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MICROBIOLOGY

Rosmarinus officinalis L. (rosemary) extract has antibiofilm effect similar to the antifungal nystatin on *Candida* samples

VANESSA M. MECCATTI, JONATAS R. DE OLIVEIRA, LEANDRO W. FIGUEIRA, AMANDIO A. LAGAREIRO NETTO, LUCAS S. ZAMARIOLI, MARIA C. MARCUCCI, SAMIRA E.A. CAMARGO, CLÁUDIO A.T. CARVALHO & LUCIANE D. DE OLIVEIRA

Abstract: *Candida* spp. are naturally opportunistic and can promote infections. These yeasts can form biofilm, after penetration and adhesion to the biotic or abiotic surfaces. Preexisting diseases, treatments with drugs and radiation therapy, medical procedures, and parafunctional habits favor the installation of a fungal infection. Increased resistance to the available antifungals has become a concern. Therefore, alternative methods to control them have been evaluated, including the use of plant substances. In this study, the antibiofilm effect of *R. officinalis* L. extract was analyzed on *C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. krusei*, and *C. tropicalis*. A phytochemical analysis of the extract was performed. Biofilms were formed for 48 h and exposed to the different concentrations of the extract (50, 100, and 200 mg/mL) for 5 min or 24 h. The effect of the plant extract was compared to the antifungal nystatin. *Rosmarinus officinalis* L. extract was constituted of phenols and flavonoids, highlighting the presence of chlorogenic acid derivatives in its composition. Biofilm reductions were observed after exposure to the plant extract for both periods. The plant extract provided a reduction similar to the antifungal. Thus, *R. officinalis* L. extract showed antibiofilm effect on *Candida* spp. comparable to the nystatin.

Key words: Antifungal effect, Biofilms, *Candida* spp., Nystatin; *Rosmarinus officinalis* L.

INTRODUCTION

Biofilms are microecosystems composed of microorganisms involved by an extracellular matrix of own production, being adhered to a surface (Ammons et al. 2014, Harriott & Noverr 2009).

Candida spp. can form biofilm and germ tubes, have adhesion proteins, as well as phospholipases, proteases, and other extracellular enzymes that contribute to their diffusion through the host tissue, because these enzymes are located at the extremity of hyphae (Gauch et al. 2014). These fungi are opportunistic and can cause diseases known as candidiasis, using factors that promote their penetration, diffusion, installation, and development in the host (Soll 2002). Some conditions such as acquired immunodeficiency syndrome (AIDS), diabetes, antibiotic therapy, corticoid therapy, chemotherapy, radiotherapy, surgeries, the presence of catheters, and probes may favor the development of candidiasis. About 80% of the hospital fungal infections are caused by *Candida* spp. Besides, cases of resistance to the antifungal therapy and to the host defenses have been reported (Ramage & López-Ribot 2005).

In the oral mucosa, the presence of *Candida* spp. is common and related to the adhesion

capacity of the yeast to the epithelial cells, considered as an essential factor of virulence to the biofilm formation after microbial adhesion (Thein et al. 2006). Oral biofilms are polymicrobial, composed by different species of microorganisms as well as of various *Candida* species, such as *C. parapsilosis*, *C. tropicalis*, *C. krusei*, *C. glabrata* and C. *guilliermondii* (Vidotto et al. 2003).

Candida albicans is the primary species responsible for causing oral candidiasis. Its ability of adhesion to the epithelial cells and the possibility of transformation from yeast to hyphae are important factors of virulence of this species (Shapiro et al. 2009, Silva et al. 2011). This yeast can invade the gingival sulcus and grow in anaerobiosis, which may aggravate the symptoms of existing periodontal disease. Moreover, *C. albicans* can form a complex microbial community with *Streptococcus mutans*, facilitating its adhesion to the polymicrobial biofilm (Ten Cate et al. 2009, Gregoire et al. 2011). Additionally, the cariogenic potential of *Candida* spp. has been reported (Nikawa et al. 2003). The yeast can also inhibit the action of polymorphonuclear leukocytes (Maccarinelli et al. 2001) and produces enzymes that degrade immunoglobulins (Hägewald et al. 2002).

Candida dubliniensis may be more prevalent in patients infected with human immunodeficiency virus (HIV) (Loreto et al. 2010). It is an emerging pathogen that shares many phenotypic characteristics with *C. albicans*. Besides, *C. dubliniensis* can produce germ tubes and lipolytic enzymes, which are considered important virulence factors. The establishment of the infection by *C. dubliniensis* is triggered by the release of lipolytic enzymes used for dissemination by the host's tissue. Adhesion accompanies this fact; synergistic interaction with other hydrolytic enzymes; non-specific activation of inflammatory reaction; and self-defense, mediated by the elimination of the competing microbiota (Park et al. 2013, Sardi et al. 2013, Trofa et al. 2008).

Candida glabrata is frequently found in cases of candidemia (Oren & Paul 2014). This species does not present polymorphism, being only found in yeast form in the oral cavity. In polymicrobial infections, *C. glabrata* can penetrate the oral epithelium with the aid of *C. albicans* (Silva et al. 2011). Prolonged exposure to antifungals may increase the probability of infections by *C. glabrata* and *C. krusei*. In addition, cases of infections by these yeasts have increased in patients with debilitating conditions (Oren & Paul 2014, Sampaio & Pais 2014). Both species have shown resistance to the fluconazole and amphotericin B (Shirani et al. 2017).

Candida tropicalis is usually associated with infections, once presents some characteristics related to its virulent potential, such as high adhesion capacity and biofilm formation (Negri et al. 2010). Patients with cancer, infections, neutropenia, or treated by radiotherapy are the most affected by this species. Gamma radiation causes increased virulence of *C. tropicalis*, since morphological and physiological alterations may occur (Silva et al. 2017).

Alternative methods to prevent and treat diseases orally transmitted are needed due to: (i) incidence of cases; (ii) increased resistance of microorganisms to the available antimicrobials; (iii) financial conditions; and (iv) harmful effects of some drugs. Phytotherapy medicines could be one of these methods once they can present biocompatibility and efficacy (Palombo 2011). Studies related to the alternative therapies with plant products have consistently appeared. Substances isolated from medicinal plants, such as extracts, have stimulated the interest of the medical field, since they can be less aggressive to the organism.

