Antifungal effect of plant extracts on Candida albicans biofilm on acrylic resin

Efeito antifúngico de extratos vegetais sobre biofilme de Candida albicans em resina acrílica

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ABSTRACT

Objective: Evaluating the antifungal potential of Equisetum arvense L. (horsetail), Glycyrrhiza glabra L. (licorice), Punica granatum L. (pomegranate) and Stryphnodendron barbatamam Mart. (barbatimão) extracts, after Candida albicans biofilm formation on acrylic resin. Material and methods: C. albicans standard strain was cultured on Sabouraud-dextrose agar for 24 h at 37 °C. After standardized in a spectrophotometer, 100 µL of the inoculum (10⁶ cells/mL) and a sterile acrylic resin disc were maintained in Brain Heart Infusion broth supplemented with sucrose (5%), for 5 days at 37 °C. The samples of the treated groups (n = 10) were separately exposed to a concentration of 50 mg/mL of each extract for 5 minutes or to nystatin (48.83 IU/mL). For the untreated group (control, n = 10), it was used sterile saline (0.9% NaCl). Biofilms were disaggregated from the acrylic resin discs by an ultrasonic homogenizer for 30 s. After decimal dilutions, sowings in Sabouraud-dextrose plates were made with incubation for 48 h at 37°C. Later, CFU/mL was verified and the values were converted to log₁₀ and they had their statistical analysis done (ANOVA and Tukey Test; p ≤ 0.05). Results: It was found that all plant extracts and nystatin resulted in significant reduction of C. albicans biofilm (p < 0.01) compared to the control group (0.9% NaCl). However, all of them showed similar reductions to each other (p = 0.1567). Conclusion: There was biofilm formation of C. albicans on acrylic resin and all plant extracts were effective against this yeast, acting similarly to nystatin.

KEYWORDS

Biofilm; Candida albicans; Medicinal plants; Nystatin; Plant Extracts.

RESUMO

Objetivo: Avaliar potencial antifúngico dos extratos de Equisetum arvense L. (cavalinha), Glycyrrhiza glabra L. (alcaçuz), Punica granatum L. (romã) e Stryphnodendron barbatamam Mart. (barbatimão) sobre biofilme de Candida albicans em resina acrílica. Material e métodos: Cepa-padrão de C. albicans foi cultivada em ágar Sabouraud-dextrose por 24 h a 37°C. Após padronização do inóculo (10⁶ células/mL) em espectrofotômetro, foram mantidos em caldo Brain Heart Infusion suplementado com sacarose (5%) um disco de resina acrílica estéril com 100 µL do inóculo padronizado, por 5 dias a 37 °C. As amostras dos grupos tratados (n = 10) foram expostas separadamente à concentração de 50 mg/mL de cada extrato por 5 min e ao antifúngico nistatina (48,83 UI/mL). Para o grupo não tratado (controle, n = 10) foi utilizada solução fisiológica estéril (NaCl 0,9%). Os biofilmes foram desagregados dos discos de resina acrílica por homogeneizador ultrassônico por 30 s. Após diluições decimais, foram feitas semeaduras em placas de Sabouraud-dextrose e incubação por 48 h a 37 °C. Posteriormente, foram contadas as UFC/mL e os valores foram convertidos em log₁₀ e realizada análise estatística (ANOVA e Tukey Test; p ≤ 0,05). Resultados: Todos os extratos naturais e a nistatina proporcionaram reduções significativas (p < 0,01) do biofilme de C. albicans em comparação ao grupo controle (NaCl 0,9%), no entanto, não houve diferença estatística entre os extratos (p = 0,1567). Conclusões: Houve formação de biofilme de C. albicans em resina acrílica e todos os extratos vegetais foram efetivos para esta levedura, atuando semelhantemente à nistatina.

PALAVRAS-CHAVE

Biofilme; Candida albicans; Plantas medicinais; Nistatina.
INTRODUCTION

Biofilms are well structured microbial communities where cells are embedded in an extracellular polysaccharide matrix, with the majority of the cells anchored to a substrate and these sessile cells are phenotypically different from planktonic cells or free floating cells [1].

