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Antifungal activity of components used for decontamination of dental prostheses on the growth of *Candida albicans*

Atividade antifúngica de produtos utilizados na descontaminação de próteses dentárias sobre o crescimento de Candida albicans

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Resumo

Introdução: A efetividade de soluções antimicrobianas empregadas na descontaminação de próteses ainda é incerta. **Objetivo:** Avaliar a atividade antifúngica de soluções empregadas na descontaminação de próteses sobre o crescimento de *Candida albicans*. **Material e método:** Foram avaliados os produtos: Corega Tabs Branqueador® (S1), Hipoclorito de Sódio 1% (S2), Bicarbonato de Sódio 1% (S3), Peróxido de Hidrogênio 1% (S4), Digluconato de Clorexidina 0,12% - Periogard® (S5), Enxaguatório bucal a base de óleos essenciais - Listerine® (S6), e óleo essencial de *Rosmarinus officinalis* (alecrim) nas concentrações 1% (S7) e 2% (S8). A atividade antifúngica foi avaliada por meio da técnica de difusão em ágar e da determinação da curva de morte microbiana de amostras de *C. albicans* (ATCC 90028) na concentração $1,5\times10^6$ UFC/mL. Os testes foram realizados em triplicata e a análise estatística se deu pelos testes ANOVA Two-Way e Tukey, sendo adotado nível de confiança de 95%. **Resultado:** A média dos halos de inibição do crescimento, em milímetros, obtidos para os produtos foram: 0,0 (S1); 44,7 (S2); 0,0 (S3); 21,6 (S4); 10,0 (S5); 6,1 (S6); 0,0 (S7) e 2,4 (S8). Para curva de morte microbiana, todos os produtos apresentaram diferença estatisticamente significante ($p<0,05$) do grupo controle (cloreto de sódio 0,85%) e do grupo S3. Verificou-se crescimento fúngico inferior a 2×10^4 UFC/mL e acentuação na curva de morte microbiana após 30 minutos de ação, a exceção do grupo S3. **Conclusão:** As substâncias analisadas, a exceção do Bicarbonato de Sódio, possuem ação antifúngica frente *C. albicans*, podendo contribuir para higienização de próteses.

Descritores: Produtos com ação antimicrobiana; *Candida albicans*; descontaminação.

Abstract

Introduction: The effectiveness of antimicrobial solutions employed in dental prosthesis decontamination is still uncertain. **Aim:** To evaluate the antifungal activity of cleaners used in the decontamination of dental prostheses on the growth of *Candida albicans*. **Material and method:** The evaluated products were: Corega Tabs ® (S1), Sodium Hypochlorite 1% (S2), Sodium Bicarbonate 1% (S3), Hydrogen Peroxide 1% (S4), Chlorhexidine Digluconate 0.12% - Periogard® (S5), Mouthrinse based on essential oils - Listerine® (S6), essential oil from *Rosmarinus officinalis* (rosemary) at concentrations of 1% (S7) and 2% (S8). The antifungal activity of the products was evaluated by agar diffusion technique and the determination of microbial death curve of samples of *C. albicans* (ATCC 90028) in concentration 1.5×10^6 CFU/mL. The tests were performed in triplicate and statistical analysis was made by ANOVA Two-Way and Tukey tests, with the confidence level of 95%. **Result:** The average of the zones of inhibition growth, in millimeters, obtained for the products were: 0.0 (S1), 44.7 (S2), 0.0 (S3), 21.6 (S4), 10.0 (S5), 6.1 (S6), 0.0 (S7) and 2.4 (S8). Considering the determination of microbial death curve, all products showed a statistical difference ($p<0.01$) from control (0.85% sodium chloride) and S3 groups. Fungal growth less than 2×10^4 CFU/mL and an accentuation of the microbial death curve were observed after 30 minutes, with exception for S3 and control groups. **Conclusion:** The studied compounds, with the exception of Sodium Bicarbonate, have antifungal effect against *C. albicans*, which contribute for dental prostheses hygiene.

Descriptors: Products with antimicrobial activity; *Candida albicans*; decontamination.

