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ORIGINAL ARTICLE

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Evaluation of the antifungal activity of plant extracts and oral antiseptics against *Candida albicans*

Avaliação da atividade antifúngica de extratos vegetais e antissépticos bucais em *Candida albicans*

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ABSTRACT

Objective: Oral candidiasis is the most common fungal infection of the oral cavity, and *Candida albicans* is the most frequently isolated species. **Material and Methods:** In this study, the potential antifungal effect of extracts from *Gossypium hirsutum* L., *Arctium lappa*, *Equisetum* sp., *Cecropia pachystachya* Trécul and *Pogostemon heyneanus* plants were evaluated on non-adhered cells of C. albicans, and the effect of oral antiseptics A (cetylpyridinium chloride 0.500 mg), B (chlorhexidine gluconate 0.12%), C (hydrogen peroxide 1.5%) and D (thymol, eucalyptol, menthol and methyl salicylate) were evaluated on non-adhered cells and biofilms of *C. albicans*, using turbidimetry and minimal inhibitory concentration (MIC) of the extracts and the maximal inhibitory dilution (MID) of the antiseptics. **Results:** The most promising results on non-adhered cells were obtained with *Cecropia pachystachya* Trécul extracts, with MIC values between 7.81 and 3.91 μg/mL. Antiseptics A and B showed the lowest MID values, between 0.20 and 0.10% (Kruskal-Wallis, $p < 0.0001$). Regarding biofilm inhibition, the MID values found were similar for the tested antiseptics, varying from 50% to 0.20% (Kruskal-Wallis, $p = 0.6915$). **Conclusion:** These results show that some plant extracts has potential use in the prevention and treatment of oral candidiasis.

KEYWORDS

Candida albicans; Oral antiseptics; Plant extracts; Antifungal activity; Biofilm.

RESUMO

Objetivo: A candidíase oral é a infecção fúngica mais comum na cavidade oral e *Candida albicans* é a espécie frequentemente relacionada. **Material e Métodos:** Neste estudo foi avaliado o potencial efeito antifúngico de extratos das plantas *Gossypium hirsutum* L., *Arctium lappa*, *Equisetum* sp., *Cecropia pachystachya* Trécul. e *Pogostemon heyneanus* sobre células não aderidas de *C. albicans* e o efeito dos antissépticos bucais A (cloreto de cetilpiridínio 0,500 mg); B (gluconato de clorexidina 0,12%); C (peróxido de hidrogênio 1,5%) e D (timol, eucaliptol, mentol e salicilato de metila) sobre células não aderidas e biofilmes de *Candida albicans*. A susceptibilidade dos isolados clínicos de *C. albicans* foi avaliada através da determinação da inibição de crescimento celular por turbidimetria e por determinação da Concentração Inibitória Mínima (CIM) dos extratos e da Máxima Diluição Inibitória (MDI) dos antissépticos. **Resultados:** Os resultados mais promissores dos testes com células não aderidas foram obtidos com extratos de *Cecropia pachystachya* Trécul., com valores de CIM entre 7,81 e 3,91 μg/mL. Os antissépticos A e B apresentaram os menores valores de MDI, entre 0,20 e 0,10% (Kruskal-Wallis, $p < 0,0001$). Em relação a inibição de formação de biofilmes, os valores de MDI encontrados foram similares para os antissépticos testados, variando de 50% a 0,20% (Kruskal-Wallis, $p = 0,6915$). **Conclusão:** Os resultados mostraram que todos os antissépticos bucais e extratos vegetais analisados apresentaram atividade antifúngica contra os isolados de *C. albicans*, e as espécies medicinais apresentam potencial uso na prevenção e tratamento de candidíase oral.

PALAVRAS-CHAVE

Candida albicans; Antissépticos bucais; Extratos vegetais; Atividade antifúngica; Biofilme.

INTRODUCTION

Candida albicans is considered the most frequent Candida species detected in human infections and can be isolated from the oral cavity of 25 to 50% of healthy individuals. This species also coexists with other saprophytic microorganisms of the oral microbiota [1], and local or systemic alterations can provide favorable conditions for the development of oral candidiasis [2].