Rosmarinus officinalis L. was evaluated in this study due to its significant anti-*Candida* effect demonstrated on planktonic culture, monomicrobial biofilm, and in interactions with *Staphylococcus aureus*, *Enterococcus faecalis*, *Streptococcus mutans*, and *Pseudomonas aeruginosa* (de Oliveira et al. 2017). Popularly known as rosemary, *R. officinalis* (Lamiaceae) is originated from the Mediterranean region but is worldwide distributed. It has been used in the cosmetics industry, culinary, and complementary and alternative medicine, mainly for its aromatic essence and beneficial pharmaceutical effects (Machado et al. 2009, Rašković et al. 2014). Its main constituents, such as 1,8-cineole (52.2%), camphor (15.2%) and α-pinene (12.4%), are the responsible for the several biological activities (Silva et al. 2015), including antibacterial, antifungal, antibiofilm, antiproliferative, anti-inflammatory, antimutagenic, and antioxidant effects (Bozin et al. 2007, Pozzatti et al. 2010, Chifiriuc et al. 2012, Rašković et al. 2014, Silva et al. 2015, de Oliveira et al. 2017, Ksouri et al. 2017, Liakos et al. 2017).

In the interaction of *R. officinalis* L. with fungi, the medicinal plant has caused a destabilization in the cytoplasmic membrane and cell wall of these microorganisms, providing a loss of internal contents of the cell fungal to the medium. This fact contributes to generate dysfunctional cells in the biofilm (da Silva et al. 2015). Besides, inhibition of germ-tube formation by *C. albicans* has also been cited. This mechanism impairs its dissemination though organic tissues (Gauch et al. 2014). By means of these mechanisms, *R. officinalis* L. extract can provide an effective control of *Candida* spp. biofilms.

Thus, the antifungal effect of *R. officinalis* L. extract was evaluated in this study, both on planktonic cultures and biofilms of *C. albicans*, *C. dubliniensis, C. glabrata, C. krusei*, and *C. tropicalis*, by exposure to the plant extract in different concentrations for 5 min or 24 h, comparing its effects with the antifungal nystatin.

MATERIALS AND METHODS

Plant extract

Rosmarinus officinalis L. extract was commercially purchased at 200 mg/mL in propylene glycol (Seiva Brazilis, São Paulo, Brazil). Plant extract was obtained from leaves and flowers of the vegetable, being chemically composed of acid saponin, borneol acetate, camphene, camphor, cineol, free borneol, glucosidic compounds, little tannin, oleanolic acid and pinene, according to the manufacturer.

Phytochemical analysis

Determination of soluble solids

The plant extract (5 mL) was dried at 80°C until total elimination of liquids. Then, the solid compounds were cooled in a desiccator and the amount of soluble solids from the extract was calculated.

Determination of total phenol

A stock solution of extract diluted in ethanol and distilled water was prepared (1:100). After, 0.2 mL of this solution was added in 5 mL of distilled water. To this new solution, 0.8 mL of Folin-Ciocalteau reagent (Merck, Germany) was added. Posteriorly, 1.2 mL of 20% sodium carbonate-tartrate buffer was added between 1 and 8 min. The solution was kept in a water bath at 20°C for 2 h. Absorbance of this solution was read in a spectrophotometer (760 nm) and the amount of total phenol was determined by the straight equation of gallic acid (Bankova &

Marcucci 2000). The procedure was performed in triplicate.

Determination of total flavonoid

A stock solution of extract in methanol was prepared (1:100) and 0.2 mL was added in 5 mL of methanol. To this solution, 0.2 mL of aluminum chloride was added and the volume was completed to 10 mL with methanol. This new solution was kept in a water bath at 20°C for 30 min. Absorbance of this solution was read in a spectrophotometer (425 nm) and the concentration of total flavonoid was determined by the straight equation of quercetin (Bankova & Marcucci 2000). The procedure was performed in triplicate.

Antioxidant activity

Elimination of free radicals by the extract was verified using 2,2-diphenyl-1-picrylhydrazyl (DPPH - Sigma-Aldrich, St. Louis, USA), according to Brand-Williams et al. (1995), with some modifications. Then, 0.5 mL of DPPH (0.2 mmol/L of methanol) was added in 0.5 mL of extract at different concentrations suspended in methanol. The samples were maintained at room temperature for 30 min, with absence of light. The absorbance of this solution was read in a spectrophotometer (517 nm). The optical density (OD) values were converted to µg/mL and the concentration that eliminated 50% of free radicals (EC_{50}) was determined by non-linear regression (Veiga et al. 2017). The procedure was performed in triplicate.

Chromatographic analysis

High-performance Liquid Chromatography (HPLC) was used to characterize and quantify the content of markers in the plant extract. Thus, a chromatograph with a photodiode detector (HPLC-DAD) and an automatic injector (D-7000 Merck-Hitachi) was used. The mobile phase of the chromatography was composed of water-formic acid solution (Merck) diluted in the ratio of 95:5 (solvent A) and methanol HPLC grade (Merck) (solvent B). The flow was 1 mL/min and the linear gradient started with 0% B and ended with 70% B in a running time of 50 min at 280 and 340 nm (Veiga et al. 2017).

Yeast strains

Reference strains (ATCC - American Type Culture Collection) of *C. albicans* (ATCC 18804), *C. dubliniensis* (ATCC MYA646), *C. glabrata* (ATCC 9030), *C. krusei* (ATCC 6258), and *C. tropicalis* (ATCC 13803), from the Laboratory of Microbiology and Immunology (ICT/UNESP), were used in this study. Yeasts were stored at -80°C in Yeast Extract Peptone Dextrose broth (YPD - Himedia, Mumbai, India) with 16% glycerol.

