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Rosmarinus officinalis l. (Rosemary) extract decreases the biofilms viability of oral health interest

Extrato de *Rosmarinus Officinalis* l. (Alecrim) reduz a viabilidade de biofilmes de interesse para saúde bucal

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ABSTRACT

Objective: This study evaluated the effect of rosemary extract on *Candida albicans*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Streptococcus mutans* and *Pseudomonas aeruginosa* monomicrobial biofilms viability, as well as on *C. albicans* associated with *S. aureus*, *E. faecalis*, *S. mutans* or *P. aeruginosa* in polymicrobial biofilms. **Material and Methods:** In microtiter plate, mono- and polymicrobial biofilms for 48 h were formed. Then, they were exposed for 5 min to rosemary extract (200 mg/mL). Saline (0.9% NaCl) was used as control. After, washes were done with saline to remove the non-adhered cells. Biofilm viability was checked by MTT colorimetric assay, after treatment. Absorbance of the wells was read in microplate spectrophotometer (570 nm) and data were converted to reduction percentage and statistically analyzed by ANOVA and Tukey test ($P \leq 0.05$). **Results:** After application of rosemary extract, with exception of the *E. faecalis* biofilm, significant reductions in mono- and polymicrobial biofilms viability were observed. **Conclusion:** *C. albicans*, *S. aureus*, *S. mutans* and *P. aeruginosa* monomicrobial biofilms were affected by rosemary extract, as well as *C. albicans* associated with *S. aureus*, *E. faecalis*, *S. mutans* or *P. aeruginosa* in polymicrobial biofilms, presenting significant viability reductions.

KEYWORDS

Monomicrobial biofilm; Polymicrobial biofilm; Rosemary; Rosmarinus officinalis; Viability.

RESUMO

Objetivo: No presente estudo foi avaliado o efeito do extrato de alecrim sobre a viabilidade de biofilmes monomicrobianos de *Candida albicans*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Streptococcus mutans* e *Pseudomonas aeruginosa*, bem como, sobre biofilmes polimicrobianos de *C. albicans* associada com *S. aureus*, *E. faecalis*, *S. mutans* ou *P. aeruginosa*. **Material e métodos:** Em placa de microtitulação foram formados os biofilmes mono e polimicrobianos por 48 h. Em seguida, foram expostos por 5 min ao extrato de alecrim (200 mg/mL). Solução salina (NaCl 0,9%) foi utilizada como controle. Após, foram realizadas lavagens com salina para remoção de células não aderidas. Para verificação da viabilidade dos biofilmes, após o tratamento, foi aplicado o teste colorimétrico MTT. A absorbância dos poços foi lida em espectrofotômetro de microplacas (570 nm) e os dados foram convertidos em percentual de redução e analisados estatisticamente por ANOVA e Tukey Test ($P \leq 0,05$). **Resultados:** Após aplicação do extrato de alecrim, com exceção do biofilme de *E. faecalis*, foram observadas reduções significativas da viabilidade dos biofilmes monomicrobianos e polimicrobianos. **Conclusão:** Biofilmes monomicrobianos de *C. albicans*, *S. aureus*, *S. mutans* e *P. aeruginosa*, foram afetados pelo extrato de alecrim, bem como, os biofilmes polimicrobianos de *C. albicans* associada com *S. aureus*, *E. faecalis*, *S. mutans* ou *P. aeruginosa* em biofilmes polimicrobianos, apresentando significativas reduções de viabilidade.

PALAVRAS-CHAVE

Alecrim; Biofilme monomicrobiano; Biofilme polimicrobiano; Rosmarinus officinalis; Viabilidade.

INTRODUCTION

R. officinalis L. (Lamiaceae), popularly known as rosemary, is a plant species originated from Mediterranean region, however can be found and cultivated in all continents. It is an aromatic and ornamental plant and its leaves are commonly used as condiment and medicinal purposes [1]. Rosemary presents several constituents which are responsible for the pharmacological activities, such as 1,8-cineole, camphor and α-pinene [2]. Some biological activities have been attributed to this plant, including antimicrobial [3], antibacterial [4], antifungal [2,5], antimycobacterial [6], anti-inflammatory [7,8], antitumor [9], antioxidant [3,10,11], antimutagenic [12] neuroprotective [13,14], cardioprotective [15], oxidative stress modulator [16,17] and DNA-protective [18] activities.

Biofilms are composed by a microbial community surrounded by a protein extracellular matrix and polysaccharides produced by them, they can be adhere on dental materials, prostheses, implants, endotracheal tube, pacemakers and catheters, or a biotic surface, such as host tissues [19-21]. Microorganisms in biofilm are naturally found in interspecific associations that may favor or hinder the development of each other, interfere with antimicrobial susceptibility and on the genes expression [22,23].