Denture-induced stomatitis is highlighted among the various clinical forms of oral candidiasis. This condition is an inflammatory reaction of the alveolar and palatal mucosa caused by the continuous use of removable prostheses that show poor hygiene or improper adjustment. The prevalence of this type of infection ranges from 15 to 65% in users of total removable prostheses [3]. The rough surface of the prosthesis contributes to adhesion and biofilm formation by *C. albicans* [4], and such biofilms formed in a dental prosthesis serve as an important predisposing factor for oral candidiasis [5].

Antiseptic mouthwashes are composed of chemical substances that assist in the removal of oral biofilms and the control of microbial growth. Moreover, oral antiseptics disrupt microbial cell walls and block other enzymatic activities, in addition to preventing microbial aggregation and decreasing their multiplication [6]. As a result, these mouthwashes play an important role in the prevention and treatment of oral candidiasis. Chlorhexidine, cetylpyridinium chloride and essential oils are among the most common active compounds in antiseptic mouthwashes.

In addition to conventional and pharmacologically proven therapeutic measures, solutions based on natural products are used as antimicrobials [7], and medicinal plants constitute an important source of new biologically active compounds [8]. Associations between medicinal plants, dentifrices and oral antiseptics have been reported in commercial formulations, and various vegetal extracts are currently being tested with the aim of reducing the activity of microorganisms in the oral cavity [9,10].

Based on an ethnobotanical survey of plants used by the Brazilian population in the treatment of signs and symptoms related to fungal infections, which was performed by Fenner et al. [11], certain species were selected for evaluation regarding their potential antifungal effect against cell suspensions and biofilms of *C. albicans*. In addition, oral antiseptics were selected to evaluate their potential inhibitory effect on biofilms of *C. albicans*.

MATERIALS AND METHODS

1. Crude plant extracts

The species *Gossypium hirsutum* L., *Arctium lappa*, *Equisetum* sp., *Cecropia pachystachya* Trécul and *Pogostemon heyneanus* were selected to obtain crude extracts. The leaves were collected, dried and ground separately. The leaves were collected, oven-dried at temperature under 40 ºC, and powdered using knife mill. The powdered material was extracted by maceration using 95% etanol as solvent at room temperature. The extracts were then filtered and concentrated under vacuum in rotatory evaporator. All the extracts were kept in tightly stoppered bottles at 4 ºC until used for the biological tests.

2. Oral antiseptics

The oral antiseptics A (cetylpyridinium chloride 0.500 mg), B (chlorhexidine gluconate 0.12%), C (hydrogen peroxide 1.5%) and D (thymol, eucalyptol, menthol and methyl salicylate) were used.

3. Microorganisms

A total of 62 clinical isolates of *C. albicans* (oral mucosa: 25; vaginal mucosa: 6; blood: 20; urinary tract: 11), which belong to the collection

kept frozen at -70 ºC at the Center for Infectious Diseases (UFES), were used. The standard strain of *C. albicans* from the American Type Culture Collection (ATCC®), number 90028, was also used in biofilm experiments.

4. Biofilm

4.1 Inoculum preparation

The inoculum preparation was performed according to the methods of Pierce et al. [12], with some modifications. The isolates were cultured in Sabouraud dextrose agar (SDA) at 37 ºC for 24 h before each assay. With the aid of an inoculation loop, five colonies were collected, inoculated in a flask containing 20 mL of yeast extract-peptone-dextrose (YPD) broth and grown in an orbital incubator with 120 rpm at 35 ºC for 18 h (overnight). After this time, the suspension was centrifuged and the supernatant removed. The cells were then washed, and the pellet was resuspended in RPMI 1640 medium to obtain a suspension with a final concentration of 1.0 x 10^6 cells/mL. This concentration was equivalent to an optical density (OD) of 0.1 and was adjusted in a spectrophotometer at a wavelength of 600 nm.

4.2 Biofilm formation

Inocula (200 μL) were placed in 96-well microplates; RPMI 1640 medium was used as a negative control, and the standard ATCC® strain 90028 was used as a positive control. The plates were sealed and incubated in an incubator at 35 ºC for 24 h. All assays were performed in duplicate. After the incubation time, the medium was carefully aspirated, and the wells washed three times with sterile PBS buffer. Safranin dye (0.1%) was added and incubated for 15 min and then discarded. The plates were washed by immersion into distilled water and kept for 1 h at room temperature to dry. To solubilize the dye, ethanol-ether 97% (v/v) and PBS were added. The OD was measured using an ELISA reader at a wavelength of 492 nm. The average of the positive control values was established as the cut-off. The isolates that showed values (expressed as percentage) equal or higher than the average values of the positive control were considered biofilm producers, and those with lower values were considered non-producers.