Antifungal effect on planktonic cultures

Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of *R. officinalis* L. extract were determined by broth microdilution method, according to the Clinical and Laboratory Standards Institute (CLSI 2002, 2012). Initially, strains were grown on Sabouraud Dextrose agar (SD - Himedia) at 37°C for 24 h. Fungal suspensions were prepared in sterile saline solution (0.9% NaCl), whose turbidity was adjusted in a spectrophotometer (Micronal, São Paulo, Brazil) with the parameters of 530 nm and optical density (OD) of 0.284 ± 0.02, to obtain 10^6 colony-forming units per milliliter (CFU/mL). After dilution of this solution, a concentration between 5×10^2 and 2.5×10^3 CFU/mL was used. Ten serial dilutions of the plant extract were performed in microplates (TPP, Trasadingen, Switzerland). For this purpose, RPMI 1640 broth with glutamine, red phenol indicator, and bicarbonate-free (Himedia), buffered at pH 7 ± 0.1 by 3-(N-morpholino) propanesulfonic acid

(MOPS - Sigma-Aldrich, St. Louis, USA) was added in each well (100 µL/well). Microdilution was initiated from the first well, adding 100 µL of plant extract to the RPMI 1640. The ten dilutions of the plant extract were obtained by successive transfers of 100 µL/well. After, standardized inoculum was added in each well (100 µL/well). Thus, evaluated concentrations were from 50 to 0.09 mg/mL. Wells for growth control (medium plus inoculum) and medium alone were added. After incubation (37°C/24 h), MIC was determined in the first well with absence of microbial turbidity, next to the well with apparent microbial growth. For determination of the MFC, MIC and concentrations above it were added on SD agar. After incubation (37°C/48 h), MFC was determined in plate with absence of colony growth, containing the lowest concentration of the plant extract. Propylene glycol was also checked on the yeasts.

Biofilm formation

Yeasts were grown on SD and after in Yeast Nitrogen Base broth (YNB - Sigma-Aldrich) at 37°C for 24 h each growth. Fungal suspension was centrifuged at 2000 rpm/10 min (MPW-350, Warsaw, Poland), supernatant was replaced with saline (0.9% NaCl) and another centrifugation was performed, this procedure was performed twice. Turbidity of the fungal suspension was adjusted in a spectrophotometer (530 nm; 0.381 ± 0.02) to obtain an inoculum of 10^7 CFU/mL. This standardized suspension was added in microplate wells (200 µL/well) and incubation (37°C/90 min) under agitation (75 rpm - Quimis, Diadema, Brazil) was performed. After pre-adherence of the fungal cells, supernatant was replaced with YNB broth and biofilm was formed for 48 h. Culture medium was exchanged after 24 h of incubation (de Oliveira et al. 2017).

Antibiofilm effect

Wells were washed with saline solution (200 µL/well) to remove cells not adhered to the biofilm. Then, biofilms were exposed to the effective concentrations of *R. officinalis* L. extract (200 µL/well), previously determined on planktonic cultures. Nystatin (100,000 IU/mL) and saline solution (0.9% NaCl) were used as controls. Ten replicates were performed in each experimental group. Biofilms were exposure to the products for 5 min or 24 h. Posteriorly, wells were washed with saline solution (200 µL/well) to remove affected cells. Biofilms were disaggregated by ultrasonic homogenizer (Sonopuls HD 2200 - Bandelin Eletronic, Berlin, Germany) for 30 s and 25% power. The suspension generated was diluted and 20 µL of each dilution was added on SD agar in triplicate. After drying the drops (~ 5 min), the plate was inverted and incubated (37°C/24 h). Colonies from each drop were counted and mean values were presented in CFU/mL.

Statistical analysis

Data were presented in mean values (± standard deviation) and checked by one-way analysis of variance (ANOVA) and Tukey's test, for comparison between treated groups and control groups (CFU/mL), or T-test, for comparison between exposure times in each treated group (reduction percentage). It was considered significant statistical difference when $P \leq 0.05$. Ten replicates were performed in each experimental group. GraphPad Prism 5.0 program was used for this purpose.

RESULTS

Phytochemical analysis

Rosmarinus officinalis L. extract showed in its composition: (i) 2.28 ± 0.00% of solids soluble

in ethanol; (ii) 3.37 ± 0.00 µg/mL of total phenol; (iii) 2.08 ± 0.37 µg/mL of total flavonoid; and (iv) eliminated free radicals with EC_{50} of 84.30 ± 2.44 µg/mL. By chromatographic analysis, it was observed some chlorogenic acid derivatives at the retention time of 12.61 min (Figure 1).

Antifungal effect on planktonic cultures

Rosmarinus officinalis L. extract showed MIC of 50 mg/mL on *C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. krusei*, and *C. tropicalis*. Therefore, MIC (50 mg/mL), 2 x MIC (100 mg/mL) and 4 x MIC (200 mg/mL) were evaluated on biofilms. Propylene glycol did not show antifungal effect, since microbial growth was observed at all evaluated concentrations. Thus, absence of interference of this vehicle on the yeasts was confirmed.

Antibiofilm effect after 5 min exposure

Rosmarinus officinalis L. extract provided significant reductions of *C. albicans* biofilm (CFU/mL), in relation to the control group ($4 \times 10^4 \pm 2.03 \times 10^4$) after exposure to 50 ($2.11 \times 10^4 \pm 1.2 \times 10^4$) and 100 mg/mL ($7.3 \times 10^2 \pm 6.12 \times 10^2$). At 200 mg/mL, a total elimination of the biofilm was observed. These concentrations of the extract had antifungal effect similar to the nystatin ($1.67 \times 10^3 \pm 0$) (Figure 2a). Thereby, concentrations of 100 and 200 mg/mL showed higher reduction, when compared to the concentration of 50 mg/mL (Figure 2b). These results were similar to the reductions presented by the nystatin (Table I).

Candida dubliniensis biofilm was significantly affected by the plant extract at 100 ($5.38 \times 10^6 \pm 1.56 \times 10^6$) and 200 mg/mL ($2.25 \times 10^6 \pm 2.33 \times 10^6$), compared to the control group ($9.38 \times 10^6 \pm 1.69 \times 10^6$). However, at 50 mg/mL ($6.56 \times 10^6 \pm 2.08 \times 10^6$), the reduction did not statistically differ from the control group. On the other hand, the concentrations of the plant extract were similar to the nystatin ($4.36 \times 10^6 \pm 1.53 \times 10^6$) (Figure 2c). Thereby, the highest rate of biofilm reduction was found after exposure to 200 mg/mL. Comparable values were observed at 50 and 100 mg/mL (Figure 2d). The groups treated with plant extract presented similar reduction percentages concerning the groups treated with nystatin, as shown in Table I.

