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ORIGINAL ARTICLE

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Total protein level reduction of odontopathogens biofilms by *Rosmarinus officinalis* L. (rosemary) extract: an analysis on *Candida albicans* and *Streptococcus mutans*

Redução do nível de proteína total de biofilmes de odontopatógenos por extrato de *Rosmarinus officinalis* L. (alecrim): uma análise sobre *Candida albicans* e *Streptococcus mutans*

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ABSTRACT

Objective: The resistance of fungi and bacteria to the available antimicrobials has increased and the development of alternative products to control them has become a very requirement. The use of plant products could be a viable option due to the efficacy, viability, and availability they present. Thereby, this study evaluated the effect of *R. officinalis* L. extract on *C. albicans* and *S. mutans* biofilms, by the total protein level analysis presented by the microorganisms. **Material and Methods:** For this purpose, monomicrobial biofilms were formed for 48 h and exposed to the *R. officinalis* L. extract for 5 min. Then, total protein quantification was performed by Lowry method. **Results:** The analysis showed significant total protein reduction of the biofilms after exposure to the plant extract, with 39 ± 11%, for *C. albicans*, and 32 ± 11%, for *S. mutans*. **Conclusion:** *R. officinalis* L. extract decreased the total protein level in both biofilms. Thus, *C. albicans* and *S. mutans* protein composition could be a target for action of antimicrobial agents.

KEYWORDS

Biofilms; *Candida albicans*; Proteins; *Rosmarinus officinalis; Streptococcus mutans.*

RESUMO

Objetivo: A resistência de fungos e bactérias aos antimicrobianos disponíveis tem se elevado e o desenvolvimento de produtos alternativos para controlar micróbios tem se tornado uma necessidade real. A utilização de produtos de origem vegetal poderia ser uma opção viável, devido à eficácia, viabilidade e disponibilidade que apresentam. Sendo assim, este estudo avaliou o efeito do extrato de *R. officinalis* L. (alecrim) sobre biofilmes de *C. albicans* and *S. mutans*, analisando o nível de proteína total apresentada pelos micro-organismos. **Material e Métodos:** Para tanto, biofilmes monomicrobianos foram formados por 48 h e expostos ao extrato de *R. officinalis* L. por 5 min. Então, a quantificação de proteína total foi realizada por método de Lowry. **Resultados:** A análise demonstrou reduções significativas de proteína total de cada biofilme após exposição ao extrato, sendo 39 ± 11% no biofilme de *C. albicans* e 32 ± 11%, no caso de *S. mutans*. **Conclusão:** O extrato de *R. officinalis* L. diminuiu o nível de proteína total em ambos os biofilmes. Com isso, a composição proteica de *C. albicans* e *S. mutans* poderia ser um alvo para ação de agentes antimicrobianos.

PALAVRAS-CHAVE

Biofilmes; *Candida albicans*; Proteínas; *Rosmarinus officinalis L.; Streptococcus mutans.*

INTRODUCTION

Microbes can establish themselves in organized and structured communities called biofilms. These three-dimensional structures remain adhere to an abiotic or biotic surface. The role of each member is carefully defined in these communities; for example, the synthesis of chemical elements (carbohydrates and proteins) responsible for forming a safe, consistent, and highly adhesive casing throughout the microecosystems, known as extracellular matrix. The formed biofilm has channels responsible for transporting water and nutrients, as well as, for eliminating toxic products from microbial cell metabolism. The structural arrangement of the biofilm protect the microorganisms against external actions, such as host's defense mechanisms, mechanical actions, and antimicrobials [1-4].

Both *C. albicans* and *S. mutans* are opportunistic microorganisms capable of forming biofilm [5-7]. *C. albicans* biofilm formation is initially characterized by the presence of yeasts. Posteriorly, in the intermediate stage, hyphal forms will become more elongated, and the extracellular matrix formation of glycoprotein characteristic will occur. Thereby, a mature biofilm will be constituted by hyphae attached to the yeasts, and all these elements will be involved by a casing of polysaccharide and protein [8]. In the oral cavity, *C. albicans* causes infections in the oral mucosa, tongue, palate, tooth, and periodontal tissue. Besides, this yeast can trigger a significant inflammatory response in the host. Pseudomembranous and erythematous are forms of candidiasis can be developed in susceptible patients [4,9,10].

