Camellia sinensis (green tea) promotes antifungal activity against *Candida* spp. isolated from HIV-positive patients and biocompatibility in murine macrophages

https://doi.org/10.56238/sevened2024.007-029

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ABSTRACT

The aim of this study was to evaluate the antifungal activity and cytotoxicity of the plant extract from Camellia sinensis (green tea) in vitro, as well as to assess the antifungal effect of amphotericin B and fluconazole on 22 strains of Candida spp. isolated from the oral cavity of HIV-positive patients. The minimum fungicidal concentration (MFC) and minimum inhibitory concentration (MIC) were determined in planktonic cells using serial dilutions of green tea extract and antifungals. After determining the extract concentration at MIC and MFC, biofilm was prepared for each strain. Cytotoxicity in mouse macrophages (RAW 264.7) was evaluated to assess the cellular viability of this substance. Colony-forming units (CFU/mL) were counted, and the data were statistically evaluated using the Mann-Whitney test ($p<0.05$) for biofilm, visual observation for MIC and MFC, and ANOVA and Tukey for cytotoxicity. The results demonstrated viability of green tea extract in the analyzed cells. It was concluded in this study that C. sinensis (green tea) extract showed antifungal activity in planktonic cells and in biofilm for all Candida strains evaluated, with no cytotoxic effects on RAW 264.7. Fluconazole exhibited fungicidal effect in planktonic cells, while amphotericin B showed antifungal effect on C. albicans strains and microbial resistance in non-albicans strains.

Keywords: Amphotericin B, Biofilms, *Camellia sinensis*, *Candida*, Fluconazole.

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INTRODUCTION

The yeasts of the *Candida* genus are responsible for most fungal infections in immunocompromised patients, such as HIV-infected patients, transplant recipients, or those undergoing chemotherapy, both due to the use of immunosuppressants and individual factors such as advanced age and systemic diseases like diabetes mellitus. The oral cavity represents one of the preferred sites for the development of *Candida* yeast infections (Samaranayake et al., 2001; Serrano-Granger et al., 2005; Coleman, 2010, Wang, 2015). Oropharyngeal candidiasis is the most common fungal infection among HIV-infected patients, often presenting recurrent episodes, especially when the CD4 lymphocyte count is low (Wengeter et al., 2007).

Azole derivatives, particularly fluconazole, are effective in treating candidiasis in patients with advanced immunodeficiency and constitute the first step in treating these infections. Polyenes such as amphotericin B are used in severe cases of systemic infections, although they present various disadvantages such as nephrotoxicity (Blakenship & Michell, 2006; Nadagir et al., 2008). Enwuru et al. (2008) studied the prevalence of yeast species in HIV-positive patients with oropharyngeal candidiasis and assessed their antimicrobial susceptibility. The most prevalent species was *C. albicans* followed by *Candida glabrata*, *Candida tropicalis*, *Candida krusei*, *Candida parapsilosis*, and *Candida dubliniensis*.

Susceptibility to antifungals may vary among yeast isolated from the same individual, revealing resistance to various azole antifungals. Some authors, faced with the increasing resistance to conventional antifungals, have been seeking alternatives for the treatment of oral candidiasis lesions, such as the use of herbal medicines among other alternative therapies (Vendruscolo et al., 2005; Nakata et al., 2007; Colleman, 2010; Ramage et al., 2011; Oliveira et al., 2013).

Medicinal plants have been widely used by humanity since ancient times. The indiscriminate and prolonged use of synthetic chemical drugs has led to the selection of mutant pathogenic microorganisms resistant to these compounds, making the use of antimicrobials of natural origin an effective alternative (Antunes, 2014). The plant *Camellia sinensis* belongs to the Theaceae family, popularly known as green tea or Indian tea. It contains substances such as flavonoids and catechins, which are potent therapeutic components such as antioxidants and inhibitors of lipid peroxidation (Narotzki, 2012; Mollashahi, 2015). Sitheeque et al. (2009) evaluated the antimicrobial effect of polyphenols and catechins found in *C. sinensis* on various *Candida* species, reporting that the therapeutic substances found in this plant had antifungal activity in all analyzed species, mainly in *C. albicans*, and emphasized the importance of more detailed studies for clinical application.

