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***Corresponding author:**
Nívea Pereira de Sá;
**Email:** niveasap@gmail.com;
**Tel.:** +55 31 3409 2739



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# Anti-*Candida* Activity of Cinnamon Inhibition of Virulence Factors of Clinical Strains of *Candida albicans* by Essential Oil of *Cinnamomum zeylanicum*

**Priscila Cordeiro de Lima Carvalho[1], Nívea Pereira de Sá[1]*, Inayara Cristina Alves Lacerda[2], Carla Pataro[1], Luiz Henrique Rosa[1], Rodrigo Silva Alves[3], Juliana Pereira Lyon[4], Carlos Augusto Rosa[1], Susana Johann[1]**

[1]Microbiology Department, Biological Sciences Institute, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil.
[2]Food Department, Pharmacy School, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil.
[3]University Center UNA, Belo Horizonte, MG, Brazil.
[4]Natural Science Department, Universidade Federal de São João Del Rei, São João Del Rei, MG, Brazil

**Abstract**
Essential oil of cinnamon (*Cinnamomum zeylanicum)* has been used in medicine and food additives. In the present work, we examined the antifungal activity of essential oil extracted from *C. zeylanicum* bark against oral opportunistic strains of *Candida albicans*. In addition, we verified the capability of this essential oil to inhibit fungal adhesion to buccal epithelial cells (BECs), germ tube formation, and proteinase activities. Cinnamon oil presented minimum inhibitory concentration (MIC) values that ranged from 31.2 to 125 µg $mL^{-1}$ against the isolates of the tested *C. albicans* strains. Cinnamon essential oil could inhibit the adhesion of *C. albicans* to BECs for all of the isolates tested in present study. The *C. albicans* strains showed inhibited proteinase production after treatment with cinnamon oil, based on the MIC values determined for each *C. albicans* strain. Cinnamon oil was also able to inhibit the germ tube formation of all isolates of *C. albicans*, with the percentage of inhibition ranging from 44.7 to 82.9%. Our results showed that cinnamon oil presented potent antifungal activity and the ability to inhibit virulence factors of oral pathogenic strains of *C. albicans*.
**Keywords:** Adhesion; Cinnamon oil; Germ tube; Proteinase.

## INTRODUCTION

Essential oils are volatile, natural, complex compounds with a strong scent that are produced as secondary metabolites by aromatic plants (Bakkali *et al.,* 2008). The genus *Cinnamomum* comprises approximately 250 species that are widely distributed in China, India, and Australia (Jayaprakasha *et al*., 2003). *Cinnamomum zeylanicum* (cinnamon) bark is used worldwide as a spice. Cinnamon is employed in cooking as a condiment and flavoring material and is largely used in the preparation of certain desserts, chocolate, spicy candies, tea, hot cocoa, and liqueurs (Shan *et al*., 2005; Sathishkumar *et al*., 2009). Cinnamon oil from *C. zeylanicum* bark commonly presents antimicrobial and antifungal properties that have drawn great attention from many researchers (Singh *et al*., 1995; Hili *et al*., 1997; Ouattara *et al*., 1997; Park *et al*., 2000; Chang *et al*., 2001; Kim *et al*., 2004).

Increased fungal resistance to classical drugs, drug toxicity, and the costs involved justify the search for new approaches to developing antifungal drugs. Among those new approaches, essential oils are one of the most promising groups of natural compounds for the prevention and treatment of fungal infection (Silva *et al*., 2011). Essential oils derived from aromatic plants are well known in traditional medicine as antimicrobial agents and are characterized as food and feed preservatives, as inhibitors of mycotoxin production, and as antimycotic agents (Azzouz and Bullerman, 1982; Knobloch *et al*., 1989; Guimarães *et al*., 2010).

Oropharyngeal candidiasis (OPC) is the most common opportunistic infection in immunocompromised patients. Although *Candida albicans* is a well-known etiological agent of OPC, in addition to being responsible for most yeast infections in humans, several other emerging *Candida* species, such as *C. dubliniensis, C. glabrata, C. krusei, C. parapsilosis,* and *C. tropicalis*, have also been associated with this disease (Horn *et al*., 2009).