The plant extract was effective on *C. glabrata* biofilm after exposure to 50 ($1.36 \times 10^3 \pm 0.31 \times 10^3$), 100 ($6.33 \times 10^2 \pm 3.98 \times 10^2$) and 200 mg/mL ($6.67 \times 10^2 \pm 2.36 \times 10^2$), in relation to the control group ($2.5 \times 10^3 \pm 0.97 \times 10^3$). Antifungal effect presented by these concentrations was similar to the effect demonstrated by the nystatin (1.67

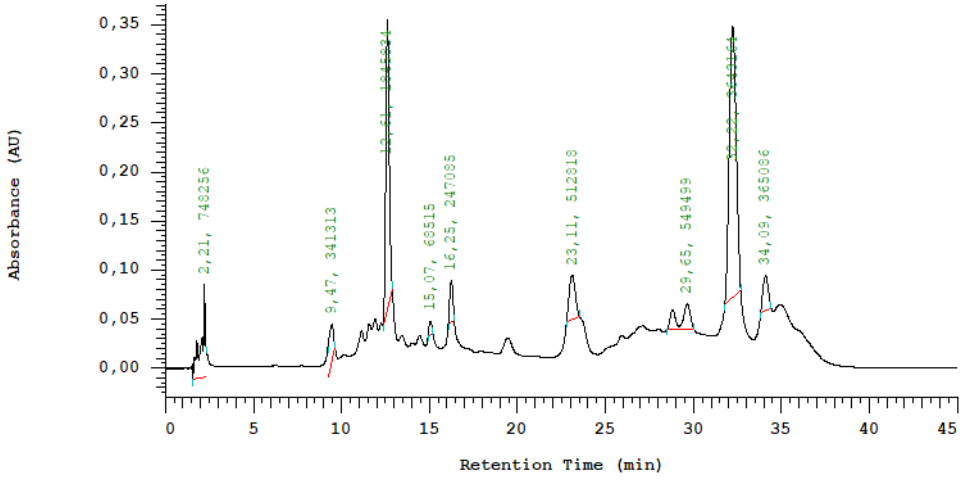

Figure 1. High-performance Liquid Chromatography (HPLC) analysis. Chromatogram of *R. officinalis* L. extract highlighted the presence of chlorogenic acid derivatives in its composition at the retention time of 12.61 min.

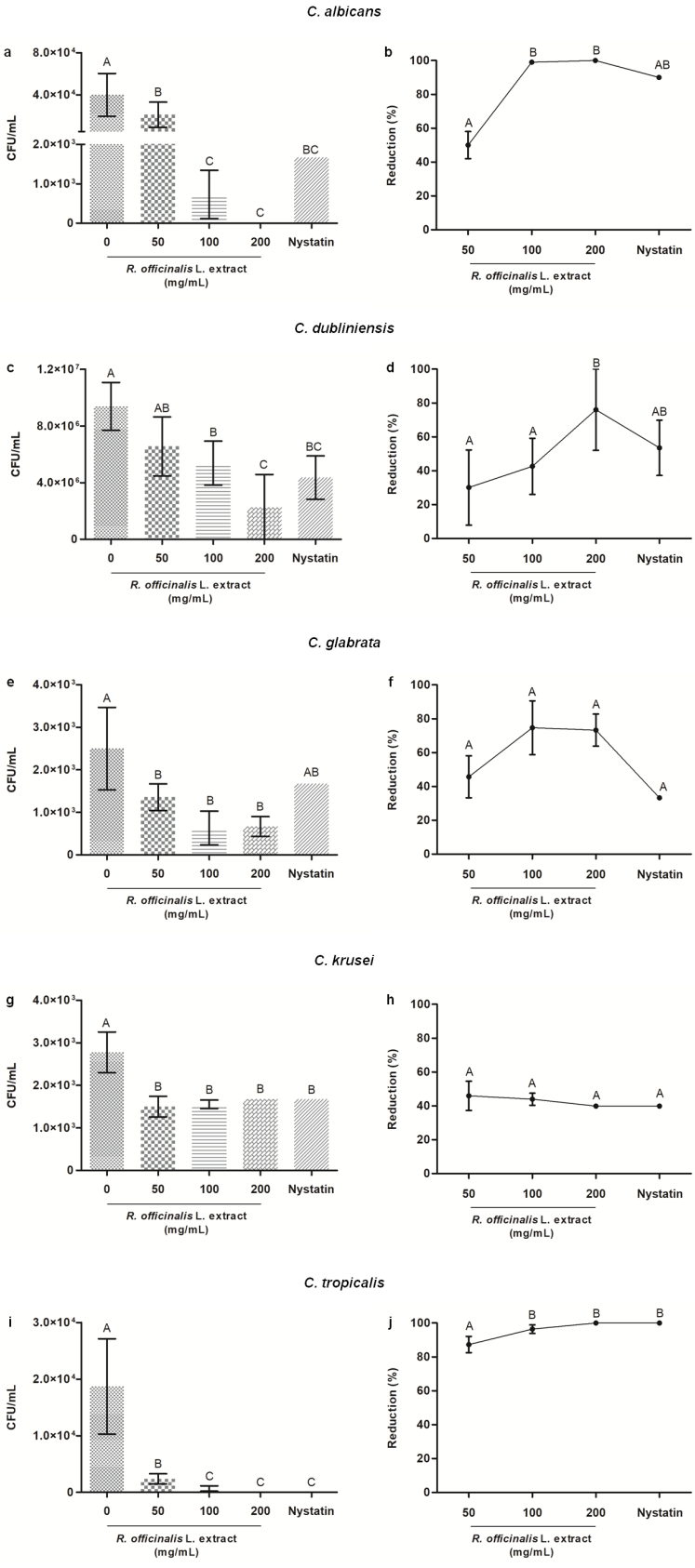

Figure 2. Mean values (± standard deviation) of CFU/mL and reduction percentage (%) of the biofilms of *C. albicans* (a,b), *C. dubliniensis* (c,d), *C. glabrata* (e,f), *C. krusei* (g,h), and *C. tropicalis* (i,j) obtained after 5 min exposure to the *R. officinalis* L. extract (50, 100, and 200 mg/mL) or to the nystatin (100,000 IU/mL), compared to the control group (0 mg/mL – 0.9% NaCl). Different letters indicate significant statistical difference. (n = 10; One-way ANOVA and Tukey's test; $P \leq 0.05$).

x 10^3 ± 0) (Figure 2e). Therefore, similarities between the reduction indices were observed in the groups exposed to the extract at 50, 100, and 200 mg/mL, as well as to the nystatin (Figure 2f; Table I).