Although it is frequently used, studying the cytotoxicity of green tea is necessary for its safer use in clinical practice as an herbal medicine. In addition to the beneficial effects, cytotoxic effects should also be considered for all plants when used for therapeutic purposes (Casaroto & Lara, 2010).

Oral cavity infections caused by *Candida* spp. are related to the formation of biofilm by these microorganisms (Blankenship & Mitchell, 2006; Thein, 2007; Tsang, 2007). The cells that make up the biofilm have distinct characteristics from planktonic cells, such as increased resistance to antifungals and the host's immune defenses (Oliveira et al., 2013; Gulati & Nobile, 2016). Biofilms are composed of microbial aggregation that can adhere to dental surfaces as well as other restorative and prosthetic materials (Eike et al., 2004; Mallashahi, 2015).

In order to expand knowledge about alternative treatments and control of oral candidiasis and reduction of biofilm, the objective of the present study was to evaluate the antimicrobial and cytotoxic activity of green tea extract and the antifungal activity of fluconazole and amphotericin B in yeasts isolated from the oral cavity of HIV-positive patients.

METHODOLOGY

The study was conducted at the Microbiology Laboratory of the São José dos Campos School of Dentistry/UNESP with the aim of quantifying and qualifying *Candida* genus yeasts as resistant or sensitive to the substances under study. The methodology employed was based on the study by Oliveira et al. (2013). Twenty-two clinical strains collected in a previous study, isolated from the oral cavity of HIV-positive patients with controlled virology, were used. The samples were stored on inclined Sabouraud agar and kept refrigerated. The strains were seeded on Petri dishes containing CHROMagar *Candida* (CHROMagar, Paris, France) and incubated at 37°C for 48 hours. Colonies were morphologically identified according to their coloration, with green suggestive of *Candida albicans*, bluish-gray of *Candida tropicalis*, and pink of *Candida krusei*. After growth, *Candida* colonies were seeded on Sabouraud dextrose agar (Difco, Detroit, USA) and incubated for 24 hours at 37°C for further use. The following phenotypic identification tests were performed: germ tube formation, microculture, and API 20 AUX biochemical test (BioMerieux Clinical Diagnostics, France). A standard sample of *C. albicans* (ATCC 18804) was used for assay standardization. The present study was submitted to and approved by the Research Ethics Committee of the School of Dentistry-UNESP, São José dos Campos campus, under protocol 028/2010. From the isolation of *Candida* species obtained on CHROMagar *Candida*, microculture, and germ tube, confirmation of species was performed by biochemical identification of the isolates. Colonies were seeded on Sabouraud dextrose agar and incubated for 24 hours at 37°C. The API20CAUX identification system (BioMerieux Clinical Diagnostics, France) was used for biochemical identification.

AQUEOUS EXTRACT OF *C. SINENSIS* (GREEN TEA)

An aqueous extract of green tea (*C. sinensis*) at a concentration of 20% was used, acquired from a compounding pharmacy (Becker - São José dos Campos, Brazil). The extract was stored in a

hermetically sealed bottle, protected from light, and at a temperature between 8 and 12°C until the preconized assays were performed.

DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC) AND MINIMUM FUNGICIDAL CONCENTRATION (MFC)

For the determination of the minimum inhibitory concentration (MIC) of the plant extract, the broth dilution method was performed. Serial dilutions of the extract were prepared in 96-well plates (Costar 3524), adding 125 µL of buffered RPMI 1640 synthetic medium at pH 7.0 with MOPS (morpholinepropanesulfonic acid), 125 µL of the plant extract, and 12.5 µL of standardized suspension (10^6 viable cells/mL) of each *Candida* spp. strain. Thus, dilutions of the extract were obtained at the following proportions: 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128 of the original concentration. After incubation for 24 h at 37°C, readings were performed by visual observation of medium turbidity. For the control group in the 96-well plates, 125 µL of RPMI culture medium and 12.5 µL of standardized suspension (10^6 viable cells/mL) of each *Candida* spp. strain were added. For the determination of the minimum fungicidal concentration (MFC), three inocula from the previous test, which showed no growth in broth, were seeded on Sabouraud Dextrose agar plates, determining the MFC as the lowest concentration of the tested extract capable of completely inhibiting microbial growth seeded in solid culture medium.