*C. albicans* is the most common opportunistic fungal pathogen agent isolated from the human body, being able to cause superficial or systemic infections, often after antibiotic treatments, more severe in immunocompromised patients [2].

One of the most important virulence factors of *Candida* species is biofilm formation, because it provides the yeast resistance to antifungal drugs and complicates the diffusion of defense substances and cells by extracellular matrix [3]. Biofilm formation of *Candida* species is very common in dental prostheses [4], however, other infections related to the formation of biofilm on implanted materials have been reported, especially in urinary catheters, intravascular devices, bone fixation materials, central venous catheters, pacemakers, vascular grafts, heart valves and mechanical prostheses [5]. There are factors that predispose to *Candida* infections, such as immunosuppressive therapy, antibiotic use, HIV infection, diabetes, advanced age and use of implanted devices for a long time [6].

With the increasing number of microbial strains that have acquired resistance to conventional antibiotics, the search for alternative methods for controlling and treating infections caused by these strains is of great importance. The use of products from plants is one of these methods because it can provide significant results in the elimination of opportunistic pathogenic microorganisms like *C. albicans*.

*Equisetum arvense* L. (Equisetaceae) is a plant from Europe that grows well in temperate areas in different parts of the world. It is popularly known in Brazil as “cavalinha” (horsetail). There are reports of antibacterial activity for *Staphylococcus aureus*, *Escherichia coli*, *Salmonella enteritidis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and antifungal activity for *Aspergillus niger* and *C. albicans* [7]. Therefore, it becomes interesting to analyze its effects on microbial biofilm.

*Glycyrrhiza glabra* L. (Fabaceae) is a native plant of the Mediterranean region, but it can develop in various parts of the world that have temperate climate [8]. His popular name in Brazil is “alçaçuz” (licorice). Its roots are indicated as responsible for lots of biological and therapeutic activities, such as antimicrobial activity for strains of *S. aureus*, methicillin-resistant *S. aureus* (MRSA), *Micrococcus luteus*, *Bacillus subtilis*, *E. coli*, *K. pneumoniae* and *P. aeruginosa* [9]. However, there are few studies on their antifungal activity.

*Punica granatum* L. (Punicaceae) had its origin recorded in the Mediterranean region [10]. In Brazil, it is called “romã” (pomegranate). Its antimicrobial activity is proven by numerous studies which suggest that the interaction of compounds of *P. granatum* L. with cell membrane of bacteria and yeast affect its integrity, promoting elimination of these microorganisms [11]. Researches with the peel and the flowers of this fruit proved its antifungal potential [12]. It was also observed antimicrobial activity of *P. granatum* L. on *B. subtilis*, *E. coli*, *Saccharomices cerevisiae* [13], *K. pneumoniae*, *Proteus vulgaris*, *Salmonella typhi* [14], *Bacillus cereus*, *B. coagulens*, *P. aeruginosa* [10] and it is of great interest to analyze its effects on biofilm.

*Stryphnodendron barbatimanum* Mart. (Leguminosae), is derived from Brazilian Cerrado and is popularly known as “barbatimão.” Its effectiveness was reported on *Streptococcus mutans*, *S. aureus*, *Actinobacillus actinomycetemcomitans*, *C. albicans*, *Cryptococcus neoformans* [15,16]. The extract of this plant can affect the integrity of its cell wall, decrease its ability to adhere, inhibit formation of its germ tube, interfere in the process of budding.
and stimulate phagocytosis by macrophages [17]. This is an extract of great importance for possible clinical applications in dental clinics.

The emergence of strains resistant to conventional antimicrobial use occurs constantly. So, it has become of great relevance to search for alternative methods that can eliminate these microorganisms. The use of medicinal plants is one of these methods, which have shown effective results in controlling pathogens. Thus, it was evaluated in the present study the antifungal activity of the extracts of *E. arvense* L., *G. glabra* L., *P. granatum* L. and *S. barbatimam* Mart. on *C. albicans* biofilm on acrylic resin.

**MATERIAL AND METHODS**

This study was approved by the Ethics Committee of the Faculty of Dentistry of São José dos Campos - UNESP / FOSJC according to the protocol 008/2010-PA/CEP.

