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Development and Quantification of Fungal Biofilm in Acrylic Resins of Dental Prostheses Pretreated with *Rosmarinnus officinalis*

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Abstract

Candida albicans is the main agent of oral candidiasis being classified as an opportunistic non-contagious yeast, also the main species involved in the development of this disease. Dentures are commonly biofilm deposits, especially the fungal biofilm that became the target of several studies, being the treatment of stomatitis with classic antifungal agents well established. However, resistance by individuals has been described. The objective of this study was to quantify the biofilm formation of *Candida albicans* in denture material pretreated with extracts of *Rosmarinus officinalis*. For the preparation of the specimens, microwave cured acrylic resin was used, being polished and sterilized with ethylene oxide, and divided into six groups according to the treatment used: nystatin, lotion of 24% of *R. officinalis*, essential oil of *R. officinalis*, hydroalcoholic extract of *R. officinalis*, carbopol and controls (untreated). The specimens were immersed in treatment for 14 days; then, the biofilm was induced and quantified. Data were statistically analyzed by ANOVA followed by Tukey test ($\alpha = 0.05$). The lotion *R. officinalis* at 24% was the most effective in preventing biofilm formation, reducing it by 96.6% compared to control, followed by the essential oil in carbopol with 83%, being those results not statistically different compared to nystatin (68.3%). The hydroalcoholic extract did not prevent biofilm formation. We conclude that the lotion at 24% and the essential oil when applied to the acrylic surface, are capable of inhibiting fungal biofilm formation.

Subject Areas

Biological Materials

Keywords

Biofilm, *C. albicans*, Plant Extracts, *Rosmarinus officinalis*

1. Introduction

Biofilms are biological structures with a high degree of organization, where microorganisms form structured, coordinated and functional communities [1]. Biofilms can develop on any moist surface, be it biotic or abiotic, such as water pipes, skin and mucous membranes, teeth, prostheses and industries [2]. Until some time, bacterial biofilms were considered of greater importance; however fungi biofilms have stood out due to pathogenic ability, highlighting yeasts of the genus Candida as the main involved, with emphasis on *C. albicans* [3].

The removal or elimination of the biofilm has been intensified due to its importance for oral health [4]. Commercially, several antifungals, mouthwashes and disinfectants are available commercially, with difficulties in controlling the biofilm [5]. *Candida albicans* biofilms are the cause of infections associated with medical devices, besides behaving like protected niches of microorganisms, not responding to treatment with antibiotics and antifungals, and may create a source of persistent infection [6]. Another worrisome fact is that the formation of biofilm triggers antifungal resistance and protection against host defense leading to an important clinical repercussion [7].

In this sense, there has been interesting in the search for plant products, such as condiment plants, due to the number of phenolic compounds present, as in the case of *Rosmarinus officinalis* (rosemary). Rosemary extracts demonstrate antiseptic properties for the airways and oral cavity, antidepressant, soothing and healing action, among others [8]. The therapeutic effects of *Rosmarinus officinalis* (rosemary) have been proven in several studies due to its high antimicrobial potential, both in the form of extracts and essential oil, mainly evaluated in bacteria and yeasts [9]-[14].

However, with regard to the control and elimination of biofilm, these extracts have not been studied, so the objective of this work was to determine the ability of plant extracts of *Rosmarinus officinalis* to inhibit the formation of biofilm by *Candida albicans* on resin surfaces used in dental prostheses.

2. Materials and Methods

2.1. Preparation of Extracts

Rosemary (*R. officinalis*), was obtained from the distributor, Luar Sul Ind. and Com. de produtos food Ltda. (lot 2011/1), with quality certificate. After that, he was referred to the Center for Chemical and Pharmaceutical Sciences to obtain

essential oil and hydroalcoholic extract. For the essential oil, 100 g of the plant and 1 L of sterile distilled water were used, which were submitted to hydrodistillation in Clevenger, after being stored under refrigeration (Brazilian Pharmacopoeia IV). For the hydroalcoholic extracts, 20 g of the plant was used with 200 mL of ethanic solution at 80%, for 1 hour at 40˚C in an oil bath, and then filtered and repeated twice by joining the filtrates and taken to the rota evaporator, obtaining the extract.