ANTIMICROBIAL ACTION OF *C. SINENSIS* EXTRACT (GREEN TEA) ON BIOFILM

For biofilm formation, acrylic resin specimens, transparent iris caps with a pin for ocular prosthesis preparation (Clássico, São Paulo, Brazil) measuring 11 mm in diameter, were used, sterilized in an autoclave for 15 minutes at 120°C. They were then divided into groups (n=10). To obtain the biofilm, each strain was seeded on culture plates containing Sabouraud dextrose agar (Difco, Detroit, USA) and incubated at 37°C/24 h. After the incubation period, a suspension of the microorganism was prepared in sterilized physiological saline solution (0.85% NaCl) and standardized in a spectrophotometer (B582, Micronal, São Paulo, Brazil), to obtain 10^6 cells/mL. The optical density and wavelength parameters were 0.284±0.02 and 530 nm. After standardizing the microorganism, the specimens were placed in 24-well cell culture plates (TPP, Europe), and 2 mL of BHI sucrose broth at 5% and 0.1 mL of the microorganism suspension were added. The 24-well plate was incubated in a bacteriological incubator for 5 days at 37°C. For each experiment, 10 specimens were treated with plant extract at the concentration defined in the MIC. This procedure was performed for all *Candida* strains, considering a control group (n=10) for each experiment.

After biofilm formation, the specimens were washed twice in physiological saline solution (0.85% NaCl). They were then placed with the aid of sterile forceps in 20% green tea extract. The 10

specimens in the control group that did not receive treatment were placed in 0.85% physiological saline solution (NaCl) and subsequently sonicated. The exposure time to the extract was 5 min. After the treatment time, the specimens were washed again and placed in tubes containing physiological saline solution, where they were agitated for 30 s in a 50W power sonicator (Sonoplus HD 2200-Bandelin Eletronic).

Subsequently, dilutions were made, 10^{-1}, 10^{-2}, 10^{-3}, and 0.1 mL of each dilution were seeded in duplicate on Sabouraud dextrose agar plates (Difco-USA). The plates were incubated for 24 h at 37°C in a bacteriological incubator. After the incubation period, the CFU/mL count was performed. The plates chosen for counting were those that presented between 30 and 300 viable colonies of each dilution. The same procedure was performed for all strains and the control group.

EVALUATION OF THE CYTOTOXICITY OF *C. SINENSIS* EXTRACT

Mouse macrophages (RAW 264.7), acquired from the Cell Culture Laboratory of FOSJC - UNESP, obtained from the cell bank of the Paul Ehrlich Technical Scientific Association (APABCAM - RJ), were used. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Sigma Chemical Company, USA), supplemented with 10% fetal bovine serum and incubated in an incubator at 37°C with atmospheric humidity containing 5% CO_2. The medium was changed every two days, and daily monitoring of each cell culture flask was performed using an inverted microscope (Nikon) until a subconfluence state of the cells was observed, characterized by more than 70% occupancy of the flask. In this case, the cell monolayer was subcultured. For subculturing, the medium from the flask was aspirated using a pipette, poured onto the cell monolayer, and then discarded. Subsequently, the cells were washed with fresh medium and again discarded. Next, fresh medium was added, and the cells were carefully removed from the flask using a cell scraper (TPP). The fresh medium containing the cell suspension was centrifuged in a falcon tube at 9,000 rpm for 5 min at 25°C. After centrifugation, the supernatant was discarded, and the cells (pellet) were resuspended in fresh medium and distributed into new cell culture flasks.