Considering increasing pathogen resistance and the toxicity of classical antifungal drugs, investigations of the antimicrobial activities, modes of action, and potential uses of essential oils and their components have garnered new attention in the search for new antimicrobial compounds (Silva *et al*., 2011). The development of new drugs with improved efficacy and safety or alternative modes of fighting infections are needed. Recent developments in fungal genomics have provided unprecedented opportunities to identify new antifungal drug targets and accomplish subsequent disease control. Targeting virulence and pathogenicity factors should provide alternatives to conventional drug targets and provide new options for the development of potential antifungal therapeutics for the treatment and/or prevention of localized or systemic fungal disease (Gauwerky *et al*., 2009). According Alksne and Projan (2001), the idea is no longer to try to kill the microorganism acting as a pathogen by any means, but rather to hinder the organism from causing any harm to the host.

In this work, we examined the effects of essential oil extracted from *C. zeylanicum* against 28 strains of *C. albicans* isolated from OPC. We also verified the ability of this essential oil to inhibit the germ tube formation, adhesion to buccal epithelial cells (BECs), and proteinase activity of the tested *C. albicans* isolates.

## MATERIALS AND METHODS

Broth microdilution testing was performed in accordance with CLSI document M27-A3 (2008). Susceptibility was determined by the microbroth dilution method, which was performed in sterile flat-bottom 96-well microplates (Difco Laboratories, Detroit, MI, USA). The cinnamon oil was diluted in synthetic RPMI medium (Sigma, St. Louis, MO, USA) supplemented with L-glutamine and buffered to pH 7.0 with 0.165 M morpholine propanesulfonic acid (MOPS; Sigma). As growth and sterility controls, RPMI alone was used, without the addition of the oil and solvent. Fluconazole (Pfizer Pharmaceuticals, USA) was included at a concentration of 0.125 to 64 µg $mL^{-1}$ as the positive antifungal control. After assembly of the plates, each yeast strain was inoculated, and the plates were incubated at 35°C for 48 h. The tests were performed in triplicate. The endpoints were determined visually by comparison with the endpoints of the drug-free growth-control well. The value of the minimum inhibitory concentration (MIC) was defined as the lowest extract concentration at which the well was optically clear and was expressed in µg $mL^{-1}$.

The preparation of HBECs for the adherence assays was performed as described by Kimura and Pearsall (1978) and Ellepola and Samaranayake (1998a,b) and modified by Lyon and Resende (2006) and Johann *et al*. (2007) The concentrations of cinnamon oil and fluconazole used in the assays were the same as those obtained in the MIC test. In the assay, 0.5 mL of the BEC suspension and 0.5 mL of yeast suspension were mixed gently in tubes and incubated on a rotary incubator at 37°C for 1 h. The yeast/BEC suspension was then harvested onto a 12 µm pore-size polycarbonate filter and washed twice with 50 mL of PBS to remove unattached yeast. Next, the filter was carefully removed with forceps and placed firmly on a glass slide, with the BECs against the glass surface. After 10 s, the filter was gently removed, leaving the BEC adherent to the glass slide. The preparations were air dried, fixed with heat, and stained with gentian violet.

The MIC values of cinnamon oil and fluconazole obtained for each strain were used to verify inhibition of the proteinase activity of these yeasts. A control consisting of SDB without any drugs was always included. A yeast suspension at an optical density of 1.5 at 520 nm was

prepared in PBS, and 1 mL of this suspension was added to 4 mL of SDB with cinnamon oil or fluconazole and to the control. This procedure yielded a suspension that presented between $10^6$ and $10^7$ cells $mL^{-1}$. The tubes were incubated at 37°C for 60 min. After this brief exposure, the cinnamon oil or fluconazole was removed by two cycles of centrifugation with PBS for 10 min at 3000 g. The pellet was suspended in 3 mL of PBS. This procedure drastically reduces the concentration of cinnamon oil and fluconazole, minimizing any residual effect. Prior to the assays, viable counts of the control and test samples were performed after drug removal by inoculating an aliquot of the suspensions in SDA. Proteinase activity was assayed on a solid medium containing bovine serum albumin (BSA), as described by Cassone *et al*. (1987). Each isolate was tested in triplicate.

Percentage of inhibition of germ tube formation (GTF) by pathogenic isolates was performed according to the method described by Brayman and Wilks (2003). The absorbance at 590 nm was determined by using a VERSAmax (Molecular Devices) and the program SoftMax® Pro 5 (Molecular Devices, USA) for SpectraMax Plus.