2.2. Making the Specimens

To make the specimens, condensation silicon blocks of zetalabor® laboratory use (1 cm × 1 cm × 0.5 cm) were included in muffle for polymerization in microwave, using stone plaster, to be later filled with thermopolymerized acrylic resin in microwave, manipulated according to the manufacturer's specifications. For polymerization of the resin, a microwave oven with a frequency of 60 Hz and maximum power of 1600 Watts was used, with the following polymerization cycle: 300 W for 3 minutes, 100 W at zero power for 4 minutes, and 600 W for 3 minutes. After cooling the muffle, the specimens were demfused and the excess acrylic was resin removed with tungsten drill. Subsequently, they were sanded with 80 granulation water sandpaper in manual metallographic circular politiz at low speed and under refrigeration. Then, the specimens were immersed in sterile distilled water at room temperature for one week for the release of the superficial monomers and sent for sterilization in ethylene oxide.

2.3. Inoculum of *Candida albicans*

The isolate of *C. albicans* (SC5314) used in the experiment came from the Integrated Research Center of the Bauru School of Dentistry of the University of São Paulo. The inoculum was prepared from frozen suspensions, soed in Petri dishes containing Agar YEPD medium and incubated at 30˚C for 36 hours. To obtain preculture, a colony of *C. albicans* was resuspended in 50 mL of YEPD broth at 30˚C, under agitation of 180 rpm overnight. After this stage, *C. albicans* cells were washed with PBS and centrifuged at 4000 rpm, and resuspended in 1 mL of PBS, being adjusted with PBS at a final concentration equal to 1.0 × 107 mL^{-1} [6].

2.4. Experimental Groups

The specimens were divided into experimental groups and submitted to treatments, for 14 days, prior to inoculation of the fungus for biofilm production, according to the distribution in Table 1.

2.5. Biofilm Development

After the 14-day treatment period, it was removed by washing with PBS, after the specimens were inoculated with 1.5 mL of the suspension of *C. albicans* containing 107 cels mL^{-1}, and incubated at 37˚C under agitation of 75 rpm, for

Table 1. Demonstrative of the distribution of experimental groups according to the treatment used to control biofilm in specimens.

Experimental groups	Treatments
G1	Untreated
G2	Treated with nystatin 0.5%
G3	Treated with *R. Oficinalis* essential oil lotion 24%
G4	Treated essential oil 21% in carbopol
G5	Treated hydroalcoholic extract of *R. Oficinalis* 20%
G6	Treatises carbopol 3%

90 minutes, for the phase of the adoption. Subsequently, the inoculated specimens were washed with PBS for removal of the non-adhered cells and 1.5 mL of RPMI-1640 medium was added, plus MOPS, incubated under the same temperature and agitation conditions for 48 hours for the formation of the biofilm [6].

2.6. Biofilm Quantification Test

Following the biofilm formation period (48 hours) the specimens were removed from the culture medium washed gently in dilution plates of 24 wells containing 1.5 mL of PBS for removal of non-adhered cells. For each plate of 24 wells of treated specimens, two specimens were made without treatment and without inoculation for colorimetric tests only (white). Then, the biofilm was fixed with 100% chilled methanol under refrigeration for 15 minutes. Subsequently, the excess methanol was removed and the specimens were colored with 1.5 mL of 0.1% violet crystal solution and incubated at 37˚C for 5 minutes under agitation of 75 rpm, for biofilm pigmentation. To remove excess dye, three washes were performed with PBS, alternated by shaker agitation for 5 minutes and after the dye of the specimens was fixed with 1.5 mL of 95% ethanol incubated at 37˚C, for 5 minutes, under agitation of 75 rpm. For each biofilm sample, a 100 μL aliquot of the decorated solution was diluted 1:10 and submitted to spectrophotometer reading with a wavelength of 570 nm to read the absorbance.

2.7. Statistical Analysis

The data were expressed by the mean ± standard deviation (SD) of the results obtained for each treatment group and were submitted to the ANOVA test followed by Tukey test ($p = 0.05$).

3. Results and Discussion

All treatments were performed with twenty replicates, and an average of the results were made. Through the spectrophotometric reading of G1 (untreated), it was demonstrated that the methodology used is applicable for the quantification of biofilm in resins, confirming observations of other authors who evaluated and quantified the biofilm by this method [6]. In addition, the results of G1 demon-

strated the efficacy of *C. albicans* in forming the biofilm, which was already expected, because in relation to the adhering of *Candida* spp. to the surfaces of dental prostheses, several studies have been developed [15], some demonstrating that the adeforeform also depends on the type of material used as the deza [16] which comes against our observations since the specimens presented roughness. Regarding nystatin, essential oil lotion of *R. officinalis* at 24%, essential oil of *R. officinalis* at 21% in carbopol, hydroalcoholic extract of *R. officinalis* at 20% and carbopol, it was observed that after spectrophotometric reading, there were statistical differences between treatments as shown in **Table 2**.

