Antifungal activity of propolis against Candida species isolated from cases of chronic periodontitis

Abstract: This research evaluated the fungistatic and fungicidal activities of red propolis alcoholic extract (RPAE) against different Candida species isolated from chronic periodontitis cases, and compared with chlorhexidine (CHX). Nineteen samples of Candida species (C. albicans \([n = 12]\), C. tropicalis \([n = 5]\) and C. glabrata \([n = 2]\)) isolated from chronic periodontitis cases were analyzed. The fungistatic and fungicidal activity of both RPAE and CHX were evaluated using fluconazole and C. parapsilosis (ATCC 6258) as a control. Fungistatic activity was analyzed based on the Clinical and Laboratory Standards Institute (CLSI) reference procedure to determine the minimum inhibitory concentrations. Fungicidal activity was established according to the absence of fungal growth on Sabouraud Dextrose Agar medium. The fungistatic and fungicidal activities of RPAE were observed, respectively, at 32-64 μg/mL and 64-512 μg/mL for C. albicans, 64 μg/mL and 64-256 μg/mL for C. glabrata, and 32-64 μg/mL and 64 μg/mL for C. tropicalis. CHX fungistatic activity was observed at concentrations of 0.003-1.92 μg/mL for C. albicans, 1.92 μg/mL for C. glabrata, and 0.03-1.92 μg/mL for C. tropicalis. Fluconazole fungistatic activity ranged between 1-64 μg/mL, and fungicidal activity occurred at 8-64 μg/mL, for the three Candida species analyzed. All the Candida species were susceptible to RPAE antifungal activity, but five samples of C. albicans, one of C. tropicalis and one of C. glabrata were resistant to fluconazole antifungal activity. CHX showed fungistatic activity against all the Candida species analyzed. The antifungal potential of these substances suggests that they can be applied as an alternative treatment for diseases affected by these species.

Keywords: Candida; Propolis; Chlorhexidine; Periodontitis.

Introduction

The presence of fungi, bacteria and viruses in dental biofilm can contribute to the progression of periodontal disease. Some authors have reported the prevalence of bacteria associated with periodontitis, but other studies have related the presence of fungi such as Candida. These fungi form part of an individual’s microbiota, especially in areas of the mucosa, such as the oral cavity, and play an important role both in health and in the development of oral diseases.
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Subgingival colonization by *C. albicans*, *C. dubliniensis*, *C. glabrata* and *C. tropicalis* has been described by other authors. Tamai et al. found that *C. albicans* may boost the infectious effect of periodontitis by acting in conjunction with anaerobic bacteria. Individuals susceptible to primary infection have been reported to use an antifungal prophylaxis to prevent cases of recurrence. Among the different chemical agents used, chlorhexidine has broad-spectrum antiseptic action. It is used in maintaining periodontal therapy, and as a treatment adjuvant in scaling and root planing procedures. The fungistatic activity of 0.12% chlorhexidine has also been reported as preventing the growth of *C. albicans* on denture acrylic resin.

The antimicrobial activities of natural derivatives, such as propolis, have been researched over recent years as alternatives for new therapeutic strategies. The presence of flavonoids, as well as phenolic, aromatic and diterpene acids, in the composition of propolis, has been associated with various biological attributes, including its antifungal properties.

The antifungal activity of Brazilian red propolis against different *Candida* species has already been reported in the literature. This propolis variety is obtained from exudates collected by bees (*Apis mellifera*) from the surface of *Dalbergia ecastophyllum* in the Northeast of Brazil, and contains a high concentration of flavonoids. The fungistatic activity of 0.12% chlorhexidine has also been reported as preventing the growth of *C. albicans* on denture acrylic resin.

The antifungal activity of Brazilian red propolis alcoholic extract, originally from Paraiba State, Brazil, proved effective against *Trichophyton tonsurans*, *T. rubrum* and *T. mentagrophytes*; however, the present study is the first to investigate the antifungal activity of red propolis against *Candida* species isolated from periodontal pockets.

The chemical composition of red propolis alcoholic extract indicates a predominance of flavonoids, such as rutin, liquiritigenin, daidzein, pinobanksin, quercetin, luteolin, dalbergin, isoliquiritigenin, pinocembrin, pinobanksin-3-acetate, biochanin A and formononetin, the last being the predominant component.

Thus, the aim of this study was to evaluate the *in vitro* fungistatic and fungicidal activities of Brazilian red propolis alcoholic extract against the *Candida* species isolated from cases of periodontitis. Its effects were compared with those of chlorhexidine, an agent considered the gold standard for antifungal periodontal treatment.