## RESULTS AND DISCUSSION

The results showed that essential oil from cinnamon (*C. zeylanicum*) inhibited the isolates of oral pathogenic *Candida* species. The MIC results for cinnamon oil are shown in Table 1. Cinnamon oil presented MIC values ranging from 31.2 to 125 µg $mL^{-1}$ against the yeast strains tested. Two isolates of *C. albicans* showed more sensitivity to cinnamon oil at an MIC value of 31.2 µg $mL^{-1}$. All tested yeast isolates were sensitive to fluconazole at an MIC value ≤16 µg $mL^{-1}$. Other authors also showed that cinnamon oil has antifungal activity. Singh *et al*. (1995) described fungitoxic properties of the vapors of bark oil from *C. zeylanicum* against fungi involved in respiratory tract mycoses, such as *Aspergillus niger, A. fumigatus, A. nidulans, A. flavus, C. albicans, C. tropicalis,* and *Histoplasma capsulatum.* According Singh *et al*. (1995), cinnamic aldehyde is an active fungitoxic constituent of *C. zeylanicum* bark oil. Unlu *et al*. (2010) analyzed cinnamon oil by gas chromatography-mass spectrometry and observed the presence of nine constituents, with (E)-cinnamaldehyde, benzaldehyde, and (E)-cinnamyl acetate as major compounds. Unlu *et al*. (2010) also tested essential oil from the bark of *C. zeylanicum* and found inhibitory activity against the tested species of *Candida*, with MIC values ranging from 40-1000 µg $mL^{-1}$, which are values similar to those found in the present work (32.5-125 µg $mL^{-1}$). Our results showed that essential oil from cinnamon has antifungal activity. Fluconazole is a therapeutic option for various types of fungal diseases, including oral candidiasis. In particular, this drug is relevant to localized candidiasis in HIV-infected patients. In this group of patients, however, the development of fluconazole resistance has become a problem, leading to treatment failure (Enwuru *et al*., 2008; Gauwerky *et al*., 2009). The use of essential oil from cinnamon could be recourse in the treatment of patients with oral candidiasis. Given that cinnamon is already used as a food additive and has high human consumption worldwide (Wang *et al*., 2009), this spice could present low or not present toxicity to people using these food products.

**Table 1. Minimal inhibitory concentrations (MICs) of essential oil from *Cinnamomum zeylanicum* (cinnamon) and fluconazole against 28 oral *Candida albicans* strains (values in µg $mL^{-1}$).**

| | MIC (µg $mL^{-1}$) | |
|---|---|---|
| **Yeast** (number of isolates) | **Cinnamon oil** | **Fluconazole** |
| *C. albicans* (2) | 31.2 | 16.0-8.0 |
| *C. albicans* (16) | 62.5 | 16.0-0.12 |
| *C. albicans* (10) | 125.0 | 4-0.06 |

Pathogenic *Candida* species produce several virulence factors that are essential for causing disease in a host. These factors include host recognition biomolecules (adhesins), morphogenesis-inducing factors (the reversible transition between unicellular yeast cells and filamentous growth forms), and aspartyl proteases and phospholipases. These virulence factors can be important targets in the development of new antifungal drugs (Calderone and Fonzi, 2001). In the present work, Cinnamon oil was also tested for its ability to inhibit the adhesion of *C. albicans* to BECs (Table 2 and figure 1A,B). Cinnamon oil inhibited the adhesion of all isolates of *C. albicans* to BECs. The percentage of inhibition ranged from 100 to 19.3%, and 14 of 29 isolates presented inhibition of the adhesion to BECs between 100 and 80%. Cinnamon oil presented a better capacity to inhibit the adhesion of isolates of *C. albicans* to