C. albicans has the ability to interact with other microorganisms such as *Streptococcus* spp. (*S. gordonii, S. sanguinis, and S. mutans*), *Staphylococcus aureus*, *Enterococcus faecalis*, and *Pseudomonas aeruginosa*, forming complex polymicrobial communities [7,11-15]. In the oral cavity, the interaction of *C. albicans* and *S. mutans* favors the development of dental caries, especially in children due to the sucrose-rich diet and poor dental hygiene [16-18]. *S. mutans* uses sucrose from the host´s diet to synthesize the extracellular polysaccharide matrix that will involve the biofilm. Besides, this bacterium produces and uses enzymes, such as glycosyltransferase or fructosyltransferase, to adsorb to the surfaces where the biofilm will be installed. In addition, *S. mutans* tolerates acidic pH; for this reason its development is not harmed, even being highly acidogenic [19,20].

The control of opportunistic microorganisms is a strategy to prevent diseases. In the case of oral biofilms, this control is mainly performed by the use of oral hygiene products, such as antiseptics and dentifrices [21,22]. Some of these products have active principles from medicinal plants, such as thymol, eucalyptol, and menthol, due these phytocompounds present recognized antimicrobial activity [22]. These organic molecules pass through the microbial cell wall and settle in the lipid bilayer, causing changes in the cell membrane and leading the microorganism to the collapse [23-25]. Thereby, the evaluation of plant products could be continuously investigated to obtain new chemical components, with the aim of eliminating microorganisms responsible for dental diseases, as well as other types of infections. In this regard, *R. officinalis* L. could be a significant candidate. Popularly known by rosemary, it is an aromatic plant originating in the Mediterranean region and used mainly for flavoring foods [26]. Additionally, this medicinal plant presents several active principles, such as α-pinene, 1,8-cineole, camphor, camphene, β-pinene, β-caryophyllene, and limonene [27] with relevant pharmacological effects, which include: antibacterial [28], antifungal [29], antiviral [30], antibiofilm [6], antitumor [31], anti-inflammatory [32], antileishmanic [33], and antioxidant [33].

Thus, this study evaluated the effect of *R. officinalis* L. extract on *C. albicans* and

S. mutans biofilms, by the total protein level analysis after exposure to the plant extract.

MATERIAL AND METHODS

Plant extract

R. officinalis L. extract (Mapric, São Paulo, SP, Brazil) was used at a concentration of 200 mg/mL of propylene glycol. According to the manufacturer, this extract was obtained from leaves of *R. officinalis* L. and was chemically composed of acid saponin, bitter substances, borneol acetate, camphene, camphor, cineol, free borneol, glucosidic compounds, oleanolic acid, pinene, sesquiterpenes, and tannin.

Microorganisms

Reference strains (ATCC – American Type Culture Collection) of *C. albicans* (ATCC 18804) and *S. mutans* (ATCC 35688) were used. These strains were obtained from Laboratory of Microbiology and Immunology, Institute of Science and Technology, São Paulo State University (ICT – UNESP), and kept frozen (-80°C) in Yeast Extract Peptone Dextrose broth (YPD – Himedia, Mumbai, Maharashtra, India) with 16% glycerol or Brain Heart Infusion broth (BHI – Himedia) with 20% glycerol, respectively.

Monomicrobial biofilm formation

C. albicans and *S. mutans* were cultured in Sabouraud dextrose agar (SD – Himedia) or BHI (Himedia) agar for 24 h (37°C; 5% CO_2 for *S. mutans*). After, *C. albicans* was grown in Yeast Nitrogen Base broth (YNB – Himedia) and *S. mutans* in BHI broth (Himedia), using same conditions and period. Then, each microbial suspension was centrifuged at 2000 rpm/10 min (MPW-350, Warsaw, Mazowieckie, Poland), the supernatant was replaced for sterile saline solution (0.9% NaCl), and a new centrifugation was performed. Posteriorly, the turbidity of the suspensions was adjusted to 10^7 colony-forming units per milliliter (CFU/mL) in a spectrophotometer (Micronal, São Paulo, SP Brazil). In 24-well plates (TPP, Trasadingen, Canton of Schaffouse, Switzerland), 300 μL of microbial suspension were added and a microbial adhesion was performed by means of incubation (37°C) under agitation (75 rpm; Quimis, Diadema, SP, Brazil) for 90 min. After, the supernatants were replaced for 300 μL/well of YNB or BHI (Himedia). Biofilms were formed for 48 h with replacement of culture medium after 24 h incubation. Posteriorly, wells were washed with saline to remove non-adherent cells (300 μL/well) and the biofilms were exposed to the *R. officinalis* L. extract (200 mg/mL) or saline (0.9% NaCl) for 5 min. New washes were performed to discard affected microbial cells by the exposure to the plant extract. Each experimental group was composed by five replicates.