INTRODUCTION

Candida spp. is a commensal yeast, which contributes to the normal microbial flora of the oral cavity, without necessarily causing infection[1,2]. However, during the loss of biological equilibrium, these microorganisms may lead to the development of oral candidiasis, which is considered one of the most common fungal infections to affect mankind. *Candida albicans, C. tropicalis* and *C. glabrata* are regarded as the most prevalent species in cases of oral candidiasis, contributing to 80% of all infections[1,2].

The use of prostheses increases the viability of yeast colonies in the oral cavity due to the affinity of *Candida* spp. for acrylic resin, to which it will adhere and colonize[3,4]. Effective oral and prosthesis hygiene is therefore essential for the maintenance of tissue viability and prevention of denture associated infections[5,6].

Topical antifungal medications, such as Nystatin and Myconazol, are recommended for the treatment of oral candidiasis, especially when associated with the use of dentures[5,7]. The use of cleaning solutions for prostheses is also recommended[5,7-9], however, they are generally only used in patients who have difficulties in maintaining the cleanliness of their dentures[10-12].

Patients with motor difficulties or inadequate mechanical hygiene are recommended to use adjuvant cleaning solutions[1,2,10-12], such as sodium hypochlorite, chlorhexidine digluconate, alkaline peroxides and enzymatic agents[8-12]. The objective of these solutions is to remove the biofilm and to prevent recolonization. However, chemical control of the biofilm must not substitute the use of mechanical methods such as brushing. The combination of these two methods contributes to an improved oral hygiene[10].

Sodium hypochlorite has the capacity to dissolve mucins and other organic substances in the biofilm matrix, inhibiting its formation and microorganism recolonization[13,14], yet promoting the degradation of the acrylic resin, depending on its concentration and immersion time[10].

Chlorhexidne digluconate is an effective wide spectrum antimicrobial, which has a bacteriostatic effect due to its capacity to precipitate and coagulate the microbial cytoplasm via low molecular weight components, such as phosphorus and potassium[15,16]. However, continued use may cause staining and alterations of color in the acrylic[17].

Alkaline peroxides are also frequently used as denture disinfectants. They are chemical agents composed of alkaline detergents and oxygen-releasing agents, such as trisodium phosphate and sodium percarbonate or perborate, respectively. Alkaline peroxides are available as tablets or in powder and when in solution they change into hydrogen peroxide, which is able to primarily attack the organic components of the deposits present in prostheses[8,9]. However, these products have been shown to be ineffective at inhibiting *Candida* spp. biofilms[9].

Although solutions for denture hygiene can inhibit *Candida* spp. activity[8,9,12], their routine use may bring some negative aspects, such as color change and lowered acrylic resistence[10,16]. The development of natural products with potential for clinical use is necessary to create new strategies to chemically control oral candidiasis, as well as to counteract the inconveniences of comercially available products[7]. It is important to highlight that studies using natural products may lead to cheaper and more practical treatment alternatives for oral diseases.

The biological activity of natural products is related to the composition of the phyto-constituents. Cavalcanti et al.[18] demonstrated that exposure to essential oils, which are liposoluble, can increase membrane permeability and cell death. In addition, the presence of components, such as terpene may contribute to the bacteriocide and fungiocide capacity of natural products[18,19]. Terpenes are hydrocarbonates of isoprene units, which are able to alter cell permeability and influence the respiratory chain cycle via enzyme inhibition[19]. Additionally, due to the presence of terpene, the essential oil of *R. officinalis* presents antimicrobial properties[19].

In view of the disadvantages of various chemical denture cleaners and the possibility of developing new products that utilize vegetable extracts, the objective of this study was to evaluate the antifungal activity of solutions for denture decontamination in terms of *C. albicans* growth.