BECs than did fluconazole. Fluconazole showed a percentage of inhibition that ranged from 96.5 to 0%, and most (21 isolates) of the yeast strains were inhibited at a percentage less than 57%. For only one isolate of *C. albicans*, fluconazole presented better adhesion inhibition than did cinnamon oil. Four isolates of *C. albicans* did not present inhibited adhesion when treated with fluconazole. However, cinnamon oil reached an inhibition of the adhesion ability of the same isolates of 88.5, 38.7, 19.2, and 79.5%. In addition, cinnamon oil also inhibited the adhesion of *C. albicans* to BECs better than fluconazole have done for 27 of the isolates tested. Ellepola and Samaranayake (1998b) also observed a very low degree of *C. albicans* adhesion inhibition by fluconazole. Several plant extracts and antifungal drugs have already been tested with the objective of inhibiting the adhesion of *C. albicans* to BECs (Ellepola and Samaranayake (1998a; Johann *et al*., 2007; Johann *et al*., 2008), but the present study is the first to examine the ability of the essential oil from the bark of *C. zeylanicum* to inhibit adhesion to BECs. Our results showed that essential oil from the bark of *C. zeylanicum* can inhibit the adhesion of *C. albicans* to BECs, therefore decreasing the virulence of oral pathogenic strains of *C. albicans*.

**Table 2. Percentage of inhibition of the adhesion of *C. albicans* to BECs of each *C. albicans* strain.**

| | Inhibition of the adhesion of *C. albicans* to BECs | |
|---|---|---|
| ***C. albicans* strains** | **Fluconazole** | **Cinnamon oil** |
| UFMG 12.2B | 43.33 | 90 |
| UFMG 34.1A | 49.12 | 84.21 |
| UFMG 4.1D | 0.00 | 88.52 |
| UFMG 30.2A | 53.77 | 97.17 |
| UFMG 4.2A | 21.62 | 91.89 |
| UFMG 7.1A | 35.05 | 91.75 |
| UFMG 11.2B | 56.74 | 97.19 |
| UFMG 29.1D | 4.35 | 91.30 |
| UFMG 23.2D | 27.03 | 78.38 |
| UFMG 21.1D | 18.33 | 75.00 |
| UFMG 31.1B | 26.00 | 100.00 |
| UFMG 1A | 0.00 | 38.71 |
| UFMG 5.2A | 0.00 | 19.23 |
| UFMG 13.1E | 40.98 | 86.89 |
| UFMG 3.1A | 65.95 | 79.46 |
| UFMG 2.1A | 16.30 | 81.52 |
| UFMG 9.1B | 33.08 | 92.48 |
| UFMG 9.2A | 0.00 | 79.59 |
| UFMG 33.1A | 51.02 | 93.88 |
| UFMG 6 | 51.16 | 56.98 |
| UFMG 1E | 29.41 | 41.18 |
| UFMG 15.3C | 96.55 | 90.80 |

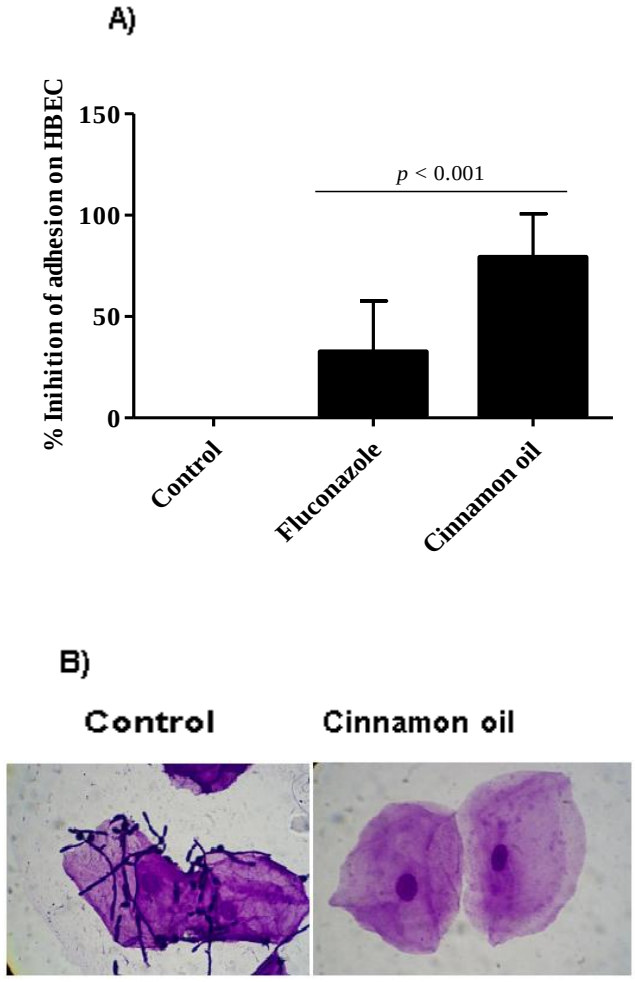


**Fig. 1. Adhesion of *Candida albicans* to human buccal epithelial cells (HBEC). A) Percentage average of inhibition of the adhesion of *C. albicans* to HBECs. B) Optical microscopy (40x) photos of *C. albicans* adhered buccal epithelial cells (gentian violet stain).**