**Experimental groups: products evaluated in biofilms of *C. albicans***

The extracts of *E. arvense* L., *G. glabra* L., *P. granatum* L. and *S. barbatimam* Mart. were prepared at a concentration of 200 mg/mL in propylene glycol, after acquisition of the dry powders from Oficina de Ervas (Ribeirão Preto, SP, Brazil). After preliminary tests on planktonic cultures of *C. albicans*, it was used at a concentration of 50 mg/mL on biofilms.

It was used the nystatin (100,000 IU/mL - Cristália, Itapira, SP, Brazil) as a control to compare the action of natural extracts. After preliminary tests on planktonic cultures of yeast, it was adopted the concentration of 48.83 IU/mL and untreated (Control - NaCl 0.9%). After 5 minutes of contact with the products, under agitation, the discs were washed again and subjected to ultrasonic agitator (Sonoplus HD 2200, Bandelin Electronic, Berlin, Germany) for 30 s in order to dissociate the biofilm. Then, it was obtained a suspension with the dissociated biofilm and from this suspension, four decimal dilutions were performed (1:10; 1:100; 1:1,000; 1:10,000) and 100 µL of each were plated in duplicate on Sabouraud-dextrose. After 48 h incubation (37 °C), plates containing 30-300 colonies were counted for colony forming units per milliliter (CFU/mL).

**Statistical Analysis**

The results were converted into log_{10}. Mean values and standard deviations were statistically analyzed by ANOVA, complemented by Tukey Test, with 5% significance (p ≤ 0.05), with the aid of statistical program BioEstat 5.0.
RESULTS

As it can be seen in Figures 1 and 2, the group treated with *E. arvense* L. showed reduction on *C. albicans* biofilm of $1.068 \pm 0.660 \log_{10}$, equivalent to 18% compared to the control group (0.9% NaCl). The group that received treatment with extract of *G. glabra* L. showed a reduction of 24% ($1.310 \pm 0.457$).

In the group treated with *P. granatum* L., reductions were of approximately 26% ($1.463 \pm 0.338$). The biofilms treated with *S. barbatimam* Mart. showed a reduction of approximately 27% ($1.549 \pm 0.418$) relative to nystatin antifungal, reductions averaged 21% ($1.257 \pm 0.265$). However, it was observed that the reductions in the biofilm of *C. albicans* presented in all treated groups were statistically similar ($p = 0.1567$).

**Figure 1** – Mean (± SD) of CFU/mL (log10) of the biofilm of *C. albicans* obtained in the untreated group (NaCl 0.9%) and treated groups with 50 mg/mL of extracts of *E. arvense* L. (Ea), *G. glabra* L. (Gg), *P. granatum* L. (Pg) and *S. barbatimam* Mart. (Sb) and nystatin (Nys) 48.83 IU/mL. In all groups there was a significant biofilm reduction when compared to the control group, non-treated ($p < 0.01$). There was no difference in the reduction between the treated groups ($p < 0.05$). ANOVA, Tukey Test ($p \leq 0.05$).

**Figure 2** – Reduction percentage of *C. albicans* biofilm after 5-minute treatment with the extracts of *E. arvense* L. (Ea), *G. glabra* L. (Gg), *P. granatum* L. (Pg) and *S. barbatimam* Mart. (Sb) and the antifungal nystatin (Nys). The reductions were statistically similar ($p > 0.05$). ANOVA, Tukey Test ($p \leq 0.05$).
DISCUSSION

Taking into consideration the time of exposure of the biofilm to treatment (5 minutes) with different plant extracts and their concentrations, all groups treated with extracts of *E. arvense* L., *G. glabra* L., *P. granatum* L. and *S. barbatimam* Mart. or nystatin exhibited significant reductions in biofilm of *C. albicans* (p < 0.05), compared to the untreated group (NaCl 0.9%) (Figures 1 and 2), however, these reductions were statistically similar (p > 0.05). Ishida et al. (2006) [17] also found that the antifungal activity of the extract fractions was similar to *Stryphnodendron adstringens* presented by the nystatin and fluconazole on *C. albicans* inhibiting the growth of the yeast.

Considering the clinical application or popular use of these products, it would be convenient to use them with less exposure time, such as 30 or 60 s for a mouth rinse, however, we extrapolated this period of exposure for five minutes, taking into consideration the possibility of adding the bioactive components of these plant extracts to dentifrices, for instance, where the exposure time is increased. In this in vitro study, we found effective reduction of the biofilm exposed to extracts for 5 minutes.