For the experiment, the same procedure was performed for subculturing the cell line; however, after resuspending the cells, viable cell counting was performed. For this, a 50 µL sample was placed in a microtube along with 5 µL of 0.5% trypan blue dye (Sigma, USA). This mixture was pipetted and transferred to the Neubauer chamber, covered with a coverslip. Viable cell counting was performed under an optical microscope. Ninety-six-well plates were used for cytotoxicity assays. In each well, 100 µL of DMEM (LGC Biotecnologia) containing 10^8 cells was added. After this procedure, the plates were incubated in a 37°C incubator with 5% CO_2 for 24 h to allow cell adhesion to the wells.

After the incubation period, the medium was removed, and 200 µL of the concentration of

green tea aqueous extract, obtained for all tested microorganisms, in culture medium was added to check its cytotoxicity. The tests were performed in duplicate, and the incubation period with the substances was 24 hours in an incubator at 37°C and 5% CO_2. After the incubation time of the 96-well plates containing the plant extract, the contents were discarded. Each well was washed with 200 µL of 3 to 5 times PBS solution. MTT was prepared at a ratio of 0.5 mg of powder/1 mL of PBS. The solution was agitated for 10 min using a magnetic stirrer. One hundred µL of MTT was placed in each well, and the plates were wrapped in aluminum foil to avoid light exposure, then incubated for 1 h. After the incubation period, the supernatant was discarded, and 100 µL of DMSO was added. The plates were incubated for 10 min with agitation for another 10 min. The plates were then taken to a spectrophotometer with OD (optical density) of 570 nm.

SENSITIVITY OF *CANDIDA* SPP. TO ANTIFUNGALS

Antifungals amphotericin B (Sigma Chemical Company, St Louis, USA) and fluconazole (Galena Química e Farmacêutica) were used. *Candida* spp. samples were tested for *in vitro* susceptibility to antifungals, following the microdilution method proposed by the Clinical Laboratory Standards Institute (CLSI, 2019). To obtain the inoculum, *Candida* spp. samples were cultured on Sabouraud dextrose agar and incubated for 24 h at 37°C. After this period, standardized suspensions were obtained in sterilized saline solution (0.85% NaCl), resulting in an initial concentration of 1.5 x 10^6 cells/mL. Subsequently, the suspension was diluted 1:2000 in synthetic RPMI 1640 medium buffered (Sigma Chemical Company, St Louis, USA) to pH 7.0 with morpholinepropanesulfonic acid (MOPS), to achieve a final concentration of 0.5 x 10^3 to 2.5 x 10^3 cells/mL. Amphotericin B antifungal was diluted in dimethyl sulfoxide (DMSO), while fluconazole was diluted in sterilized distilled water. The antimicrobials were prepared at the following concentrations: 320 µg/mL for amphotericin B, 1250 µg/mL for fluconazole. The antifungal solutions were diluted in synthetic RPMI medium to obtain final concentrations ranging from 64 to 0.03 µg/mL. For the microdilution technique, acrylic plates with 96 flat-bottomed wells and lids (Difco) were used. In each well, 100 µL of the antifungal concentration and 100 µL of the sample-test inoculum were dispensed. The plates were incubated at 37°C, and readings were taken after 24 and 48 h. Plates containing amphotericin B were covered with aluminum foil for protection against light.

Results were interpreted based on a visual turbidity scale by clouding of a control tube represented as: 0 (clear); 1 (slightly cloudy); 2 (intermediate turbidity) corresponding to 80% growth reduction; 3 (prominent turbidity) and 4 (completely cloudy). Results were expressed as ranges between minimum and maximum MIC values for each sample and also as MIC_{50} and MIC_{90} values, representing the growth of 50% and 90% of isolates, respectively. MIC for azoles was defined as the concentration resulting in approximately 80% growth reduction, whereas for amphotericin B, it

corresponded to the concentration with complete absence of growth (100%). Cut-off values for classifying tested antifungals and isolates as susceptible or resistant followed the criteria established by (CLSI, 2019).