The tested *C. albicans* strains demonstrated alteration in their proteinase production after treatment with cinnamon oil at the MIC values determined for each yeast strain. Table 3 shows that cinnamon oil possesses very similar activity to fluconazole in the alteration of proteinase secretion. Except for one isolate of *C. albicans*, cinnamon oil showed better activity than did fluconazole in the inhibition of proteinase production. One isolate did not present proteinase production inhibition by fluconazole, whereas cinnamon oil inhibited the activity of the proteinase of the same isolate by 66%. Cinnamon oil inhibited proteinase production by the *C. albicans* strains tested in the present work. The secretion of proteinase is a major virulence factor of *C. albicans* and is responsible for causing tissue damage, providing nutrition to the yeast cells, and evading immune responses (Hube and Naglik, 2001; Gauwerky *et al*., 2009). Shreaz *et al*. (2012) showed that three compounds (cinnamaldehyde, 4-hydroxy-3-methoxy cinnamaldehyd, and 3,5-dimethoxy-4-hydroxy cinnamaldehyde) that occur naturally in the bark of the cinnamon tree and other species of the genus *Cinnamomum,* such as camphor and cassia, significantly inhibited phospholipase and proteinase secretion by oral *C. albicans* isolates. The significant decrease in proteinase and phospholipase secretion by cinnamaldehyde and its derivatives can be attributed to ATPase-dependent efflux mechanisms (Shreaz *et al*., 2012). These compounds may also have been responsible for the inhibition of the proteinase activity of the *C. albicans* strains observed in the present work.

The cultivation of the isolates of *C. albicans* in polystyrene microplates resulted in germ tube attachment to the surface of each well. In the present work, 92.2% of untreated *C. albicans* cells presented germ tube formation under the conditions tested, whereas only two isolates of *C. albicans* did not (Table 4). Cinnamon oil was able to inhibit germ tube formation by the isolates of *C. albicans*. Considering all isolates tested, 16 presented inhibition of germ tube formation between 44.7 and 82.9% after treatment with cinnamon oil at concentration values ranging from 62.5-500 µg $mL^{-1}$. One isolate of *C. albicans* presented 82.9% inhibition of germ tube formation, but at a concentration of 250 µg $mL^{-1}$ cinnamon oil. The compounds cinnamaldehyde, 4-hydroxy-3-methoxy cinnamaldehyd, and 3,5-dimethoxy-4-hydroxy cinnamaldehyde, which are present in cinnamon oil, inhibits the transition of *C. albicans* cells from yeast to hyphae (Shreaz *et al*., 2012). Our results also showed that cinnamon oil inhibited the germ tube formation of the tested *C. albicans* strains, which is the initial stage of hyphal formation. The formation of germ tubes could be a new target for drugs that is more sensitive to drug intervention than the inhibition of growth is (Brayman and Wiks, 2003). According with Brayman and Wiks (2003) and Hector (1993), the formation of germ tubes is related to the activation of glucan synthase because there is extensive synthesis of cell walls during the formation of hyphae. Therefore, the inhibition of germ tube formation could be associated with inhibition of glucan synthase, which could also be an interesting target for new antifungal drugs. The inhibition of glucan synthase is related to the integrity of the fungal cell wall, and this target is not related to any other target in the mammalian host (Brayman and Wiks, 2003; Gauwerky *et al*., 2009).