Total protein quantification

Modified Lowry method [34] was used to determine the total protein level. Firstly, microbial cells were lysed by 2000 μL of 0.1% sodium lauryl sulfate (Sigma-Aldrich, St Louis, MO, USA) for 30 min (37°C). Cell lysate (1000 μL) and Lowry reagent (1000 μL) (Sigma-Aldrich) were added in a tube, which was homogenized and incubated for 20 min at 37°C. Subsequently, Folin Ciocalteu's phenol reagent (Sigma-Aldrich) was added to the solutions (500 μL/tube). After 30 min incubation, the absorbance was measured in a spectrophotometer (Micronal) at 680 nm. Optical density (OD) values were converted to micrograms per milliliter (μg/mL), considering a standard curve of bovine serum albumin (BSA, US Biological, Salem, MA, USA), from 400 to 25 μg/mL. GraphPad Prism 5.0 software (San Diego, CA, USA) was used for this conversion.

Statistical analysis

Data were presented as mean values (± standard deviation) and analyzed by T-Test ($P \leq 0.05$). GraphPad Prism 5.0 software was used for this purpose.

RESULTS

In both biofilms a lower total protein concentration was verified after exposure to the *R. officinalis* L. extract, compared to the control group.

C. albicans biofilm presented a concentration of 237 ± 22 μg/mL, in the control group (Figure 1). After exposure to the plant extract, the total protein concentration decreased significantly to 144 ± 26 μg/mL (Figure 1), totalizing a reduction of 39 ± 11%.

S. mutans biofilm was also affected by the extract (Figure 2). The control group showed a total protein level of 71 ± 10 μg/mL and the treated group had a concentration of 48 ± 8 μg/mL, demonstrating a reduction of 32 ± 11%.

Additionally, it was verified that these reductions did not statistically differ. On the other hand, a higher concentration of total protein (~ 3x) was observed in the *C. albicans* biofilm, in relation to the *S. mutans* biofilm.

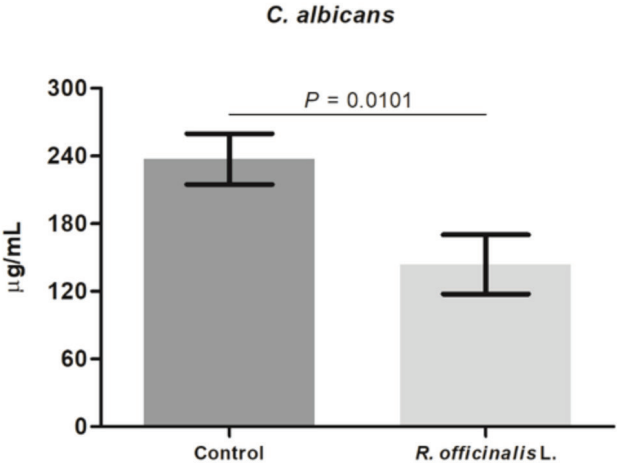

Figure 1 - Mean values (± standard deviation) of total protein level (µg/mL) obtained from *C. albicans* biofilm exposed to the *R. officinalis* L. extract (200 mg/mL) or saline solution (0.9% NaCl, control) for 5 min. (n = 5; T-Test; P ≤ 0.05).

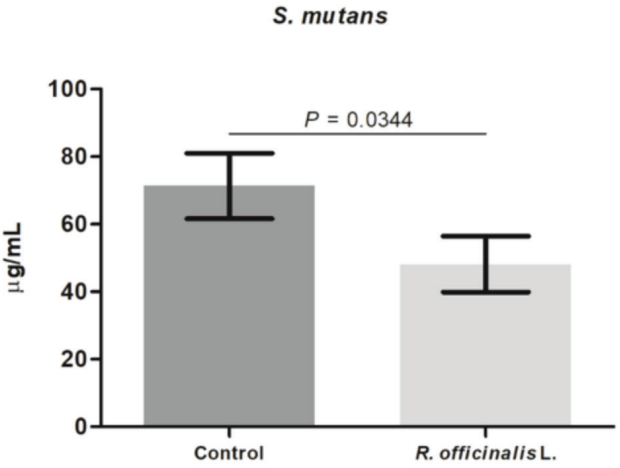

Figure 2 - Mean values (± standard deviation) of total protein level (µg/mL) obtained from *S. mutans* biofilm exposed to the *R. officinalis* L. extract (200 mg/mL) or saline solution (0.9% NaCl, control) for 5 min. (n = 5; T-Test; P ≤ 0.05).