STATISTICAL ANALYSIS

For the biofilm, the Mann-Whitney statistical test was conducted, while for cytotoxicity, analysis of variance (ANOVA) and the Tukey test for mean comparison were performed, considering a significance level of 5%.

RESULTS

MIC AND MFC OF *C. SINENSIS* (GREEN TEA) EXTRACT

It was observed in the present study that *C. sinensis* extract exhibited fungicidal effect against all yeast species studied, both in planktonic form and in biofilm. Despite the extract having a concentration of 20%, to obtain the MIC, the initial concentration used was 10%, considering dilution in buffered RPMI broth. All evaluated strains were sensitive to green tea extract at a concentration lower or equal to 10%, in planktonic form. The results obtained in MIC and MFC showed variation in the susceptibility of different *C. albicans* strains regarding the concentration of extract dilutions. It was observed that MIC ranged from 1.25% to 5% and MFC from 2.5% to 10%. From the inhibitory and fungicidal concentration in planktonic cells, a concentration of 20% was determined to evaluate the effects of green tea on biofilm adhered to the test specimens. Non-albicans strains showed lower susceptibility at the evaluated extract concentrations. MIC ranged from 1.25% to 10% and MFC ranged from 2.5% to 10%. *C. glabrata*, *C. tropicalis*, and *C. krusei* were the species least susceptible to the extract, and there was a difference in susceptibility among *C. glabrata* species.

ANTIMICROBIAL ACTION OF *C. SINENSIS* (GREEN TEA) EXTRACT ON *CANDIDA* SPP. BIOFILM

Table 1 shows that *C. albicans* strains treated with green tea extract exhibited significant reduction in CFU/mL compared to the untreated control group; however, there was one strain (*C. albicans* 24s) that did not show a significant reduction compared to the control group. The results of CFU/mL reduction in biofilm presented in Table 1 demonstrated that the 20% green tea extract had fungicidal effect on *C. albicans* strains when in biofilm. The average percentage reduction was 60%.

Table 1 - Colony-forming units (CFU/mL), p-value, and percentage reduction, obtained from the 10 strains of *C. albicans* (n=10) treated with NaCl 0.85% (Control) or green tea extract 20%.

Strain	**CFU/mL**		**p-value**	**Reduction (%)**
	Control	***C. sinensis* (green tea)**		
C. albicans 17	22,100	3,750	0.0002	83
C. albicans 4s	54,800	19,400	0.0002	65
C. albicans 52	95,500	4,650	0.0004	51
C. albicans 1s	41,300	18,300	0.0004	56
C. albicans 10s	48,000	22,750	0.0002	53
C. albicans 24s	36,000	26,000	0.0754	28
C. albicans 31s	10,450	3,850	0.0002	63
C. albicans 39s	68,300	30,200	0.0008	56
C. albicans 9	37,300	5,400	0.0002	86
C. albicans 14	68,000	32,000	0.001	53

Source: Authors.

In Table 2, there was a significant statistical difference in CFU/mL reduction in *C. tropicalis* (p=0.0002), *C. novergensis* (p=0.0002), and *C. glabrata* (p=0.0002) strains, while the other strains showed little reduction compared to the control group. Strain *C. glabrata* 45 did not experience reduction compared to the untreated group, and *C. krusei* also did not show significant statistical difference (p=0.0082). The average percentage reduction for all strains was 48.5%, indicating that non-albicans strains were more resistant in biofilm treated with green tea than C. albicans strains. The difference in CFU/mL reduction between *C. albicans* and non-*albicans* was 10.7%.

Table 2 - Colony-forming units (CFU/mL), p-values, and percentage reduction obtained from the 10 strains of non-*albicans Candida* treated with NaCl 0.85% (Control) or green tea extract 20%.