### Methodology

#### Fungi samples

*Candida* samples (twelve isolates of *C. albicans* [63%], five of *C. tropicalis* [26%] and two of *C. glabrata* [11%]) were obtained from periodontal pockets of patients with chronic periodontitis seen in the *Faculdade de Odontologia* of the Centro Universitário Cesmac [Clinical Dental School of the Cesmac University Center]. All isolates were preserved in mineral oil; taxonomically confirmed, and then seeded on CHROMagar Candida® (BD, Sao Paulo, Brazil) in a Petri dish kept at 37°C for 48 h. A sample of *C. parapsilosis* (ATCC 6258) was used as the control.

This study was approved by the Research Ethics Committee of Centro Universitário Cesmac, Alagoas, Brazil (protocol number: 723/09).

#### Evaluation of antifungal activity

The antifungal agents evaluated were fluconazole (Pfizer®, Sao Paulo, Brazil), chlorhexidine and red propolis alcoholic extract (lot code PRDE0906 – Pharmanéctar®, Belo Horizonte, Brazil). According to the certificate of analysis provided by the manufacturer of the red propolis alcoholic extract, the chemical composition of the product is consistent with that found by Daugsch et al. The procedure was performed according to standards published in document M27-A3 by the Clinical and Laboratory Standards Institute (CLSI). The culture medium used was bicarbonate-free RPMI 1640 (Sigma-Aldrich, St. Louis, USA) with L-glutamine, buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) (Sigma-Aldrich, St. Louis, USA). The medium was sterilized by filtration through a 0.22 µm Millipore membrane.

The commercial antifungal agent was dissolved in sterile distilled water and prepared in concentrations ranging from 0.125 to 64 µg/mL. The lyophilized red propolis alcoholic extract, originally from Paraiba (Brazil’s Northeast region), was solubilized in 70% ethyl alcohol (v/v), according to Sawaya et al., to obtain concentrations of from 4 to 2048 µg/mL. Chlorhexidine was prepared at concentrations from 0.003 to 1.92 µg/mL, corresponding to 0.000003 and 0.000192%, respectively.
All *Candida* isolates were plated on Sabouraud Dextrose Agar medium contained in test tubes and maintained at 28°C (± 1°C), for 48 h, to standardize the inoculum. Thereafter, suspensions were prepared in 5 ml of 0.145 mol/L sterile saline solution, then vortexed for 15 seconds. The cell density was adjusted to an equivalent of 0.5 on the McFarland scale and standardized in a spectrophotometer at 530 nm to obtain 90% transmittance. The suspensions were then diluted sequentially in a RPMI 1640 liquid medium to obtain concentrations of 1:100 to 1:20, resulting in a concentration of 2-5 x 10³ cells/mL.

Sterile microtiter plates (TPP, Trasandingen, Switzerland) containing 96 U-wells were used to perform the test procedure. 100 µL of fluconazole, red propolis and chlorhexidine were deposited on separate plates, in rows from 1 to 10, and each concentration was deposited in a row. 100 µL of RPMI 1640 medium was deposited in well rows 11 and 12, in which the controls and the sterilization medium were grown, respectively. These plates were stored at -20°C until use.

At test time, 100 µL of standardized inoculum was deposited in each well, and the microtiter plates were incubated at room temperature (28 ± 1°C) for 48 h until the interpretation of the results. The determination of the minimum inhibitory concentrations (MIC) of fluconazole, red propolis and chlorhexidine was performed by visual observation of each well to identify the reduction in fungal growth. Considering the total growth (100%) in the control well, the percentage growth reduction was attributed to the remaining wells.

The MIC for fluconazole was regarded as the lowest concentration, inhibiting growth by ≥ 50%. The interpretive CLSI breakpoints for fluconazole were: susceptible [S]: ≤ 8 µg/mL; susceptible dose dependent [SDD]: from 16 to 32 µg/mL; and resistant [R]: ≥ 64 µg/mL. The MICs for chlorhexidine and red propolis were regarded as the lowest concentrations, inhibiting fungal growth by 100%.

The minimum fungicidal concentration (MFC) was determined as follows: the contents of the wells showing 100% inhibition of fungal growth were transferred from the microtiter plate to Petri dishes containing Sabouraud Dextrose Agar medium. The dishes were kept at room temperature (28 ± 1°C) for 72 h to determine the development of *Candida* colonies. The MFC corresponded to the lowest fungistatic concentration preventing fungal growth. The MIC and MFC of fluconazole, red propolis and chlorhexidine were determined, as well as the concentration capable of inhibiting either half or all of the fungal growth (MIC₅₀ and MIC₅₀₀ respectively).

