Antimicrobial and antioxidant activity of hydroalcoholic extracts of bee propolis (Tetragonisca angustula) and/or Calendula officinalis and potential use in mouthwash formulation

Atividade antimicrobiana e antioxidante de extratos hidroalcoólicos de propolis de abelha (Tetragonisca angustula) e/ou Calendula officinalis e potencial uso na formulação de enxaguantes bucais

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ABSTRACT
Calendula officinalis and propolis extracts have a wide range of biological activities, including antimicrobial action against a variety of pathogenic microorganisms. The aim of this study was to produce a mouthwash containing extracts of propolis and/or C. officinalis, with antimicrobial and antioxidant activity against pathogenic microorganisms implicated in periodontitis. The antimicrobial activity of extracts was evaluated based on the well diffusion assay. Total phenols, flavonoids and radical...
scavenging activity were evaluated by spectrophotometric method. The extract that showed greater antioxidant and antimicrobial potential against bacterial strains isolated from patients with periodontal disease were used for the composition of five mouthwash formulations. Thus, *Staphylococcus aureus*, *Streptococcus salivarius* and *Streptococcus pneumoniae* were used as antimicrobial activity indicator microorganisms. *Staphylococcus aureus* ATCC 25923, *Streptococcus mutans* ATCC 25175 and *Enterococcus faecalis* ATCC 29212 were used as control group. Five mouthwash formulations were developed, differing in the addition or not of raw propolis extract (20%), chlorhexidine digluconate or sodium fluoride (0.05%). The results indicate that propolis extract was more efficient than the calendula extract with antimicrobial action superior compared to azithromycin against *S. pneumoniae*, isolated from patients with stage III/IV periodontitis. The propolis extract had a higher flavonoid content (0.288 ± 0.038 mg EQ/g) than marigold extract. The F5 formulation containing chlorhexidine was more efficient in inhibiting Gram-positive pathogens, followed by formulations no. 2 and no. 4 which contained propolis extract. In this perspective, the propolis extract presented not only antimicrobial activity, but also antioxidant activity. Thus, the use of propolis in pharmaceutical formulas, such as the one presented here, could be especially useful in oral hygiene, contributing to the prevention of periodontitis.

**Keywords:** mouthwash. *streptococcus pneumoniae*, azithromycin, periodontal disease.

**RESUMO**

Extratos de *Calendula officinalis* e própolis possuem uma ampla gama de atividades biológicas, incluindo ação antimicrobiana contra uma ampla gama de microrganismos patogênicos. O objetivo deste estudo foi produzir um enxaguante bucal contendo extratos de própolis e/ou *C. officinalis*, com atividade antimicrobiana e antioxidante contra microrganismos patogênicos associados à periodontite. A atividade antimicrobiana dos extratos foi avaliada com base no ensaio de difusão em poço. Fenóis totais, flavonoides e atividade sequestradora de radicais foram avaliados por método espectrofotométrico. O extrato que apresentou maior potencial antioxidante e antimicrobiano contra cepas bacterianas isoladas de pacientes com doença periodontal foi utilizado para a composição de cinco formulações de enxaguantes bucais. Assim, *Staphylococcus aureus*, *Streptococcus salivarius* e *Streptococcus pneumoniae* foram utilizados como microrganismos indicadores de atividade antimicrobiana. *Staphylococcus aureus* ATCC 25923, *Streptococcus mutans* ATCC 25175 e *Enterococcus faecalis* ATCC 29212 foram usados como grupo controle. Foram desenvolvidas cinco formulações de enxaguantes bucais, diferindo na adição ou não de extrato de própolis bruto (20%), digluconato de clorexidina ou fluoreto de sódio (0,05%). Os resultados indicam que o extrato de própolis foi mais eficiente que o extrato de calendula, com ação antimicrobiana superior à azitromicina contra *S. pneumoniae*, isolado de pacientes com periodontite estágio III/IV. O extrato de própolis apresentou maior teor de flavonoides (0,288 ± 0,038 mg EQ/g) do que o extrato de calêndula. A formulação F5 contendo clorexidina foi mais eficiente na inibição de patógenos Gram-positivos, seguida das formulações n. 2 e n. 4 que continham extrato de própolis. Nesta perspectiva, o extrato de própolis apresentou não apenas atividade antimicrobiana, mas também atividade antioxidante. Dessa forma o uso de própolis em fórmulas farmacêuticas, como a aqui apresentada, poderia ser especialmente útil na higiene bucal, contribuindo para a prevenção da periodontite.