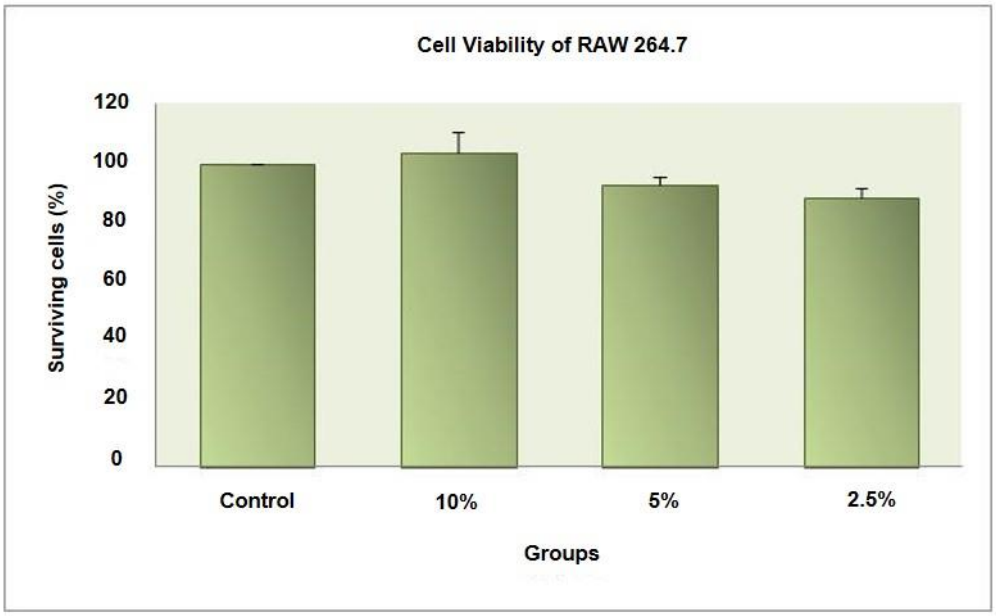
Strain	CFU/mL		p-value	Reduction (%)
	Control	*C. sinensis* (green tea)		
C. dubliniensis	12,150	7,500	0.1859	38
C. glabrata 45	32,400	18,400	0.1618	43
C. glabrata 43	60,400	28,800	0.0002	52
C. glabrata 12s	60,800	46,000	0.0139	24
C. glabrata 46	51,200	38,000	0.0017	26
C. glabrata 51	20,100	6,900	0.0257	66
C. glabrata 67	60,400	28,800	0.0002	52
C. krusei 62	53,400	48,800	0.0082	9
C. novergensis 51s	52,400	23,600	0.0002	55
C. novergensis 52s	18,550	4,300	0.0002	77
C. tropicalis 11	38,400	5,450	0.0002	86
C. tropicalis 12	12,700	5,680	0.0002	55

Source: Authors.

CYTOTOXICITY OF *C. SINENSIS* (GREEN TEA) EXTRACT

Concentrations of green tea extract (10%, 5%, and 2.5%) were analyzed in RAW 264.7 (mouse macrophages). Statistical difference was observed in the means of the concentrations. According to the results obtained, cells subjected to green tea extract showed viability at all analyzed concentrations. The viability of the 10% concentration (104%) showed statistical difference compared to the 2.5% concentration (89%). These data demonstrate that *C. sinensis* extract did not exhibit cellular cytotoxicity at the analyzed concentrations and are expressed in Figure 1.

Figure 1 - Mean values of cell viability of RAW 264.7 of the evaluated groups by MTT, after exposure to *C. sinensis* (green tea) extract at concentrations of 2.5%, 5%, and 10%.



Source: Authors.

In Figure 1, we observe that the *C. sinensis* extract did not exhibit cellular cytotoxicity at the analyzed concentrations, suggesting it is safe for continuous use.

ANTIFUNGAL SENSITIVITIES

It was observed in this study that all strains of *C. albicans* evaluated in the fluconazole MIC showed susceptibility at the tested concentrations (0.25 to 32 µg/mL), with no resistance observed in the strains studied. The values of the concentrations of the antifungal studied ranged from 0.5 to 32 µg/mL, and it was observed that there was no resistance among the various strains and species of *Candida* analyzed. The *C. albicans* strains were susceptible to amphotericin B at concentrations of 0.5 to 2 µg/mL; however, microbial resistance was observed at concentrations of 4 to 8 µg/mL in two strains.