MATERIAL AND METHOD

Antifungal activity of chemical cleaners was evaluated *in vitro* via agar diffusion and by determining the yeast cell death curve. The commercially available antimicrobial solutions and natural products tested included Corega Tabs®, 1% Sodium Hypochlorite (Milton Solution. Asfer-Ind. Química Ltda. São Caetano do Sul, SP, Brazil), 1% Sodium Bicarbonate, 1% Hydrogen Peroxide, 0.12% Chlorhexidine digluconate (Periogard®), essential oil-based mouthwash (Listerine®) and 1% and 2% *Rosmarinus officinalis* (rosemary) essential oil (Rosemary essential oil. Ferquima® Ind. e Com. Ltda. Vargem Grande Paulista, SP, Brazil). A 0.85% sodium chloride solution was used as a control. Table 1 describes the chemical cleaners evaluated and their respective active components.

Stock cultures of *C. albicans* (ATCC 90028) were reactivated and cultivated in Sabouraud-Dextrose broth (DIFICO®, Detroit, Michigan, USA), incubated at 37°C under atmospheric aerobic conditions for 18-20 hours (overnight). The cells were then centrifuged, rinsed and suspended in saline solution (0.85% NaCl) in order to determine the inoculum. The fungal suspensions were adjusted to MacFarland 0.5, corresponding to 0.1 optical density at a 600ηm wavelength. The approximate number of viable colonies from the inoculum was $1{,}5\times10^6$ UFC/mL.

Product activity was evaluated via agar diffusion. Fungal suspensions were plated in Sabouraud-Dextrose agar (DIFICO®, Detroit, Michigan, USA). Sterile paper discs impregnated with 20µL of the tested solution were distributed throughout the agar plates. Tests were performed in triplicate and the plates were kept at 37°C for 24 hours in a bacteriological incubator. Fungal growth inhibition zones were measured in millimeters, using a caliper, and descriptively analyzed.

The yeast cell death curve was determined via a kinetic test. The total viable microbial cells following contact with the

Table 1. Chemical cleaners and their respective active components

Chemical cleaner	Active component
Corega Tabs®	Sodium perborate, Potassium persulphate, Sodium polyphosphate
1% Sodium Hypochlorite	Sodium Hypochlorite
1% Sodium Bicarbonate	Sodium Bicarbonate
1% Hydrogen Peroxide	Hydrogen Peroxide
0.12% Chlorhexidine digluconate (Periogard®)	Chlorhexidine digluconate
Essential oil-based mouthwash (Listerine®)	Thymol, Eucalyptol, Sodium Fluoride 0.022%
1% *Rosmarinus officinalis* essential oil (rosemary)	1.8-Cineole, limonene, para-cymene, α-pinene, Camphor
2% *Rosmarinus officinalis* essential oil (rosemary)	1.8-Cineole, limonene, para-cymene, α-pinene, Camphor

solutions for a predetermined time was evaluated by quantitative analysis of viable yeast-like colonies. Sterile glass test tubes were filled with 1.8 mL of a double concentration of Sabouraud-Dextrose broth (DIFICO®, Detroit, Michigan, USA), 2 mL of the product to be tested and 0.2 mL of *C. albicans* solution at 1.5×10^6 UFC/mL.

Ten µL of the prepared solution was plated on Sabouraud-Dextrose agar (DIFICO®, Detroit, Michigan, EUA) after incubation for 0, 30 and 60 minutes. The plates were incubated for 24 hours at 37 °C for the quantitative analysis of viable colonies and determination of the total colony forming units per millimeter (CFU/mL) for each sample.

All tests were performed in three independents experiments with n=3 for each group for each experiment. The solutions tested were given codes in order for the study to be performed in a blind fashion in terms of data collection. The data regarding the growth inhibition zones were descriptively analyzed via the means.

The microbial death kinetic test data (total CFU/mL) was tabulated and logarithmically transformed in the GraphPad Prism 5.0 software (Program GraphPad for Windows, San Diego, CA, USA). The data presented a normal distribution. The antimicrobial solutions and times tested (0, 30 and 60 minutes) were considered study factors. The variable 'response to treatment' consisted of the viable colony forming units (UFC/mL) obtained from the subcultures.