The treatment with *R. officinalis* that proved to be most effective in inhibiting biofilm formation was 24% lotion, which reduced this formation by 96.6%, followed by essential oil in carbopol that reduced biofilm formation by 82.3% and nystatin, which reduced the same by 68.93% when compared to the untreated group, there are no statistical differences between these results. On the other hand, the specimens treated with hydroalcoholic extract and carbopol did not show efficacy in inhibiting the formation of biofilm, showing an increase in biofilm quantification.

The possibility of inhibiting the formation of biofilm on the acrylic surface with natural products would cause fewer adverse effects for the patient and would be more accessible to the population. It is known that the formation of the film in users of dental prostheses is linked to poor oral hygiene [17] [18] [19], what is closely related to the health education of patients, and that within the risk groups, we highlight users with lower socio-economic conditions who are victims of pathologies caused by biofilm, because they do not have access to information and methods of prevention and treatment [20].

Some authors have emphasized that in addition to the importance of oral hygiene of prostheses to prevent the formation of biofilm, it should be invested in the education of users in this aspect in order to promote the disinfection of

Table 2. Demonstration of the mean spectrophotometric reading of inhibition of biofilm formation by *Candida albicans*, according to the different treatment groups.

Treatments	Reading on spectrophotometer (Absorbance)	Reduction in quantification (%)
G1	4.3957^{B}	0
G2	1.3657^{C}	68.93
G3	0.1488^{C}	96.61
G4	0.7788^{C}	82.30
G5	9.0338^{A}	0
G6	7.2600^{A}	0

*a, b, c: Different letters, demontm significant statistical difference (p = 0.05); G1 Untreated, G2 Nystatin 0.5%, G3 *R. Officinalis* Essential Oil Lotion 24%, G4 Essential Oil 21% in Carbopol, G5 Hydroalcoholic Extract of *R. officinalis* 20% G6 Carbopol 3%.

dentures [17] [18] [19] [21] [22]. Mechanical removal is the most accepted method for the removal of dental biofilm by *Candida albicans*; however, the use of chemical adjuvants is quite valuable. In this sense, [23] [24] evaluated the efficacy of sanitizing substances such as alkaline peroxide containing an enzyme, 0.5% sodium hypochlorite or distilled water to prevent recolonization by Candida spp. on denture surfaces, verifying that sodium hypochlorite was the only treatment that efficiently eliminated biofilm. Other plant extracts have been used to prevent the formation of biofilm by *C. albicans*, but the use of *Rosmarinnus officinalis* extracts does not [25] have been documented, evaluating the effect of thyme tea (Thymus vulgaris Linn.) on the adhesion of C. albicans in acrylic resins, detecting the non-stick effect of this extract, [26] used sed the same line of research with the ethanolextract of the leaves of Streblus asper also demonstrating the decrease in the ability of yeast to adhere to resins. [27], obtained promising since results when they evaluated in vitro the antimicrobial, antifungal and non-stick activity of the bark extract of the stem of the hinterskin, and extracts of the leaves of malva and guava on microorganisms of the dental biofilm and oral candidosis.

The results of inhibition of fungal biofilm formation using rosemary essential oil lotion 24% as well as the use of essential oil 21% in carbopol were promising since difficulties in the elimination and control of biofilm by *C. albicans* are known. It is also known the ability of microorganisms to protect themselves from the action of antibiotics and antifungals, and maybe a source of persistent infection [6] [7]. Another problem in relation to biofilm is the precise diagnosis of infections associated with it, as it is often difficult, and the appropriate choice of treatment is complicated. As these infections contribute significantly to the morbidity of patients and the high costs of health programs, new strategies to treat them should be developed urgently [17] [27]. Thus, the importance of the results obtained is highlighted, and the extracts of *R. officinalis* represent an option for the bioprospecting of herbal medicines to prevent the formation of biofilm.

4. Conclusion

The 24% essential oil lotion of *Rosmarinus officinalis* inhibited the formation of biofilm by *C. albicans* in dental material. The essential oil in carbopol at 21% was also effective in reducing the formation of biofilm, and the two treatments were superior to the standard antifungal.

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Conflicts of Interest

The authors declare no conflicts of interest.

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