The results of the amphotericin B MIC demonstrated that some non-*albicans* species were resistant to the analyzed antifungals, with five strains being resistant (4 to 8 µg/mL) to this antifungal.

DISCUSSION

Candida spp. are opportunistic fungi responsible for infections in various sites of the body, with the oral cavity being the preferred location for the development of these diseases (Jorge et al., 1997; Appleton, 2000; Wang, 2015). Oral candidiasis is a localized infection often found in immunocompromised patients such as HIV-positive individuals, who have a deficient immune

response, which can lead to systemic infection (Hospental et al., 2006; Matsuki et al., 2006). The most common pathogen isolated from oral lesions in immunocompromised patients is *C. albicans*, however, the proportion of candidemias due to non-*albicans* species has increased considerably, with *C. glabrata*, *C. parapsilosis*, *C. krusei*, and *C. tropicalis* being emerging species (Samaranayake et al., 2001; Kcromery, 2002; Aquino et al., 2005). Of all the species analyzed in this study, *C. glabrata* was the most prevalent non-albicans species, totaling 80% of the studied isolates, with *C. glabrata* infections being frequently associated with HIV-positive patients.

In the present study, 22 clinical strains isolated from the oral cavity of HIV-positive patients obtained from a previous study were analyzed. These strains were initially morphologically identified on CHROMagar Candida medium, which presumptively identifies species by colony coloration. Green colonies suggestive of *C. albicans*, pink for *C. krusei*, and bluish-gray for *C. tropicalis* were found. Ten species of *C. albicans* and 12 non-*albicans* species were identified, including *C. glabrata*, *C. krusei*, *C. tropicalis*, and *C. novergensis*. Species confirmation was performed using the API 20AUX biochemical identification system, confirming the data obtained with the chromogenic medium. Thus, a proportional number of species were found, including *C. dubliniensis*, a pathogen phenotypically similar to *C. albicans*, and *C. novergensis*, yeasts considered emerging in HIV-positive patients. These data are consistent with various studies in the literature, which consider other *Candida* species as contributors to the oral cavity infection process, with non-*albicans* isolates being equivalent in number to the predominant species (Panackal et al., 2006; Shivaprakasha et al., 2007; Coco et al., 2008). Dental conditions are mostly of microbial origin and have been treated with phytotherapy, employed as adjunct therapy to conventional treatments (Pereira, 2009; Oliveira et al., 2013).

The antimicrobial activity of green tea has been previously studied, and according to these studies, in addition to all the benefits found in this plant, the microbicidal effect is also considerable (Miller-Hamilton, 1995; Nakata et al., 2007; Setheeque, 2009). In our study, the objective was to evaluate the fungicidal effect of 20% green tea, however, for minimum inhibitory concentration and minimum fungicidal concentration assays, the extract was diluted in RPMI culture medium at a ratio of 50%, reaching a concentration of 10% which was analyzed in clinical strains of both *C. albicans* and non-*albicans* in planktonic form. The results showed that all strains of *C. albicans* were susceptible to green tea extract, although they exhibited different susceptibility patterns for MIC (1.25% to 5%) and MFC (2.5% to 10%). It was observed that non-*albicans* strains were more resistant to green tea extract than *C. albicans*, with variations in MIC (1.25 to 10%) and MFC (2.5% to 10%), and the species showing the highest resistance were *C. glabrata*, *C. tropicalis*, and *C. krusei*.