In preliminary tests on planktonic cultures of *C. albicans*, it was determined Minimum Fungicidal Concentration (MFC) of all extracts, i.e., 50 mg/mL [19]. Based on these results, we sought in this study to verify the effectiveness of this concentration on *C. albicans* biofilm, in vitro, being confirmed its fungistatic activity for the biofilm. Watamoto et al. (2009) [20] checked the action of some antifungals as amphotericin B, nystatin, caspofungin, ketoconazole and 5-flucytosin against *C. albicans* strains, including mutant strains, and also determined inhibitory concentrations for biofilm growth above the levels found in planktonic cultures for all antifungals studied.

The extracts were prepared in propylene glycol, however, when we checked its possible influence on the results of antimicrobial plant products, we found that the concentration of 50 mg/ml propylene glycol showed no antifungal effect for *C. albicans* planktonic cultures, therefore, we didn't continue the tests on biofilm.

It was also verified that nystatin, used as control in this study, presented antifungal action against planktonic cultures and its MFC was 48.83 IU/mL. This antifungal was commercially purchased at a concentration of 100,000 IU/mL and it was indicated for oral administration of 5 mL. However, we found convenient to check its action using the same MFC presented by plant extracts. Endo et al. (2012) [21] studied microparticles composed by the extract of *P. granatum* L. and observed active role on *C. albicans* planktonic and biofilm elimination, whose controls were nystatin and fluconazole. These authors also found that nystatin has presented the minimum inhibitory concentration (MIC) of 3.9 mg/mL while *P. granatum* L. in planktonic culture showed MIC of 3.1 mg/mL, demonstrating that the antifungal effect was similar between a product of known efficacy and a plant extract. Thus, we can see a correlation between the study of Endo et al. (2012) [21] and our study, regarding the similarity of the antifungal activity presented by plant extracts (including extract of *P. granatum* L.) and nystatin, which was used at a concentration equivalent to that of plant extracts.

Products from plants such as essential oils and extracts, have several chemical compounds which may exhibit various biological functions such as anti-inflammatory, antioxidant, anti-tumor, anti-allergic, antiviral, antibacterial, antifungal activity, etc. Thymol, a compound present in *E. arvense* L., showed antimicrobial activity [22]. Besides, it was observed strong antifungal action of this compound in combination with 1,8-cineole [23]. Glabridin [9], glycyrrhizin and 18-β glycyrrhetinic acid, *G. glabra* L. compounds [24,25] were shown to be responsible for the antimicrobial activity of the plant, and 18-β glycyrrhetinic acid showed inhibitory effect for *C. albicans* [26]. It has been demonstrated by studies that the antimicrobial...
antifungal activity similar to nystatin. The inhibitory effect of the extracts of S. barbatimam is also due to the presence of tannins [17] and gallic acid [29].

The ability of biofilm formation is a major virulence factor presented by C. albicans and it gives this microorganism greater chance of resistance to the action of antifungal agents when compared to planktonic growth [6,30]. Factors such as the presence of extracellular matrix, gene expression induced by intercellular contact and antioxidant capacity provide the biofilm of C. albicans high antifungal resistance [31,32]. The success of the formation of a mature biofilm is related to the adhesion to a surface, its colonization and survival, including the competition between different microorganisms [20].

It was demonstrated in this study that the analyzed natural products performed significantly control of C. albicans biofilm on acrylic resin and it was demonstrated that they can be as efficient as the use of conventional products, such as nystatin, commonly used in the treatment of candidosis. Thus, we believe that future studies should be conducted, related to development of products for dental use as mouthwashes or toothpastes, based on isolated active principles of plants studied here. Other research that verify in vivo application of these products should also be conducted to verify their biocompatibility in humans, targeting the therapeutic use of these plant extracts.

CONCLUSION

According to the results, it was concluded that the extracts of E. arvense L., G. glabra L., P. granatum L. and S. barbatimam Mart. were effective in reducing C. albicans biofilm on acrylic resin, presenting antifungal activity similar to nystatin.

REFERENCES