Reductions of the *C. krusei* biofilm were observed after exposure to the plant extract at 50 (1.5 x 10^3 ± 0.24 x 10^3), 100 (1.56 x 10^3 ± 0.1 x 10^3), and 200 mg/mL (1.67 x 10^3 ± 0), compared to the control group (2.78 x 10^3 ± 0.48 x 10^3). Treated groups were similar to each other and nystatin (1.67 x 10^3 ± 0) (Figure 2g). Thus, similar reduction indices were observed in the groups exposed to 50, 100, and 200 mg/mL, and to the antifungal nystatin (Figure 2h; Table I).

Candida tropicalis biofilm was impaired after treatment with the plant extract. In the group treated with 200 mg/mL there was a total elimination of the biofilm, as well as in the group exposed to the nystatin. In addition, reductions of this biofilm were observed in the groups treated with extract at 50 (2.37 x 10^3 ± 0.9 x 10^3) and 100 mg/mL (6.67 x 10^2 ± 4.71 x 10^2), in relation to the control group (1.87 x 10^4 ± 0.84 x 10^4). Groups treated with extract at 100 and 200 mg/mL or nystatin demonstrated similar antibiofilm effect (Figure 2i). Therefore, reduction rates of the biofilm were similar between the groups treated with extract at 100 and 200 mg/mL and nystatin, different from the treatment with extract at 50 mg/mL (Figure 2j; Table I).

Antibiofilm effect after 24 h exposure

Rosmarinus officinalis L. extract significantly reduced the *C. albicans* biofilm after treatment with concentrations of 50 (7.65 x 10^6 ± 1.52 x 10^6), 100 (3.87 x 10^4 ± 0.46 x 10^4), and 200 mg/mL (1.87 x 10^4 ± 0.56 x 10^4), in relation to the control group (1.94 x 10^7 ± 0.55 x 10^7). Concentrations of 100 and 200 mg/mL showed effect similar to the nystatin (1.71 x 10^5 ± 0.92 x 10^5) (Figure 3a). Thus, the lowest reduction index of the biofilm was observed after exposure to the plant extract at 50 mg/mL. However, concentrations of 100 and 200 mg/mL presented indices similar to the nystatin (Figure 3b; Table I).

Candida dubliniensis biofilm was decreased after exposure to the plant extract at 50 (7.43 x 10^6 ± 1.51 x 10^6) and 100 (8.33 x 10^5 ± 0), and the total elimination of this biofilm was observed at 200 mg/mL, compared to the control group (2.98 x 10^7 ± 0.73 x 10^7). Concentrations of 100 and 200 mg/mL demonstrated effect similar to the nystatin (1.11 x 10^4 ± 0.82 x 10^4) (Figure 3c). Thereby, the highest reduction percentages were observed in the treatments with 100 and 200

Table I. Reduction percentage of the fungal biofilms after exposure to the *R. officinalis* L. extract or nystatin.

Yeast	5 min exposure				24 h exposure			
	R. officinalis L. extract (mg/mL)			Nystatin	*R. officinalis* L. extract (mg/mL)			Nystatin
	50	100	200		50	100	200	
C. albicans	50 ± 8^A	99 ± 0^B	100 ± 0^B	90 ± 1^{AB}	62 ± 8^A	100 ± 0^B	100 ± 0^B	99 ± 0^B
C. dubliniensis	30 ± 22^A	43 ± 17^A	76 ± 24^B	54 ± 16^{AB}	75 ± 5^A	97 ± 0^B	100 ± 0^B	100 ± 0^B
C. glabrata	46 ± 12^A	75 ± 16^A	73 ± 9^A	33 ± 0^A	91 ± 7^A	88 ± 0^A	100 ± 0^A	100 ± 0^A
C. krusei	46 ± 7^A	44 ± 3^A	46 ± 0^A	40 ± 0^A	95 ± 2^A	100 ± 0^A	100 ± 0^A	100 ± 0^A
C. tropicalis	87 ± 5^A	96 ± 2^B	100 ± 0^B	100 ± 0^B	78 ± 0^A	100 ± 0^B	96 ± 0^B	100 ± 0^B

Different letters in row indicate significant statistical difference for each incubation time. (n = 10; One-way ANOVA and Tukey's test; $P \leq 0.05$). These percentages were obtained by comparing with the control group (0 mg/mL – 0.9% NaCl). Nystatin was at 100,000 IU/mL.