Biofilms are considered a form of microbial virulence and resistance, formed when a

microbial community adheres and settles on a surface, an important step in the infection process, presenting greater resistance to drugs and immune defenses by not allowing the entry and flow of drugs into their structure (Thien et al., 2001; Tsang & Mcmillan, 2007; Hasan et al., 2009). Acrylic resins used in the fabrication of dentures and orthodontic appliances are sites that favor biofilm formation in the oral cavity, especially pieces subjected to mechanical abrasion and sometimes not polished, causing roughness that favors microbial adhesion and constitutes an important etiological factor in the development of candidiasis associated with prosthetic use (Coco, 2008). Acrylic resin prosthetic specimens were used to quantify biofilm formation in CFU/mL. Romeiro et al. (2009) evaluated the biofilm formation by *C. albicans*, *C. dubliniensis*, and *C. glabrata* on the surface of titanium discs, a material used for dental implants, and reported that there was adhesion in the analyzed specimens, with *C. albicans* being the microorganism that adhered most to the surface of the implants. The present study evaluated the antifungal activity of green tea extract on biofilm adhered to acrylic resin specimens, and the results obtained demonstrate fungicidal activity with the use of 20% green tea. Most strains of *C. albicans* studied were susceptible to treatment with green tea extract, except for one strain of *C. albicans* ($p=0.0754$). These data indicate that *C. sinensis* exhibited antifungal effects on the evaluated strains. Non-*albicans* species, when in biofilm, proved to be more resistant to green tea extract, with an average percentage reduction of 48.5%. *C. krusei* ($p=0.0820$), *C. dubliniensis* ($p=0.1859$), and *C. glabrata* ($p=0.1618$) were the least susceptible species compared to the control.

Although many herbal remedies are used as adjunct and alternative treatments in oral pathologies, in addition to their therapeutic properties, the toxicity of these substances should be considered. In the present study, the cytotoxicity of *C. sinensis* extract was evaluated in mouse macrophage culture. Three concentrations of the extract, 10%, 5%, and 2.5%, were studied, and cell viability was 104%, 93%, and 89%, respectively. Cell viability was observed at all evaluated extract concentrations, and it did not exhibit cytotoxicity to the studied cells. There was no statistical difference in cell viability after extract application compared to the control.

The frequent reports of resistance of *C. albicans* and non-*albicans* species have been related to chemoprophylaxis and treatment with antifungals, especially fluconazole. The efficacy of this medication in the treatment of oropharyngeal candidiasis is attributed to its characteristics such as low toxicity and broad spectrum of action, being the primary drug of choice in treatments (Krcmery, 2002; Perea et al., 2002; Aquino et al., 2005; Ramage et al., 2011; Wang, 2015). The results of the present study indicated that isolates of *C. albicans* were sensitive to fluconazole (0.5 to 32 µg/mL) at different concentrations. The non-albicans species analyzed in this study also showed sensitivity to fluconazole (0.25 to 32 µg/mL) at different concentrations, and it was observed that non-albicans species were more resistant to amphotericin B than *C. albicans*. *C. glabrata* (4 to 8 µg/mL), *C.*

tropicalis (4 µg/mL), and *C. krusei* (8 µg/mL) were more resistant to the evaluated antifungal, however, 2 strains of C. albicans (4 to 8 µg/mL) were resistant. Currently, antimicrobials are industrially produced in large numbers for the treatment of infections caused by fungi and bacteria. Antifungals fluconazole and amphotericin B are frequently used drugs in the control of fungal infections, both superficial and systemic. The data observed in this study are in accordance with the literature, as the yeasts studied were susceptible to the analyzed antifungals. Although the use of antimicrobials is widely spread, the use of herbal medicines as an alternative therapy is still cautious, especially in dentistry, which aims at patient safety in treatments applied to the oral cavity.

CONCLUSION

The green tea extract showed antifungal activity against the analyzed strains of *Candida* spp., both in biofilm and planktonic form. Fluconazole was effective against both *Candida albicans* and non-*albicans,* and the result of amphotericin B was not satisfactory in non-*albicans* strains. There was no cytotoxicity in macrophage cells exposed to *C. sinensis* extract; however, this study did not consider the possibility of the extract causing tooth staining, a common property attributed to green tea. We conclude that further studies are necessary to evaluate such side effects of *C. sinensis* tea.

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