DISCUSSION

Total protein level of the *C. albicans* (Figure 1) and *S. mutans* (Figure 2) biofilms was significantly decreased after exposure to the *R. officinalis* L. extract. Therefore, antibiofilm effect of this vegetal extract was evidenced by means of this analysis. Antifungal action of *R. officinalis* L. essential oil has also been reported on *C. albicans*, showing a minimum inhibitory concentration (MIC) of 9.14 μg/mL [27]. However, in another study the MIC_{80} of this plant product has varied from 24 to 31 μg mL as analyzed on clinical samples obtained from bovine mastitis and reference strain [35].

On biofilms, the application of concentrations above the MIC is necessary to have an effective microbial reduction. In this study, reductions of 39% (± 11), for *C. albicans*, and 32% (± 11), for *S. mutans*, were obtained using the plant extract in its higher concentration, i.e. at 200 mg/mL. Previously, this concentration was evaluated on different microbial biofilms and significant reductions of CFU/mL was found, such as 99.96% (± 0.07), for *C. albicans*, and 79.32% (± 7.34), for *S. mutans* [7]. Thus, we propose to verify if this concentration could interfere in the total protein level of *C. albicans* and *S. mutans* biofilms. After 5 min exposure, significant reductions were observed (Figures 1

and 2); however, biocompatibility tests should be performed to check the potential cytotoxic of this concentration because 200 mg/mL could be a high concentration.

The antibiofilm effect of a nanosystem composed by Fe_3O_4/oleic acid:$CHCl_3$ has significantly improved after addition of *R. officinalis* L. essential oil to the nanoparticles. Coated catheters by the nanosystem with essential oil have inhibited the *C. albicans* adhesion to the material surface, and consequently prevented the development of the fungal biofilm. Thus, reductions have been found after exposures for 48 h (85%) and 72 h (98%) [5].

Total protein concentration of antifungal-resistant *C. albicans* samples has varied from 1.05 to 3.22 mg/mL after exposure to the different therapeutics agents, such as fluconazole, itraconazole, amphotericin B, and ketoconazole. These levels have been lower than those presented by untreated samples [36]. Similarly, in our study the total protein level of the *C. albicans* and *S. mutans* biofilms was decreased by the treatment with *R. officinalis* L. extract (Figures 12).

In this study, *S. mutans* biofilm was affected by the *R. officinalis* L. extract (Figure 2). This antibiofilm effect of the plant extract has also been observed on infected bovine teeth with *S. mutans* [6]. In this case, the MIC of the plant extract (0.25 mg/mL) has prevented the biofilm formation on the dental surface, in about 87.5% of the tooth area. Additionally, the bacterial adhesion has been impaired by the MIC (OD = 0.04 ± 0.01) and 0.5 x MIC (OD = 0.24 ± 0.04), compared to the control group (OD = 1.54 ± 0.03), as analyzed at 480 nm. The glucan production by *S. mutans* has also been reduced, presenting a percentage of 30 ± 2 (MIC), 68 ± 5 (0.5 x MIC), and 6.5 ± 2 (2 x MIC). Thus, *R. officinalis* L. has acted on *S. mutans* surface molecules, preventing the adhesion and biofilm formation by this pathogen.

Phytocompounds from *R. officinalis* L. have shown significant effect on *S. mutans*, such as rosmarinic acid, carnosol, and carnosic acid, which have presented MIC of 200, 75, and 30 μg/mL, respectively. Therefore, they have been reported as responsible for the antimicrobial effect of this plant [37]. Interestingly, toothpaste with *R. officinalis* L. essential oil (200, 400, 600 and 800 ppm) has potentialized the anti-*S. mutans* effect. Besides, this plant product has shown a superior antimicrobial effect to the chlorhexidine. At 400 ppm, a higher *S. mutans* biofilm inhibition has been observed, in relation to the chlorhexidine. Thereby, the contribution of a plant product to improve the antimicrobial effect of an oral hygiene product has been proven [38].

Plant products, such as extracts, essential oils, and phytocompounds, have presented a significant antimicrobial effect on opportunistic pathogens, both on planktonic cultures and biofilms. In the latter case, these products have shown capacity to inhibit biofilm formation, by interfering in the microbial adhesion molecules synthesis. Moreover, these products have also acted on formed biofilms, by inhibiting the cell metabolism regulatory proteins production.

CONCLUSION

Thus, *C. albicans* and *S. mutans* biofilms were affected by the exposure to the *R. officinalis* L. extract. Since, the total protein level of both biofilms was significantly decreased. Therefore, the protein composition of these microorganisms could be a potential target for antimicrobial agents.

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