Two-way analysis of variance (ANOVA) was used to verify the influence of the study variables. The test hypothesis (h1) was that the products and times would quantitatively influence the CFU of viable *C. albicans*. Interaction between the study variables was also investigated. The Tukey multiple comparison test was applied and described as 95% confidence intervals. In order to perform the Tukey test, the sample size and data distribution were confirmed as sufficient to reach a power of 80%, which is considered satisfactory.

RESULT

Table 2 describes the mean diameter of the growth inhibition zones in millimeters for the tested products against *C. albicans*.

Table 2. Mean diameter of growth inhibition zones, in millimeters, for the products tested against *C. albicans*

Product	Zone of inhibition (mm)
0.85% Sodium Chloride (Controle)	0.0 ± 0.0
Corega Tabs®	0.0 ± 0.0
1% Sodium Hypochlorite	44.7 ± 4.9
1% Sodium Bicarbonate	0.0 ± 0.0
1% Hydrogen peroxide	21.6 ± 2.5
0.12% Chlorhexidine digluconate (Periogard®)	10.0 ± 0.8
Essential oil-based mouthwash (Listerine®)	6.0 ± 1.8
1% *Rosmarinus officinalis* essential oil (rosemary)	0.0 ± 0.0
2% *Rosmarinus officinalis* essential oil (rosemary)	2.3 ± 3.2

Table 3 presents the ANOVA of the quantitative CFU of viable *C. albicans* colonies according to the study variables (antimicrobial solution and time). Following the quantitative viable colonies kinetic test, a significant effect was observed for both study variables. In addition, a significantly different interaction was demonstrated between the study variables.

Table 4 presents the means of the quantitative CFU of viable colonies per millimeter ($CFU/mL\times10^4$) for the products evaluated against *C. albicans*, at 0 (T0), 30 (T30) and 60 (T60) minutes. At T0, chlorhexidine and essential oil-based mouthwashes presented the largest reduction in total number of viable colonies ($p<0.05$). However, after 30 minutes, all solutions, with the exception of 1% Sodium Bicarbonate, presented similar antifungal potential in terms of quantitative viable *C. albicans* colonies ($p>0.05$).

DISCUSSION

The results of this study confirm the antifungal potential of peroxide-based chemical cleaners, sodium hypochlorite and the antimicrobial-based mouthwash solutions containing chlorhexidine and essential oils, which corroborate the findings

Table 3. Analysis of variance of the viable colony forming units of *C. albicans*, according to the study variables: antimicrobial solutions and time

Variable	Degrees of freedom	Sum of the squares	Mean of the squares	F	P
Interaction (Antimic. Sol. X Time)	16	5959	372.4	93.14	< 0.05
Antimicrobial solution	8	14753	1844	461.2	< 0.05
Time	2	5238	2619	655.1	< 0.05
Residual	54	215.9	3.998		

Table 4. Mean of the total Colony Formation Units per millimeter (CFU/mL x 10^4) of the evaluated products against *C. albicans*, at 0 (T0), 30 (T30) and 60 (T60) minutes

Product	T0	T30	T60
0.85 Sodium Chloride (Control)	39.0 ± 4.4 ^{aA}	42.0 ± 5.3 ^{aA}	42.0 ± 2.5 ^{aA}
Corega Tabs®	42.0 ± 3.6 ^{aA}	0.6 ± 0.5 ^{bB}	0.0 ± 0.0 ^{bB}
1% Sodium Hypochlorite	42.0 ± 2.2 ^{aA}	0.0 ± 0.0 ^{bB}	0.0 ± 0.0 ^{bB}
1% Sodium Bicarbonate	34.9 ± 2.8 ^{aA}	42.0 ± 2.9 ^{aA}	42.0 ± 2.3 ^{aA}
1% Hydrogen Peroxide	33.6 ± 3.9 ^{aA}	0.0 ± 0.0 ^{bB}	0.0 ± 0.0 ^{bB}
0.12% Chlorhexidine digluconate (Periogard®)	0.1 ± 0.2 ^{bA}	0.0 ± 0.0 ^{bA}	0.0 ± 0.0 ^{bA}
Essential oil-based mouthwas (Listerine®)	0.1 ± 0.1 ^{bA}	0.0 ± 0.0 ^{bA}	0.0 ± 0.0 ^{bA}
1% *Rosmarinus officinalis* essential oil (rosemary)	15.4 ± 2.6 ^{cA}	0.4 ± 0.2 ^{bB}	0.0 ± 0.0 ^{bB}
2% *Rosmarinus officinalis* essential oil (rosemary)	17.4 ± 1.9 ^{cA}	1.9 ± 0.7 ^{bB}	0.0 ± 0.0 ^{bB}