The microbial species selected to realization of this study are of interest to oral health, since they may cause serious disorders throughout the oral cavity and furthermore they can be disseminated systemically and induce significant infections in other organs. *C. albicans* may cause pseudomembranous and erythematous candidiasis [24], besides angular cheilitis [25]. *S. aureus* from supra and subgingival biofilm may be responsible for periodontitis [26]. *E. faecalis* can also be associated with periodontal disease, once was identified in root canal infections and apical periodontitis [27]. The presence of *P. aeruginosa* in subgingival biofilm can induce a more aggressive form of periodontitis [28].

R. officinalis L. has been extensively studied in relation to its action on microorganisms, however its effect on microorganisms grouped in biofilms and on polymicrobial associations has not been evaluated. In addition, in the present study, it will be possible to note how much the plant extract could affect the metabolism of microbial cells in mono- and polymicrobial communities.

The emergence of resistant strains to antimicrobial of conventional use in medical fields has challenged the research groups in the investigation of new products and methods for their control. One of these alternative methods could be the application of medicinal plant products such as extracts, essential oils and phytochemicals in medications and also in toothpastes, mouthwashes, intracanal medication, ointments, soaps, in order to eliminate these microorganisms which can cause serious local and systemic infections. Thus, the present study aimed to analyze the antimicrobial effect of rosemary extract on *C. albicans*, *S. aureus*, *E. faecalis*, *S. mutans* and *P. aeruginosa* monomicrobial biofilms viability, as well as on polymicrobial biofilms of *C. albicans* associated with *S. aureus*, *E. faecalis*, *S. mutans* or *P. aeruginosa*.

MATERIAL AND METHODS

Plant extract

Rosemary extract was commercially acquired (Mapric, SP, Brazil) at 200 mg/mL propylene glycol. This extract was obtained from leaves of the plant, chemically composed by pinene, camphene, free borneol and borneol acetate, cineol, camphor, sesquiterpenes, oleanolic acid, little tannin, bitter substances, acid saponin, and glucosidic compounds, according to the manufacturer.

Microbial strains

Reference strains (ATCC - American Type Culture Collection) of *C. albicans* (ATCC18804), *S. aureus* (ATCC 6538), *E. faecalis* (ATCC 4083),

S. mutans (ATCC 35688) and *P. aeruginosa* (ATCC 15442) obtained from Institute of Science and Technology/UNESP, were used in this study. Strains were kept frozen at -80°C in Brain Heart Infusion broth (BHI - Himedia, Mumbai, India) with 20% glycerol, for bacteria, and Yeast Extract Peptone Dextrose broth (YPD - Himedia) with 16% glycerol, for *C. albicans*.

Biofilms formation

Microbial suspensions adjusted to 107 CFU/mL (colony-forming unit per milliliter) were added in 96-well plates (200 μL/well). After 90 min incubation (37°C; 75 rpm - Quimis, Diadema, Brazil), the supernatant was discarded and BHI or Yeast Nitrogen Base(YNB, Himedia) broth was added (200 μL/well). After 24 h, the medium was replaced by fresh medium and the biofilms were formed for 48 h. For polymicrobial biofilms, equal parts of each suspension and medium were added. Posteriorly, biofilms were exposed to extract (200 mg/mL) for 5 min and saline (0.9 % NaCl) was used as negative control (n =10/group).

MTT assay

Reductases present in viable cells break MTT [bromide of 3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide] (Sigma Aldrich) generating formazan, which may be quantified by spectrophotometer. Therefore, MTT solution was prepared at 0.5 mg/mL phosphate-buffered saline (PBS) and 100 μL/well were added. After 1 h incubation, under protection from light, the supernatant was discarded and dimethyl sulfoxide (DMSO - Sigma Aldrich) was added (100 μL/well). Ten minutes incubation was performed, followed by agitation of the 96-well plate in shaker for more 10 min. Then, the absorbance of the wells was measured in microplate spectrophotometer (Bio-Tek, Vermont, USA) at 570 nm. Data were converted to reduction percentage.

Statical analysis

The results were presented in mean values (± standard deviation) and were analyzed by ANOVA and Tukey Test with aid of GraphPad Prism 5.0 software, considering statistically significant when $P \leq 0.05$.

RESULTS

Rosemary extract provided significant reductions of the viability of *C. albicans*, *S. aureus*, *S. mutans* and *P. aeruginosa* monomicrobial biofilms. However, the reduction demonstrated by *E. faecalis* biofilm was not significant when compared to the control group (Figure 1). In the polymicrobial biofilms was found that the plant extract reduced significantly their viability (Figure 2). Reduction percentages can be observed in Figure 3.