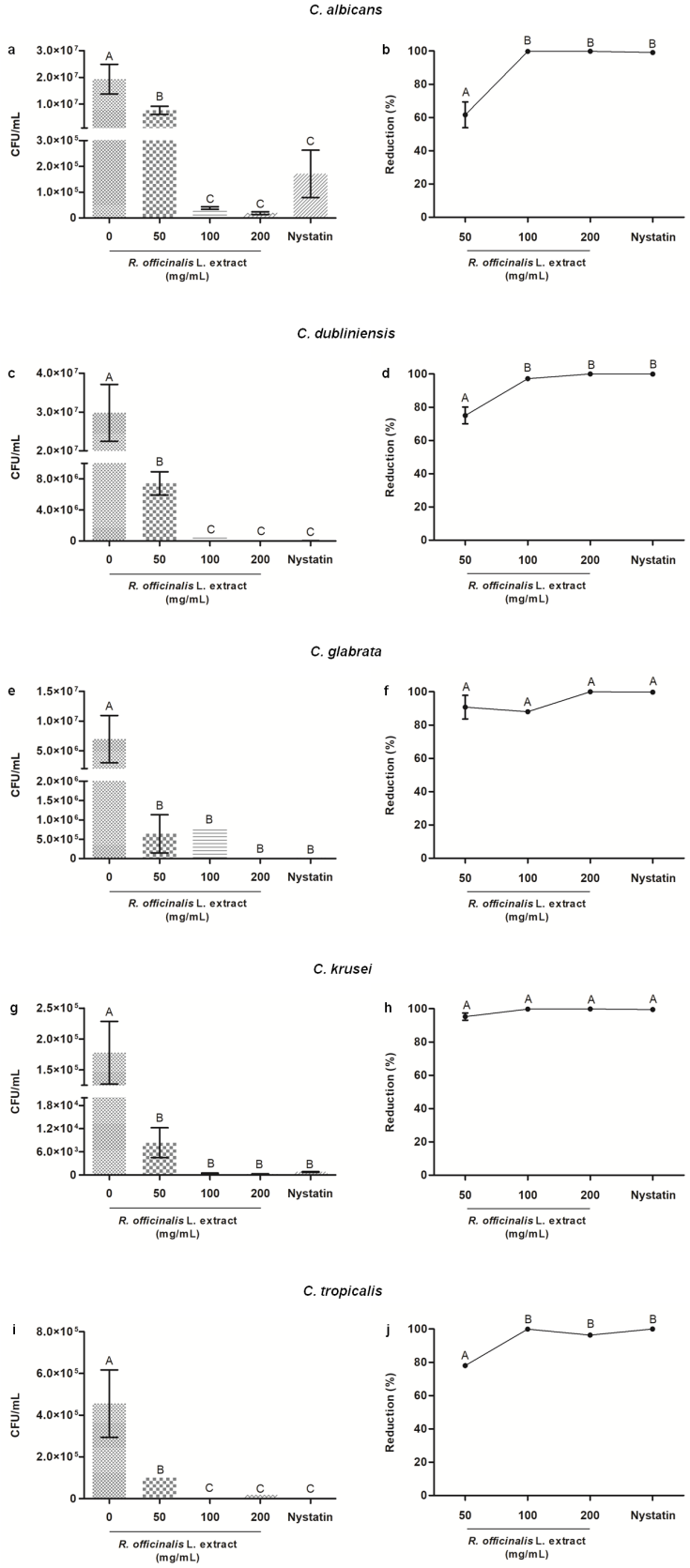

Figure 3. Mean values (± standard deviation) of CFU/mL and reduction percentage (%) of the biofilms of *C. albicans* (a,b), *C. dubliniensis* (c,d), *C. glabrata* (e,f), *C. krusei* (g,h), and *C. tropicalis* (i,j) obtained after 24 h exposure to the *R. officinalis* L. extract (50, 100, and 200 mg/mL) or to the nystatin (100,000 IU/mL), compared to the control group (0 mg/mL – 0.9% NaCl). Different letters indicate significant statistical difference. (n = 10; One-way ANOVA and Tukey's test; $P \leq 0.05$).

mg/mL, which were similar to the nystatin. The lowest index was obtained at 50 mg/mL (Figure 3d; Table I).

Rosmarinus officinalis L. extract showed antibiofilm action on *C. glabrata* after exposure to concentrations of 50 ($6.4 \times 10^5 \pm 4.93 \times 10^5$), 100 ($8.33 \times 10^5 \pm 0$), and 200 mg/mL ($1.67 \times 10^2 \pm 0$), demonstrating an effect similar to the nystatin ($1.58 \times 10^4 \pm 0.12 \times 10^4$). In the control group, $6.97 \times 10^6 \pm 3.97 \times 10^6$ CFU/mL were obtained (Figure 3e). Thereby, the reduction percentages were similar between the groups treated with extract at 50, 100, and 200 mg/mL, and treated with nystatin (Figure 3f; Table I).

Candida krusei biofilm was affected by the plant extract at 50 ($8.33 \times 10^3 \pm 3.85 \times 10^3$), 100 ($3.33 \times 10^2 \pm 1.67 \times 10^2$), and 200 mg/ml ($2.08 \times 10^2 \pm 0.83 \times 10^2$), compared to the control group ($1.78 \times 10^5 \pm 0.51 \times 10^5$). The plant extract had antibiofilm effect similar to the nystatin ($7.78 \times 10^2 \pm 0.96 \times 10^2$) (Figure 3g). Thus, the reduction rates observed in the groups exposed to the extract at 50, 100, and 200 mg/mL did not differ from the indices presented by the group treated with nystatin (Figure 3h; Table I).

The plant extract effectively contributed to eliminate the *C. tropicalis* biofilm after exposure to the concentrations of 50 ($1 \times 10^5 \pm 0$), 100 ($1.67 \times 10^2 \pm 0$), and 200 mg/mL ($1.67 \times 10^4 \pm 0$), in relation to the control group ($4.56 \times 10^5 \pm 1.62 \times 10^5$). The antibiofilm effect showed by the concentrations of 100 and 200 mg/mL was similar to that showed by the nystatin, which completely eliminated this biofilm (Figure 3i). Thus, the reduction percentages of the biofilm were similar between the groups exposed to the extract at 100 and 200 mg/mL, and to the nystatin. The lowest reduction rate was observed at the concentration of 50 mg/mL (Figure 3j; Table I).

5 min versus 24 h

A comparison between the reduction percentages obtained from the experimental groups after exposure to *R. officinalis* L. extract for 5 min or 24 h was shown in Figure 4. A higher reduction percentage of the *C. albicans* biofilm was observed after 24 h exposure to the extract (50 mg/mL) and to the nystatin, compared to the exposure for 5 min. Similar reduction percentages were observed after exposure to the plant extract at 100 and 200 mg/mL (Figure 4a).

