. antibiotic, antitumor and anti-inflammatory) can be explored, including application to cytotoxicity tests to ensure its use in possible formulations, broadening the antifungal therapeutic scope. Thus, it was demonstrated that *S. terebinthifolius* extract

presented antifungal effect on *C. albicans*, *C. dubliniensis*, *C. glabrata* and *C. krusei* both in planktonic cultures and biofilms, being of great interest for the development of new antifungal pharmaceuticals.

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