Different letters indicate a significant difference (p<0.05– Two way-ANOVA and Tukey). Small letters: products. Capital letters: Time.

from other studies[8,9,12,18]. Although other studies have widely evaluated the antimicrobial potential of these solutions against mature biofilms, the results of the present study suggests that these substances are capable of inhibiting biofilm formation. Additionally, it is important to highlight that many of these substances do not exhibit an immediate action, instead, a long contact time is required between the antimicrobial solution and the microorganisms present in the biofilm. The objective of the agar diffusion test was to identify which products presented the potential to inhibit the studied microorganism, without establishing a comparison between the measurments of the inhibiton zone, due to the different diffusion speeds of the products tested and the possibility of interference by the Sabouraud-Dextrose culture medium with substance diffusion.[20] In addition, the low sensitivity of the agar diffusion test resulted in differences in antifungal activity for some products in two microbiological tests (Tables 2 and 4).

The microbial death kinetic test is more reliable due to a greater contact between fungal cells and the test products, as well as a reduced interference by the culture medium. Additionally, it allowed a visual assessment of the magnitude of the antimicrobial potential at the different times for each solution tested.

The results of the present study confirmed that only 1% Sodium Bicarbonate did not demonstrate antimicrobial activity against *C. albicans*, whilst all other substances were capable of inhibiting the growth of *C. albicans.* In addition, time was an important factor in terms of substance activity, with the majority demonstrating a larger effect after 30 minutes. These results suggest that these products have a satisfactory antimicrobial potential, demonstrating an antimicrobial effect in both the removal of biofilms,[9,12-14] and the prevention of colonization by *C. albicans*[16,18,20-22].

The best result was shown for chlorhexidine and essential oil-based mouthwash solutions, which may be justified by their potent antimicrobial effects that interfere with cellular permeability and plasma membrane intergrity[15,16,18].

Sousa et al.[8] evaluated the effects of the disinfectant solutions chlorhexidine, sodium bicarbonate, vinegar and alkaline peroxide in terms of adherence of *C. albicans* to heat-activated acrylic resin. 0.12% chlorhexidine digluconate presented the best results, decreasing the number of adhered microorganisms by 86%, which implies that it is efficiently potent to eliminate contamination by *Candida* spp. The present study corroborates the results by Sousa et al.[8], thus confirming an inhibitory effect against *C. albicans*, even at T0, which means that the product has an immediate action, as shown in Table 4.

Chlorexidine is a cationic agent, which is adsorbed by oral surfaces and negatively charged microbes, interfering with osmotic equilibrium, the formation of acquired films and the microbial adsorption of oral surfaces. Therefore, it has the capacity to reduce biofilm formation and inhibit the synthesis of insoluble polysaccharides in the microbial matrix of the dental biofilm[3,13,14]. This biochemical behavior may be explained by its excellent antimicrobial activity and its clinical effectiveness

at inhibiting the proliferation of microorganisms and biofilm formation.

Vieira et al.[9] evaluated denture cleaning products in terms of recolonization of surfaces by *Candida* spp. biofilms. Biofilms containing *C. albicans* or *C. glabrata* were placed at the surfaces of denture reliners for 48 hours. The samples were then designated to treatment groups for cleaning: immersion in two alkaline peroxides for three or 15 minutes; immersion in 0.5% Sodium Hypochlorite for 10 minutes; or immersion in distilled water for 15 minutes. Sodium Hypochlorite was the only treatment that efficiently removed the biofilm, with no detection of viable colonies following its use. Alkaline peroxides were ineffective at removing *Candida* spp. form the surface of reliners, allowing recolonization of the specimens. Therefore, the present study corroborates the findings of Vieira et al.[9] in demonstrating the antifungal activity of 1% Sodium Hypochlorite when faced with *C. albicans*.