**Table 3. Percentage of inhibition of proteinase activity of *Candida albicans* by essential oil from *Cinnamomum zeylanicum* (cinnamon).**

| | Inhibition of proteinase activity | |
|---|---|---|
| ***C. albicans* strains** | Fluconazole | Cinnamon oil |
| UFMG 12.2B | 50 | 50 |
| UFMG 34.1A | 34 | 34 |
| UFMG 4.1D | 0 | 0 |
| UFMG 30.2A | 0 | 0 |
| UFMG 4.2A | 0 | 0 |
| UFMG 7.1A | 40 | 40 |
| UFMG 11.2B | 34 | 34 |
| UFMG 29.1D | 25 | 25 |
| UFMG 23.2D | 34 | 34 |
| UFMG 21.1D | 34 | 34 |
| UFMG 31.1B | 0 | 0 |
| UFMG 1A | 0 | 0 |
| UFMG 5.2A | -* | - |
| UFMG 13.1E | 50 | 50 |
| UFMG 3.1A | - | - |
| UFMG 2.1A | 0 | 0 |
| UFMG 9.1B | - | - |
| UFMG 9.2A | 0.00 | 66 |
| UFMG 33.1A | 0 | 0 |
| UFMG 6 | 25 | 25 |
| UFMG 1E | 30 | 40 |
| UFMG 15.3C | - | - |

*****: not tested; UFMG: strains of *C. albicans* from the culture collection of the Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil.

In the present work, we characterized the antifungal activity of cinnamon oil and its potent inhibition of virulence factors of oral pathogenic *C. albicans*. Cinnamon oil showed a better ability to inhibit the adhesion of *C. albicans* to BECs than did fluconazole. In addition, cinnamon oil decreased proteinase secretion and inhibited the formation of germ tubes by *C. albicans* treated with this essential oil. This work suggests options for expanding the utility of the essential oil of the bark of *C. zeylanicum* as an antifungal agent against oral infection by *C. albicans*.

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## CONFLICT OF INTEREST

There is no conflict of interest.

**Table 4. Percentage of inhibition of germ tube formation (GTF) by pathogenic isolates of *Candida albicans* in the presence of essential oil from *Cinnamomum zeylanicum* (cinnamon) at different concentrations.**

| | Cinnamon oil | | Fluconazole | |
|---|---|---|---|---|
| ***C. albicans* strains** | **Concentration (μg mL$^{-1}$)** | **% of inhibition of GTF** | **Concentration (μg mL$^{-1}$)** | **% of inhibition of GTF** |
| UFMG 32.1A | 250 | 54.9 | - | - |
| UFMG 3.2 B | ni | ni | ni | ni |
| UFMG 12.2B | 250 | 50- | - | - |
| UFMG 34.1A | 500 | 64,8 | - | - |
| UFMG 4.1D | - | - | - | - |
| UFMG 8.1B | ni | ni | ni | ni |
| UFMG 30.2A | - | - | - | - |
| UFMG 4.2A | 125 | 59.8 | 62.5 | 55.4 |
| UFMG 7.1A | 250 | 66.7 | - | - |
| UFMG 11.2B | ni | ni | 62.5 | 20.1 |
| UFMG 23.1C | 500 | 61.8 | 62.5 | 90 |
| UFMG 29.1D | 250 | 54.7 | - | - |
| UFMG 23.2D | ni | ni | - | - |
| UFMG 21.1D | 125 | 49.7 | 62.5 | 43.9 |
| UFMG 31.1B | ni | ni | ni | ni |
| UFMG 1A | 125 | 50 | 62.5 | 51.7 |
| UFMG 5.2A | - | - | - | - |
| UFMG 13.1E | 62.5 | 61.6 | 62.5 | 100 |
| UFMG 3.1A | - | - | - | - |
| UFMG 25.1A | 250 | 58.8 | ni | ni |
| UFMG 2.1A | 62.5 | 60 | ni | ni |
| UFMG 24.1A | N | N | N | N |
| UFMG 9.1B | N | N | N | N |
| UFMG 9.2A | 500 | 44.7 | 31.2 | 55 |
| UFMG 9.3A | ni | ni | ni | ni |
| UFMG 33.1A | 500 | 50.2 | - | - |
| UFMG 6 | 500 | 65.4 | 62.5 | 50 |
| UFMG 19.1E | 250 | 82.9 | 62.5 | 37.2 |

ni: not inhibited under the tested conditions; -: not tested; N: no germ tube formation under the tested conditions; UFMG: strains of *C. albicans* from the culture collection of the Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil.

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