5. Inhibition of biofilm formation with oral antiseptics

The ability of oral antiseptics to inhibit biofilm formation (anti-adhesion) was assessed in clinical isolates classified as biofilm producers. The oral antiseptics were individually tested in microplates and distributed in a two-fold serial dilution. After the addition of the inoculum, the plates were sealed and placed in an incubator at 37 ºC for 24 h. All assays were performed in duplicate.

6. Antifungal activity of plant extracts and oral antiseptics

The inoculum was obtained according to the broth dilution method of the document M27- A3 [13].

To determine the minimal inhibitory concentration (MIC) of the plant extracts, ten two-fold serial dilutions were performed in RPMI 1640 medium, starting from the concentration of 1.95 μg/mL. The assays were performed in duplicate. After incubation for 24 h at 35 ºC, the MIC of the extract was determined as the lowest concentration capable of totally inhibiting fungal growth. In parallel, the activity of the solvent dimethylsulfoxide (DMSO), used in the first solubilization of the extracts, was evaluated.

Ten twofold serial dilutions in RPMI 1640 medium, from solutions of predetermined concentrations of commercial products, were used to determine the maximal inhibitory dilution (MID) of the oral antiseptics. The MID values of the oral antiseptics were established when there was 100% inhibition of growth of the clinical isolates compared to the control. In cases in which there was partial inhibition of biofilm formation (less than 100%), the results were considered negative.

7. Data analysis

For analysis of the data, the nonparametric analysis of variance (ANOVA) (Kruskal-Wallis) was used with the aid of the GraphPad Prism 5.0b software. The significance level was established at 5%.

RESULTS

Biofilm

Among the 62 clinical isolates tested, only 19 isolates, which showed values greater than or equal to the average values of the positive control (standard strain), were considered biofilm producers.

Inhibition of biofilm formation was not observed in four isolates; four isolates were inhibited only by one antiseptic, and one isolate was inhibited by two antiseptics. The lowest MID values were obtained for antiseptic A, ranging from 0.39% and 0.20% for eight isolates, and between 25% and 12.5% for four clinical isolates. Antiseptic D showed MID vales between 0.39% and 0.20% for eight isolates, 3.13% for one isolate and 12.5% for two clinical isolates. The MID values of antiseptic B were between 0.39% and 0.20% for seven isolates, 1.56% for four isolates and 6.25% and 3.13% for two isolates. Antiseptic C demonstrated a MID value between 0.78% and 0.20% for ten isolates, 3.13% for one isolate and 50% and 25% for two clinical isolates.

Regarding the inhibition of biofilm formation, there were no significant differences among the oral antiseptics analyzed (Kruskal-Wallis, $p = 0.6915$).

Antifungal activity (MIC - μg/mL) of the plant extracts

Lower MIC values (between 7.81 and 3.91 μg/mL) were observed for the *C. pachystachya* Trécul extract (Figure 1). The *G. hirsutum* L. extract showed MIC values between 250 and 62.5 μg/mL, the *A. lappa* and *Equisetum* sp. extracts showed a MIC value of 250 μg/mL, and the *P. heyneanus* extract MIC values varied between 125 and 62.5 μg/mL. The solvent DMSO inhibited fungal growth until a concentration of 10% (v/v), which was equivalent to an extract concentration of 500 μg/mL; therefore, MIC values below this concentration were not considered to show interference from solvent action (Figure 3).

Antifungal activity (MID -% v/v) of the oral antiseptics

Antiseptics A and B showed lower MID values (Figure 2 and Figure 4), ranging between 0.20 and 0.10% (Kruskal-Wallis, $p < 0.0001$); antiseptic C showed varied MID values between 1.56% and 0.39%; and antiseptic D showed values between 6.25% and 0.39%.