Exposure for 24 h to the extract at 50 and 100 mg/mL or to the nystatin promoted higher reduction percentages of the *C. dubliniensis* biofilm in relation to the exposure for 5 min. However, similar percentages were observed after treatment with extract at 200 mg/mL (Figure 4b).

Candida glabrata biofilm had the highest reduction percentages after 24 h exposure to the plant extract at 50 and 200 mg/mL, as well as to the nystatin, compared to the 5 min exposure. Similarities were found in the treatment with extract at 100 mg/mL (Figure 4c).

All the experimental groups showed a higher reduction percentage of the *C. krusei* biofilm after 24 h exposure, than in the exposure for 5 min (Figure 4d).

Concentrations of 50 and 200 mg/mL showed higher reduction percentage of the *C. tropicalis* biofilm after 5 min exposure. The highest reduction percentage was found in the group exposed to the extract at 100 mg/mL for 24 h. In addition, exposure to the nystatin provided a reduction percentage similar between both exposure times (Figure 4e).

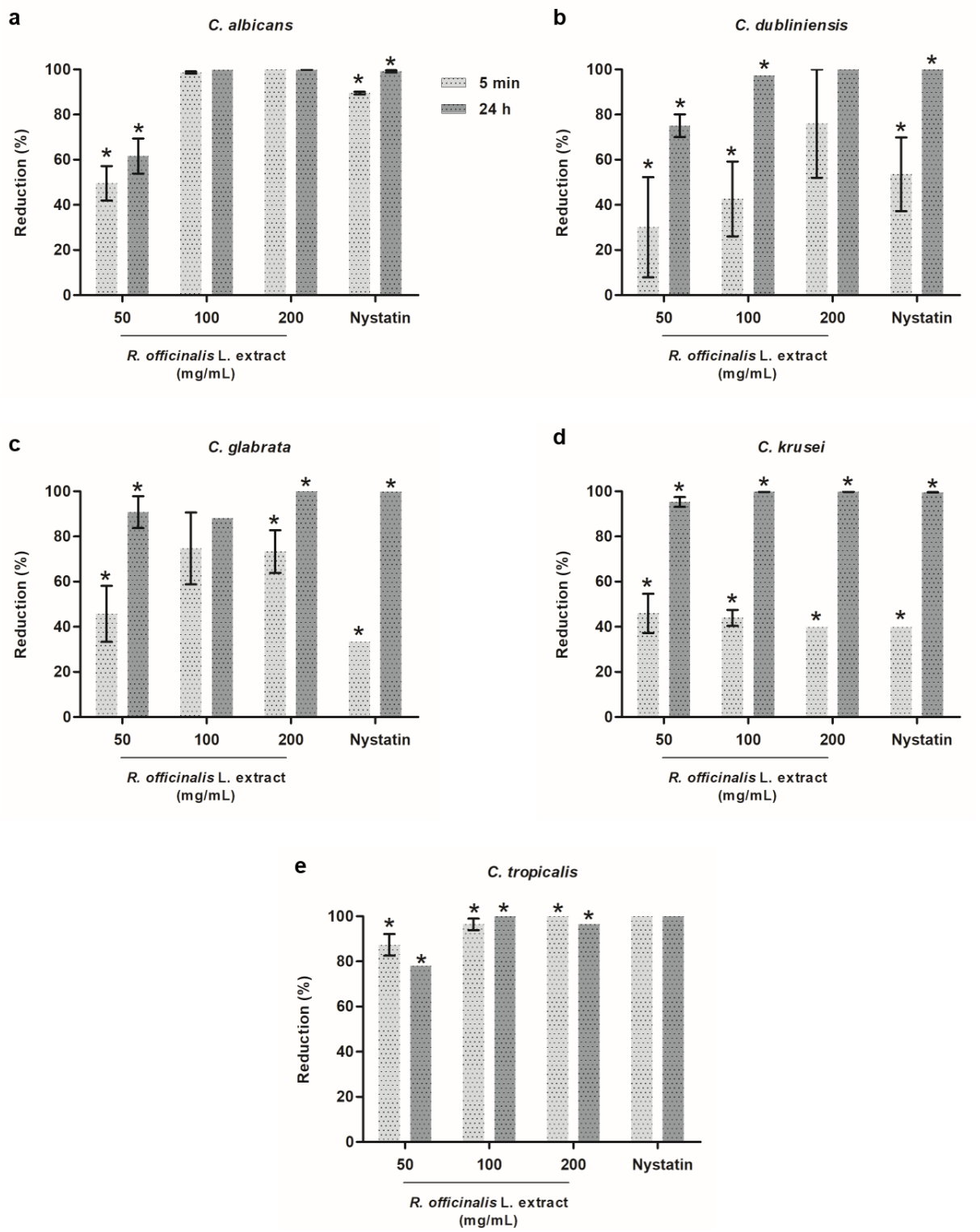

Figure 4. Comparison of mean values (± standard deviation) of reduction percentage of the biofilms of *C. albicans* (a), *C. dubliniensis* (b), *C. glabrata* (c), *C. krusei* (d), and *C. tropicalis* (e) between the exposure times of 5 min and 24 h to the *R. officinalis* L. extract (50, 100, and 200 mg/mL) or to the nystatin (100,000 IU/mL). Asterisks indicate significant statistical difference between the exposure times in each experimental group. (n = 10; T-test; $P \leq 0.05$).

DISCUSSION

In this study, the MIC of *R. officinalis* L. extract was 50 mg/mL on planktonic cultures of *C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. krusei*, and *C. tropicalis*. Therefore, MIC (50 mg/mL), 2 x MIC (100 mg/mL) and 4 x MIC (200 mg/mL) were evaluated on the fungal biofilms, once higher concentrations than the MIC is necessary to affect biofilms, compared to planktonic cultures (Lewis 2001).