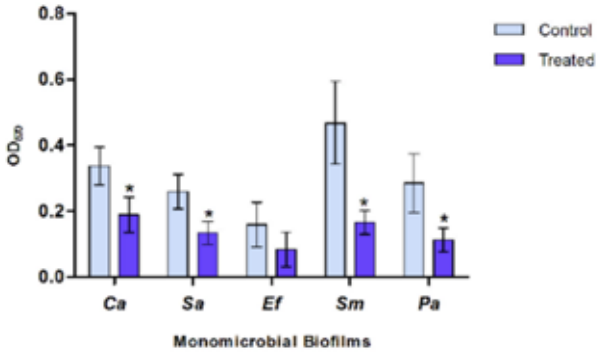

Figure 1 - Mean (± standard deviation) of OD (570 nm) obtained on C. albicans (*Ca*), S. aureus (*Sa*), E. faecalis (*Ef*), S. mutans (*Sm*) and P. aeruginosa (*Pa*) monomicrobial biofilms. Asterisks indicate statistically significant difference comparing treated group with control group in each biofilm. (ANOVA, Tukey Test, $P \leq 0.05$).

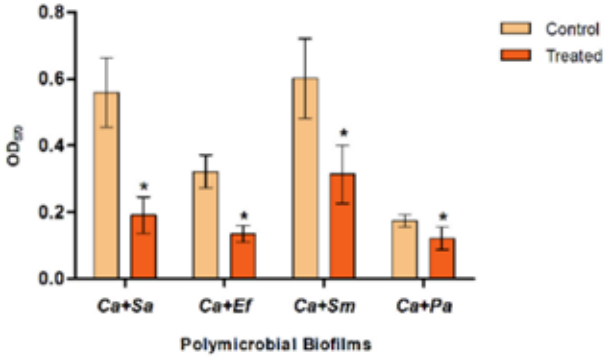

Figure 2 - Mean (± standard deviation) of OD (570 nm) obtained on polymicrobial biofilms composed by C. albicans and S. aureus (*Ca+Sa*), C. albicans and E. faecalis (*Ca+Ef*), C. albicans and S. mutans (*Ca+Sm*) and C. albicans and P. aeruginosa (*Ca+Pa*). Asterisks indicate statistically significant difference comparing treated group with control group in each biofilm. (ANOVA, Tukey Test, $P \leq 0.05$).

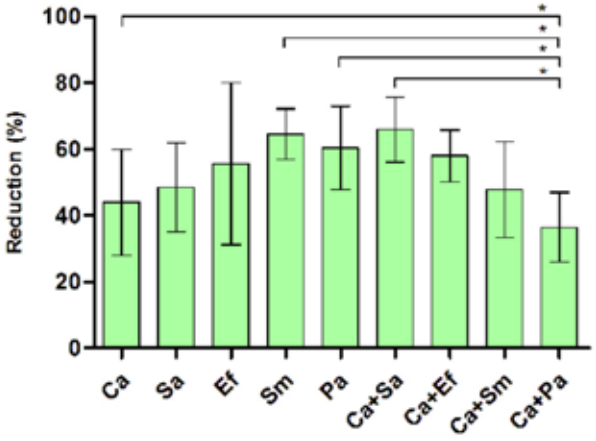

Figure 3 - Mean (± standard deviation) of percentage reduction of C. albicans (*Ca*), S. aureus (*Sa*), E. faecalis (*Ef*), S. mutans (*Sm*) and P. aeruginosa (*Pa*) monomicrobial biofilms and on polymicrobial biofilms composed by C. albicans and S. aureus (*Ca+Sa*), C. albicans and E. faecalis (*Ca+Ef*), C. albicans and S. mutans (CaÜSm) and C. albicans and P. aeruginosa (*Ca+Pa*). Asterisks indicate statistically significant difference. (ANOVA, Tukey Test, P ≤ 0.05).

DISCUSSION

In this study it was found that the rosemary extract provided antimicrobial effect on different species of bacteria and *C. albicans*. This plant product promoted significant reductions of the mono- and polymicrobial biofilms viability.

Rosemary extract reduced significantly the *C. albicans* biofilm viability (44 ± 16%). Similarly, the antibiofilm effect of rosemary essential oil was also reported by Chifiriuc et al. [29], who prepared a nanobiological system, formed by the union of rosemary essential oil, nanoparticles comprising a core of iron oxide (Fe3O4) and an oleic acid coating (CHCl3), which was analyzed on clinical isolates of *C. albicans* and Candida tropicalis. Catheters were coated or not with this system and the ability of the fungal biofilm development was in vitro observed. It was verified a significant reduction in adhesion of fungal cells to the material, as well as interference in the biofilm development, with complete absence of adhesion in periods of 48 and 72 h. On uncoated catheters the biofilm formation occurred initially by yeast (24 h) and subsequently by filamentous forms (72 h). There was *C. albicans* biofilm reduction of approximately 85% after 48 h, and 98% after 72h. Additionally, rosemary essential oil was also able to interfere on the in vitro filamentation of clinical isolates of *C. albicans*, considered like the major virulence factor of this yeast [30].