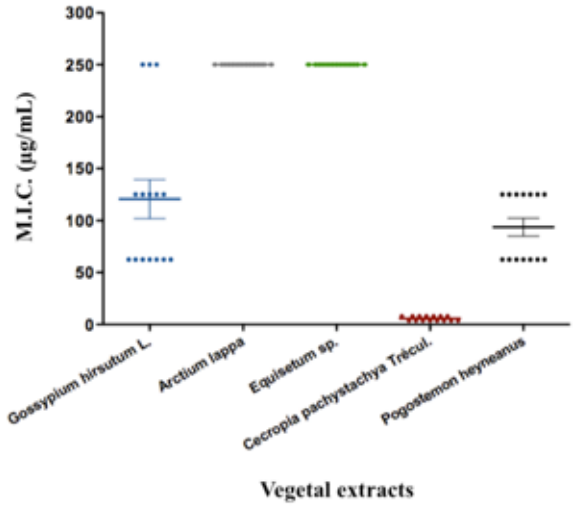

Figure 1 - MIC values (μg/mL) of the vegetal extracts

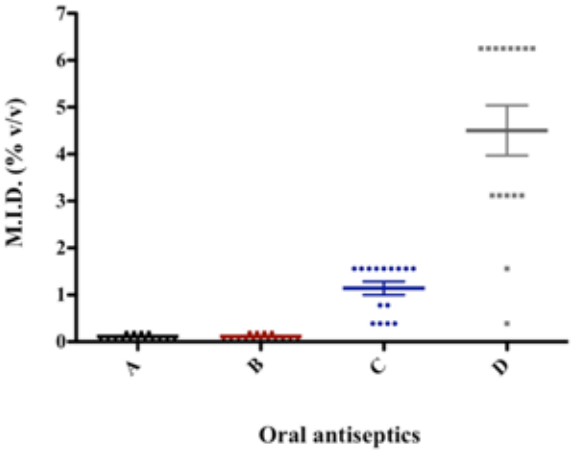

Figure 2 - MID values (%) of the oral antiseptics

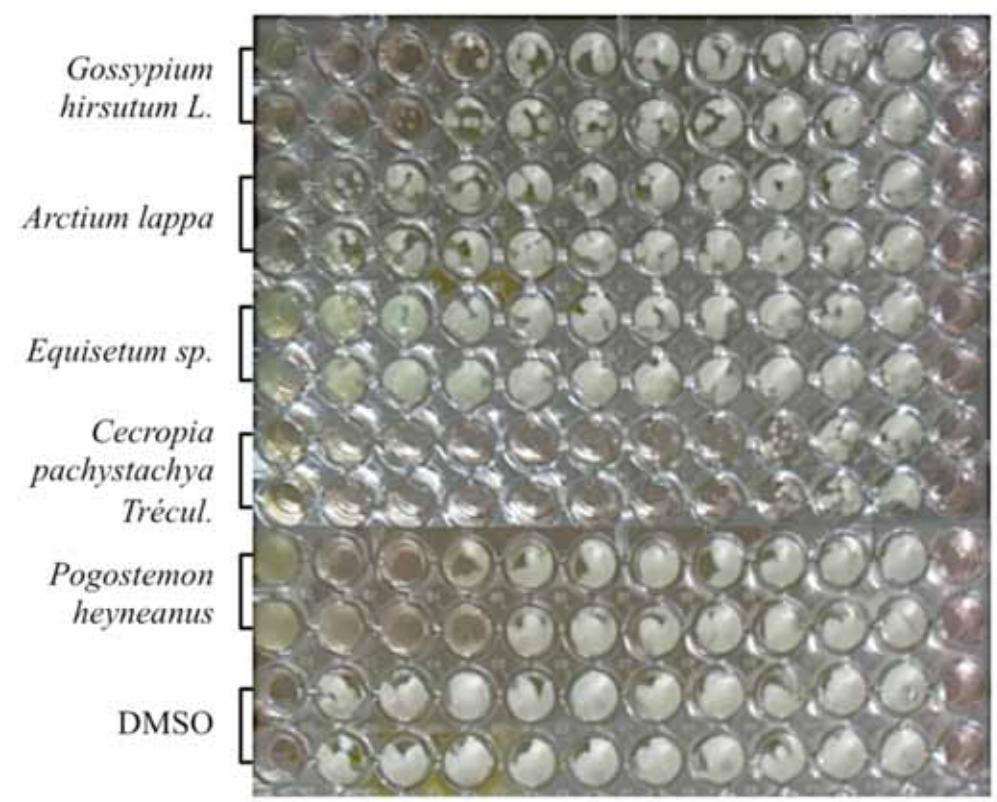

Figure 3 - Activity of the vegetal extracts

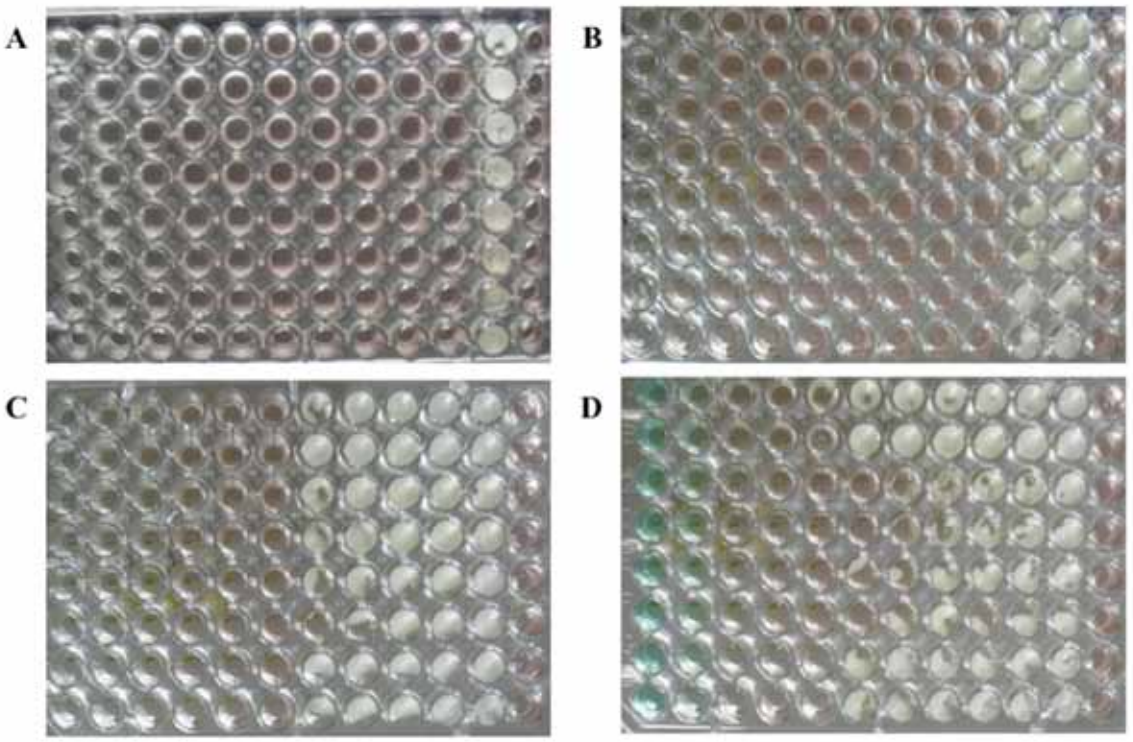

Figure 4 - Activity of the oral antiseptics: (A); (B); (C); (D)

DISCUSSION

Most studies concerning the antifungal activity of drugs or substances of natural origin are performed using plankton cells (non-adherent cells). Increasing data on resistance associated with biofilms highlight the need to evaluate chemical agents capable of preventing or reducing cell adhesion and biofilm formation, such that these compounds can be effectively used in the prevention and treatment of infections caused by *Candida* spp. [14].

In our study, all oral antiseptic analyses showed antifungal action against *C. albicans* isolates, with antiseptics A and B showing the best results with MID values between 0.20% (26.7% of the isolates) and 0.10% (73.3% of the isolates). The manufacturers of these antiseptics recommend their use without previous dilution, although in our study, the greatest MID value observed was 6.25%, which represents a product dilution of 1:16.

The antimicrobial action of cetylpyridinium chloride was previously reported in the literature [6,15], which confirms our results. In a study performed by Rocha et al. [16], the antiseptic containing this antimicrobial compound presented MIC values between 0.003 and 0.025 μg/mL for yeast isolated from the oral cavity of adults with HIV.

Matos et al. [17] reported MID values for a solution containing 0.12% chlorhexidine that varied between 0.2% and 0.1% for 72.7% of the *C. albicans* isolates analyzed. The antimicrobial effects of chlorhexidine on Candida spp. have previously been reported in the literature [2,18]. For example, Maekawa et al. [19] evaluated a chlorhexidine-based oral antiseptic without alcohol in comparison to a control antiseptic containing alcohol using *C. albicans* isolates and found no significant differences between the groups.