On the *C. albicans* biofilm, concentrations of 50, 100, and 200 mg/mL of *R. officinalis* L. extract showed effect similar to the nystatin, after only 5 min exposure (Figure 2a). Similar reduction rates to the nystatin were observed after exposure for 24 h at 100 and 200 mg/mL (Figure 3a). Studies have reported that the *R. officinalis* L. essential oil have been able to act on clinical isolates of *C. albicans*, affecting the formation of germ tubes (Pozzatti et al. 2010, Gauch et al. 2014, Ksouri et al. 2017). Additionally, the antibiofilm effect of this essential oil against *C. albicans* has been demonstrated. This plant product was incorporated into cellulose acetate nanofibers once it was an excellent carrier with the absence of biological activity. The union of these products resulted in a uniform structure, whose highest concentrations of the essential oil were more effective against *C. albicans* (Liakos et al. 2017). Ethanolic and aqueous extracts from leaves of *R. officinalis* L. and *Echinophora platyloba* were evaluated and compared. *R. officinalis* L. ethanolic extract demonstrated effective antifungal activity on *C. albicans*, showing superior effect to the *E. platyloba* ethanolic extract. On the other hand, the aqueous extracts did not have significant impact against *C. albicans* (Sepehri et al. 2016).

Candida dubliniensis biofilm suffered significant reductions when treated with *R. officinalis* L. extract. at 200 mg/mL (Figure 2c), this concentration similarly acted to the nystatin after 5 min exposure. However, at 100 and 200 mg/mL, an action similar to the antifungal was also observed after 24 h exposure (Figure 3c). The effect of *R. officinalis* L. extract against *C. dubliniensis* biofilms have not been reported, but susceptibility of this yeast to that plant extract has been demonstrated on planktonic cultures (Höfling et al. 2010).

The effect of *R. officinalis* L. essential oil has been evaluated, as well as other aromatic plants, such as *Origanum vulgare*, *Cinnamomum zeylanicum*, *Lippia graveolens*, *Thymus vulgaris*, and *Salvia officinalis* on planktonic cultures of fluconazole-susceptible or -resistant *C. glabrata*. Only *O. vulgare* and *L. graveolens* inhibited fluconazole-susceptible *C. glabrata* samples, and *C. zeylanicum* was capable of acting on fluconazole-resistant *C. glabrata* isolates (Soares et al. 2015). In our study, inhibitory effect on planktonic culture (MIC = 50 mg/mL) and significant antibiofilm activity in both exposure times (5 min and 24 h) were found on *C. glabrata,* with an action similar to the nystatin (Figures 2e and 3e).

Candida krusei biofilm showed a significant reduction after exposure to the *R. officinalis* L. extract for 5 min and 24 h, being similar to the nystatin (Figures 2g and 3g). Action of *R. officinalis* L. extract on *C. krusei* biofilm has not yet been reported. However, an inhibitory effect was reported on planktonic cultures, using 3 µg/mL, for a dichloromethane extract, and 1 µg/mL, for a methanol extract (Höfling et al. 2010).

Candida tropicalis biofilm was significantly reduced after 5 min and 24 h exposure to the *R. officinalis* L. (Figures 2i and 3i), presenting concentrations with effects similar to the nystatin. Antifungal effect of *R. officinalis* L extract has also been reported on planktonic culture of *C. tropicalis* by MIC of 15 µg/mL, using a dichloromethane extract, and 3 µg/mL, for a

methanol extract (Höfling et al. 2010). Besides, the ability to inhibit the adhesion and formation of polymicrobial biofilms composed by *C. albicans* and *C. tropicalis* has been demonstrated by the *R. officinalis* L. essential oil conjugated with nanoparticles (Chifiriuc et al. 2012).

CONCLUSIONS

Rosmarinus officinalis L. extract had a significant antibiofilm effect at the evaluated concentrations, after 5 min and 24 h exposure. However, the extract at 100 and 200 mg/mL demonstrated the highest reduction rates for biofilms of *C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. krusei*, and C. *tropicalis*, showing action similar to the nystatin.

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VANESSA M. MECCATTI[1]
https://orcid.org/0000-0003-3297-2288

JONATAS R. DE OLIVEIRA[2]
https://orcid.org/0000-0003-2398-6506

LEANDRO W. FIGUEIRA[1]
https://orcid.org/0000-0002-8504-2183

AMANDIO A. LAGAREIRO NETTO[3]
https://orcid.org/0000-0001-7337-9435

LUCAS S. ZAMARIOLI[4]
https://orcid.org/0000-0003-4710-431X

MARIA C. MARCUCCI[1]
https://orcid.org/0000-0002-8065-5618

SAMIRA E.A. CAMARGO[5]
https://orcid.org/0000-0002-2527-0651

CLÁUDIO A.T. CARVALHO[6]
https://orcid.org/0000-0003-0987-5594

LUCIANE D. DE OLIVEIRA[1]
https://orcid.org/0000-0001-9956-7768

[1]Universidade Estadual Paulista(UNESP), Instituto de Ciência e Tecnologia, Departamento de Biociências e Diagnóstico Bucal, Av. Engenheiro Francisco José Longo, 777, 12245-000 São José dos Campos, SP, Brazil

[2]Universidade Anhembi Morumbi, Escola de Medicina, Av. Dep. Benedito Matarazzo, 4050, 12230-002 São José dos Campos, SP, Brazil

[3]Universidade Anhanguera, Departamento de Farmácia, Av. Raimundo Pereira de Magalhães, 3305, 05145-200 São Paulo, SP, Brazil

[4]Universidade Federal de São Paulo(UNIFESP), Instituto de Farmacologia e Biologia Molecular, Departamento de Modo de Ação de Drogas, Rua Três de Maio, 100, 04044-020 São Paulo, SP, Brazil

[5]University of Florida, College of Dentistry, Department of Restorative Dental Sciences, Gainesville, FL, 32610, USA

[6]Universidade Estadual Paulista(UNESP), Instituto de Ciência e Tecnologia, Departamento de Odontologia Restauradora, Av. Engenheiro Francisco José Longo, 777, 12245-000 São José dos Campos, SP, Brazil

Correspondence to: **Jonatas Rafael de Oliveira**
E-mail: jroliveira16@hotmail.com

Authors contributions

Conception and design - JRO, LDO; Acquisition of data - VMM, LWF, AALN, LSZ; Analysis and interpretation of data - VMM, JRO, MCM; Writing of the manuscript - VMM; Critical review of the manuscript - MCM, SEAC, CATC, LDO. Funding acquisition - CATC, LDO.