### Results

All the samples of *C. albicans*, *C. tropicalis* and *C. glabrata* were confirmed in regard to aspects of purification and taxonomy. No fungistatic activity of fluconazole was observed for any isolate of any species analyzed; for this reason, these results are not shown in Table 1. Sensitivity to fluconazole was observed

<table>
<thead>
<tr>
<th><em>Candida</em> species (number of samples tested)</th>
<th>Antifungal (growth inhibition)</th>
<th>MIC₅₀ (µg/mL)</th>
<th>MIC₅₀₀ (µg/mL)</th>
<th>MIC₅₀₀₀ (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Candida albicans</em> (11)</td>
<td>Fluconazole</td>
<td>1 - 32</td>
<td>4</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>Red propolis</td>
<td>32 - 64</td>
<td>32</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>Chlorhexidine</td>
<td>0.003 - 1.92</td>
<td>0.12</td>
<td>1.92</td>
</tr>
<tr>
<td><em>Candida glabrata</em> (1)</td>
<td>Fluconazole</td>
<td>2</td>
<td>NDᵃ</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Red propolis</td>
<td>64</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>Chlorhexidine</td>
<td>1.92</td>
<td>1.92</td>
<td>1.92</td>
</tr>
<tr>
<td><em>Candida tropicalis</em> (4)</td>
<td>Fluconazole</td>
<td>1 - 8</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Red propolis</td>
<td>32 - 64</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>Chlorhexidine</td>
<td>0.003 - 1.92</td>
<td>0.48</td>
<td>1.92</td>
</tr>
</tbody>
</table>

ᵃ MIC: Minimum Inhibitory Concentration
ᵇ MIC₅₀: Minimum inhibitory concentration able to inhibit 50% of the *C. albicans*, *C. glabrata* and *C. tropicalis* samples.
ᶜ MIC₅₀₀: Minimum inhibitory concentration able to inhibit 100% of the *C. albicans*, *C. glabrata* and *C. tropicalis* samples.
ᵈ ND: Not determined
in nine isolates of *C. albicans*, four of *C. tropicalis* and one of *C. glabrata* (MIC ≤ 8 μg/mL). Two isolates of *C. albicans* were SDD, demonstrating MICs of 16 and 32 μg/mL. In addition, five samples of *C. albicans*, one of *C. tropicalis* and one of *C. glabrata* were resistant to the fungicidal activity of fluconazole.

The fungicidal activity of chlorhexidine was observed in concentrations of 0.003-1.92 μg/mL, 1.92 μg/mL and 0.03-1.92 μg/mL for *C. albicans*, *C. glabrata* and *C. tropicalis*, respectively.

The fungicidal activity of red propolis alcoholic extract was 32-64 μg/mL for *C. albicans*, 64 μg/mL for *C. glabrata* and 32-64 μg/mL for *C. tropicalis*. The fungicidal activity was 64-512 μg/mL for *C. albicans*, 64-256 μg/mL for *C. tropicalis*, and 64 μg/mL for *C. glabrata*.

The fungicidal activity of red propolis alcoholic extract and of chlorhexidine was observed for all Candida species tested. The MFC was 0.003 μg/mL for chlorhexidine. The results are shown in Tables 1 and 2.

**Discussion**

The frequency rates of *C. albicans*, *C. tropicalis* and *C. glabrata* in oral infections have been previously reported by other authors, who confirmed the susceptibility of the Candida species associated with chronic periodontitis to fluconazole. Similar results were observed in the present study.

The variation in the MICs obtained for fluconazole for samples of *C. albicans* (≥ 64 μg/mL, and a resistance profile) have been previously reported by other authors. The decrease in the susceptibility profiles of non-*albicans* samples to fluconazole may be attributed to this antifungal agent, used both prophylactically and as a treatment. A national survey in Taiwan showed increasing rates of fluconazole resistance, from 1.9% in 2002 to 17.1% in 2006, mainly among non-blood or non-*albicans* isolates.

A similar susceptibility of *C. albicans* to Brazilian red propolis alcoholic extract in different concentrations (32-512 μg/mL) was observed in this study for *C. albicans* to Mexican propolis (at higher concentrations of 0.06 to 32 mg/mL), unlike the susceptibility of *C. glabrata* to Iranian propolis (approximately 5000 μg/mL) and of *C. tropicalis* to Chilean propolis (197-476 μg/mL).