It was observed that on *S. aureus* biofilm (49 ± 13%) and on association of *C. albicans* and *S. aureus* (66 ± 10%) the rosemary extract promoted significant antibiofilm effect. There are reports that the rosemary essential oil may also be effective against *S. aureus* and Staphylococcus xylosus strains, in growth inhibition of these bacteria, as demonstrated by disc-agar diffusion test, where halos of 6.3 mm and 8 mm were generated respectively [31]. Besides of essential oil, some rosemary phytochemicals like α-pinene, β-pinene and 1.8-cineole also showed antibacterial effect against *S. aureus*, being found sharp decline in the concentration of CFU/mL after 12 h exposure and total elimination after 24 h, using essential oil. Regarding biocompounds, α-pinene showed inhibitory effect after 8 h contact and bactericidal effect after 12 h, β-pinene was bactericidal after 24 h and showed inhibitory effect after 12 h, and 1.8-cineole, showed sharp decline of CFU/mL from 24 h exposure and total elimination after 30 h [32].

S. mutans monomicrobial biofilm and its association with *C. albicans* were significantly affected by the rosemary extract presenting reductions of 65 ± 8% and 48 ± 14%, respectively. Likewise, it was also reported that the extract from rosemary leaves demonstrated significant in vitro activity on *S. mutans* in relation to the biofilm formation, reduction of virulence factors and also on planktonic cultures [33]. The authors showed that after 1 h incubation in liquid medium plus plant extract there was decrease of the *S. mutans* biofilm viability in 10-fold, increasing to 100-fold after 6 h, compared to the control group. Additionally, they also found inhibitory effect on other microbial species such as *C. albicans*, *S. aureus*, *E. faecalis*,

P. aeruginosa, Actinomyces spp., Streptococcus spp., Escherichia coli, Lactobacillus acidophilus and Veillonella spp.

Significant reductions in the *P. aeruginosa* biofilm viability (60 ± 13%) and in the associations of *C. albicans* and *P. aeruginosa* (36 ± 10%) and *C. albicans* and *E. faecalis* (58 ± 8%) were observed. However, the reduction shown by *E. faecalis* monomicrobial biofilm was not significant. On the other hand, it was reported that the rosemary hydroalcoholic extract from its leaves and fractions provide inhibitory effect and, in some cases, bactericidal effect on *E. faecalis* and *P. aeruginosa* strains [34]. By broth microdilution test, the crude extract and its n-hexane (F1), hexane/ethyl acetate (75:25 v/v) (F2), hexane/ethyl acetate (50:50 v/v) (F3), ethyl acetate (F4), ethyl acetate/ethanol (75:25 v/v) (F5), ethyl acetate/ethanol (50:50 v/v) (F6) and ethanol (F7) fractions were evaluated on a reference strain and a clinical isolate for each species. The results demonstrated that the crude extract and its fractions (F3, F2, F4, F7) inhibited the growth of the *P. aeruginosa* reference strain and only F1 and F3 inhibited the growth of the clinical isolate. Regarding *E. faecalis* strains, the crude extract and fractions (F4, F5 and F3) presented bactericidal effect on the reference strain and clinical isolate, however, only the F2 fraction afforded inhibitory effect on clinical isolate.

According to outcomes of present study and with reports in the literature on antimicrobial effect of rosemary, it was noted the importance of investigation on biological effects of medicinal plants, which can be an effective alternative for the control of bacteria and yeasts able to provide in human beings important infections that can start in the oral cavity and spread systemically and generate morbidities and, in extreme cases, death of patients. Nonetheless, in this study evaluations of the effect of a plant product were conducted in vitro and the confirmation of its effectiveness in the control of important microorganisms that cause infections was obtained. Even though, projections, as in vivo assays and clinical trials should be conducted in the future in order to enhance the study of medicinal plant products in search for an effective and biocompatible alternative method.

CONCLUSION

C. albicans, *S. aureus*, *S. mutans* and *P. aeruginosa* monomicrobial biofilms were affected by rosemary extract, as well as *C. albicans* associated with *S. aureus*, *E. faecalis*, *S. mutans* or *P. aeruginosa* in polymicrobial biofilms, presenting significant viability reductions.

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