Studies regarding anti-adhesion activity have demonstrated the ability of an antimicrobial agent to control infections associated with biofilms formed on biological or artificial surfaces. However, although the ability of *C. albicans* to form hyphae is essential for biofilm development, not all clinical isolates are capable of forming biofilms at equivalent levels. In our study, the ability for biofilm formation was evaluated in 62 clinical isolates of *C. albicans*, among which only 19 were classified as biofilm producers when compared to the standard strain.

Regarding the inhibition of biofilm formation, we considered only MID values that inhibited 100% of biofilm formation. Our results revealed that the MID values of antiseptic A were between 25% and 0.20%; those of antiseptic D were between 12.5% and 0.20%; those of antiseptic B were between 6.25% and 0.20%; and those of antiseptic C were between 50% and 0.20%. Although the methodology used in our study was used to analyze the inhibition of biofilm biomass, the data show some agreement with studies evaluating the reduction of cell viability in biofilms.

Ramage et al. [18] showed that an oral antiseptic containing 0.2% chlorhexidine and essential oils (thymol, eucalyptol, menthol and methyl salicylate) had the ability to reduce the viability of *in vitro* pre-formed biofilms by 80% after washing for 30-60 seconds, according to the recommendations of each manufacturer. Other previous studies also reported similar data, showing that antiseptics containing chlorhexidine at 0.12% and essential oils were effective against fungal biofilms [20,21]. In addition, Pusateria et al. [20] showed that acrylic discs previously treated with chlorhexidine at 0.12% resulted in a significant reduction of *C. albicans* biofilm growth on their surface over the course of 72 hours of treatment.

All plant extracts evaluated showed antifungal action, even at the highest concentrations of cells. The most promising results were obtained with *C. pachystachya* Trécul. In addition, the antifungal activity observed with *A. lappa* in this study is consistent with the report by Ferracane et al. [23], which showed antifungal and antibacterial activity

in extracts from *Arctium* minus and *A. lappa*. Lubian et al. [10] and Cavalcanti et al. [24] also showed that the aqueous extract of A. minus had an *in vitro* fungistatic effect against oral species of Candida.

Glenh and Rodrigues [25] tested the potential effects of vegetal products on Candida sp. and observed antifungal activity of the hydroglycolic extract of *A. lappa*, which may have been related to the presence of active principle compounds such as tannins.

Yu-Cui et al. [26] evaluated the *in vitro* and in vivo antifungal activity of pogostone, a natural product isolated from *P. cablin* (Blanco) Benth, and this product showed potent *in vitro* activity against isolates of Candida spp.

Studies on the clinical application of vegetal extracts for the treatment of oral candidiasis have also shown promising results. For example, Sabzghabaee et al. [27] performed a randomized study with 80 patients who used 1% *Satureja hortensis* gel or placebo treatment of denture stomatitis for 14 consecutive days, and the intervention group showed significant improvement in mucosal erythema and Candida spp. colony counts. In another study, Sabzghabaee et al. [28] evaluated the efficacy of 1% *Pelargonium graveolens* gel and found that it was more effective than a placebo in the treatment of patients with denture stomatitis.

Adwan et al. [29] tested various formulations of herbal toothpaste and observed antifungal activity, especially in formulations containing plant extracts. In particular, synergistic interactions between the main components were considered important for this efficacy. Furthermore, the combination of sodium fluoride and plant extracts showed greater antifungal activity than these formulations alone against *C. albicans*.

The results obtained in this study show that constituents of the plant extracts evaluated can interfere with the growth and metabolism of *C. albicans*. Thus, studies involving the fractionation of potentially active plant extracts and in vivo testing are needed to confirm the potential therapeutic use of these species as an alternative and low-cost method in the dental clinic, specifically for the prophylaxis and treatment of oral candidiasis and the prevention of biofilm formation in dental prostheses.

CONCLUSION

The oral antiseptics analyzed showed antifungal activity against isolates of *C. albicans*, with the solutions of chlorhexidine gluconate and cetylpyridinium chloride showing the best results. In addition, all plant extracts showed inhibitory activity against *C. albicans*, particularly *C. pachystachya* Trécul. These results support the potential use of these compounds and medicinal species in the prevention and treatment of oral candidiasis.

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