Sforcin et al. reported that *C. albicans* was more susceptible to propolis from Sao Paulo (Brazil) than *C. tropicalis*. Our results were similar for MIC<sub>50</sub>; however, in regard to fungicidal activity, the susceptibility of *C. tropicalis* was higher. The antifungal effect of Brazilian propolis from the northeastern region, as observed in this study, is consistent with the findings of other studies that used Brazilian propolis from the southeastern region; however, the antifungal activity was observed at higher concentrations for the propolis from the Southeast of Brazil (between 3 and 12 μg/mL).

Most biological activities of propolis are related to the alcoholic extract, because when the extract is prepared in ethyl alcohol, a greater amount of active compounds are extracted and the inhibitory effect is greater. When water is used as the solvent, the antifungal and antibacterial activities are weaker, compared with the alcoholic extract.

**Table 2.** Mean results of fungicidal activity of fluconazole and red propolis alcoholic extract against Candida species isolated from cases of chronic periodontitis.

<table>
<thead>
<tr>
<th>Candida species (number of samples tested)</th>
<th>Antifungal (growth inhibition)</th>
<th>MFC&lt;sub&gt;s&lt;/sub&gt; (μg/mL&lt;sup&gt;a&lt;/sup&gt;)</th>
<th>MFC&lt;sub&gt;50&lt;/sub&gt; (μg/mL&lt;sup&gt;b&lt;/sup&gt;)</th>
<th>MFC&lt;sub&gt;100&lt;/sub&gt; (μg/mL&lt;sup&gt;c&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Candida albicans</em> (12)</td>
<td>Fluconazole</td>
<td>8 - 64</td>
<td>32</td>
<td>ND&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Red propolis</td>
<td>64 - 512</td>
<td>ND</td>
<td>512</td>
</tr>
<tr>
<td><em>Candida glabrata</em> (5)</td>
<td>Fluconazole</td>
<td>8 - 32</td>
<td>16</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Red propolis</td>
<td>64 - 256</td>
<td>ND</td>
<td>256</td>
</tr>
<tr>
<td><em>Candida tropicalis</em> (2)</td>
<td>Fluconazole</td>
<td>64</td>
<td>64</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Red propolis</td>
<td>64</td>
<td>64</td>
<td>ND</td>
</tr>
</tbody>
</table>

<sup>a</sup> MFC: Minimum Fungicidal Concentration  
<sup>b</sup> MFC<sub>50</sub>: Minimum fungicidal concentration able to inhibit 50% of the *C. albicans*, *C. glabrata* and *C. tropicalis* samples.  
<sup>c</sup> MFC<sub>100</sub>: Minimum fungicidal concentration able to inhibit 100% of the *C. albicans*, *C. glabrata* and *C. tropicalis* samples.  
<sup>d</sup> ND: Not determined
Flavonoids are largely responsible for antifungal activity. The susceptibility of different Candida species is dependent on the chemical composition of the propolis; however, Sforcin et al. found no change in the susceptibility of C. albicans and C. tropicalis. Thus, the fungicidal activity of red propolis alcoholic extract found in this study could be attributed to its chemical composition.

The use of propolis in dentistry has been proposed as an aid in the treatment of oral infections. Koo et al. evaluated the antimicrobial activity of red propolis alcoholic extract from Minas Gerais, southeastern Brazil (10% w/v) and concluded that propolis may have an effect on periodontal disease, because of its antifungal activity against C. albicans and other microorganisms, and because it inhibits the formation of biofilm in vitro.

Chlorhexidine is known to have excellent antifungal activity in vitro against all isolates of C. albicans, C. glabrata and C. tropicalis. Furthermore, fungistatic and fungicidal activities were found at a concentration of 1.92 µg/mL. Dodwad and Kukreja found that chlorhexidine (0.2%) can inhibit dental biofilm formation better than propolis contained in mouthwash solutions. The fungistatic activity of chlorhexidine has also been reported recently in the literature. Whereas the MIC for different Candida species, including C. albicans, C. glabrata and C. parapsilosis, was 6.25 mg/L, our results showed an activity of 1.92 µg/mL. Ellepola et al. found that chlorhexidine interferes with the formation of the germ tube of C. albicans, in subtherapeutic concentrations (0.00125, 0.0025 and 0.005%). According to Pizzo et al., chlorhexidine causes changes in the epithelial cell surface of the oral mucosa, thus interfering in the colonization of different Candida species, and indicating a reduction in the adhesion of these fungi.

**Conclusion**

The results of this study show that, similar to chlorhexidine, red propolis alcoholic extract has good fungistatic and fungicidal activity against most samples of Candida species. This antifungal activity may hold a promise for future applications as an alternative treatment for infections caused by these fungi. Further investigation into the use of red propolis for the prevention and treatment of periodontal diseases is required, including microbiological, randomized controlled trials and longitudinal studies.

**References**

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