A similar study by Vieira et al.[9], which used peroxide-based solutions (Corega Tabs ® and 1% Hydrogen Peroxide), did not show an inhibitory effect at T0. However, after 30 minutes of contact with the microorganism suspension, a significant reduction in quantitative viable colonies of *C. albicans* was observed, confirming their antimicrobial activity. As discussed by Vieira et al.[9], alkaline peroxide solutions are more effective against biofilms when in constant use, mainly when dentures are immersed for a prolonged period.

The absence of antimicrobial activity in 1% Sodium Bicarbonate contradicts the results demonstrated by Sousa et al.[8], who confirmed the efficacy of this solution in reducing biofilm formation on acrylic resin surfaces. However, these differences can be explained by their use of a higher concentration of Sodium Bicarbonate, equivalent to 5%, compared to 1% in this study. Silhacek, Taake[21] demonstrated that Sodium Bicarbonate solution presents an inhibitory effect against microorganism in the oral cavity when used in high concentrations.

The essential oil *R. officinalis*, evaluated in this study, inhibited *C. albicans* activity, which corroborates the studies by Pozzatti et al.[20] and Cavalcanti et al.[18], who evaluated its antifungal activity *in vitro* in order to determine the minimum inhibitory concentration (MIC) of *Candida* spp., in both adherence inhibition tests and biofilm formation on the acrylic surface. The same authors also determined the MIC of the essential oil *R. officinalis* at low concentrations (1 mg/mL[18] and 0.56 mg/mL[20]), which demonstrated adherence inhibition[18]. Menezes et al.[22] demonstrated that natural products with a MIC of less than 100 mg/mL presented acceptable antimicrobial activity, with the potential for pharmacological use[22]. The present study also demonstrated the antifungal activity of this natural product, which, due to its inhibitory effect at low concentrations, has the potential for clinical use, such as for decontamination of dentures.

Cavalcanti et al.[18] evaluated the antifungal, anti-adherence and morphological effects the essential oil *R. officinalis* on *C. albicans* strands. The authors confirmed its inhibitory effect at a concentration of 0.56 mg/ml, similar to nystatin. Therefore, the results of the present study corroborate the literature in confirming the antifungal activity of the essential oil *R. officinalis*. Sikkema et al.[19] confirmed that the fungicide and bactericide action of the essential oil based on *R. officinalis* on *C. albicans* strands occurs due to the high concentration of terpene in the plant. This substance is capable of reducing cell growth by enzymatic inhibition, as well as microbial death by increasing cell permeability[19].

The results of the present study suggest that substances used for denture decontamination presented satisfactory antimicrobial activity, potentially inhibiting colonization by *C. albicans*. Time was also confirmed at an important factor in the action of these products, with the need for dentures to stay in contact with the antimicrobial solutions for a longer time in order to guarantee their effect. Further studies are needed, however, in order to compare the antifungal activity of these products against other microorganisms, evaluating the efficacy of these substances at preventing biofilm formation on the surface of dentures[12].

It is also important to highlight the effect observed by the natural product-based solutions. Thus, further studies must be performed in order to compare the antimicrobial potential of these products against other commercially available products. Research that focuses on the application of natural products, as antimicrobial agents are important in terms of their benefits to health, aiming to minimize the adverse effects that are found with currently used products.

CONCLUSION

The substances evaluated, with the exception of 1% Sodium Bicarbonate, have antifungal action against *Candida albicans*, which may be beneficial towards denture hygiene. A longer contact time between antimicrobial solutions and microorganisms may enhance antimicrobial effect.

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CONFLICTS OF INTERESTS

The authors declare no conflicts of interest.

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ERRATUM

Due to a desktop publishing error the article "Antifungal activity of components used for decontamination of dental prostheses on the growth of Candida albicans", published on volume 43, issue 2, 2014, had one of its authors names misspelled, please find below the correct authors names list.

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