**Palavras-chave:** enxaguante bucal, *streptococcus pneumoniae*, azitromicina. doença periodontal.
INTRODUCTION

Propolis is a substance produced by bees whose consumption dates back to antiquity (PASUPULETI et al., 2017). Due to the numerous biological properties of propolis, it is used in folk medicine, in humans and animals as a natural therapeutic agent (TIVERON et al., 2016; IBRAHIM; ALQURASHI, 2022). In beehives propolis serves to align the alveoli and chambers where the queens lay eggs (KLHAR et al., 2019) and has a protective function against natural enemies. The antimicrobial potential of propolis contributes to hive hygiene and disease prevention (PASUPULETI et al., 2017; AFROUZAN et al., 2018).

*Calendula officinalis* L. is an annual herbaceous vegetable of the family *Asteraceae* originating in the Mediterranean and currently grown in various regions of the world (SANTOS et al., 2015). The flower has a soft smell, considered safe as a seasoning, and used on a large scale in cosmetology (FATIMA et al., 2018). From this perspective, studies report that extracts of *Calendula officinalis* flowers and propolis extracts have a wide range of biological activities, antimicrobial potential against several pathogenic microorganisms, with high potential to be explored (SHANKAR et al., 2017; FATIMA et al., 2018; ABDULLAH et al., 2020). Thus, their applications in the health area should be further researched, because there is evidence that they may prevent oral pathologies, gingivitis and periodontitis, due to the antimicrobial and antioxidant properties that present (SHANKAR et al., 2017; NAKAO et al., 2020; TANIDEH et al., 2020).

Periodontitis or periodontal disease is one of the main causes of tooth loss, affecting some 20 to 50% of the world's population (HAQUE; SARTELLI; HAQUE, 2019; SAMISTRARO, 2018). It originates in gingival tissue and, if left untreated, affects the deeper tissues responsible for supporting the teeth, altering bone homeostasis and causing loss of the dental element (MEHROTRA; SINGH, 2020). Despite the multifactorial origin, periodontitis is initiated by microorganisms that are the surface of the teeth and gums, forming the biofilm, which interact with the host's immune defenses, leading to inflammation and disease (KWON; LAMSTER; LEVIN, 2021).

Periodontitis can be classified according to the severity of the disease, i.e stage I is an initial stage, stage II is a moderate stage, while stage III and IV are considered severe stages, including loss of teeth (CATON et al., 2018).

According to Martins et al. (2019) mouthwashes containing chlorhexidine are often used as adjuvants in oral hygiene measures due to their ability to control biofilm
formation. However, many side effects such as changes in the staining of the tongue and teeth, irritation in the oral mucosa and taste disorder have been widely reported (GÜRGAN et al., 2006; BRAGA et al., 2019).

The use of natural substances in dentistry has as advantage the affordable cost, easy handling, large amount of raw material available, in addition to presenting low adverse effects, as they make the body's immune reaction more favorable (FRANCISTO et al., 2010; OLIVEIRA et al., 2018).

In addition, the resistance to antimicrobials by microorganisms of human oral interferes with periodontal prevention and treatment (MARTINO, 2018). Ansiliero et al. (2021) observed multiresistance to antimicrobials in *Streptococcus* sp. strains and *Enterococcus avium*, of oral cavity origin, which constitutes a risk factor for human health. Biofilm can block the action of several antibiotics, hindering the permeability of drugs (SANZ et al., 2017). Thus, studies are needed to assess the potential of compounds as an alternative measure for the prevention and treatment of periodontitis (ÁLVAREZ-MARTÍNEZ; BARRAJÓN-CATALÁN; MICOL, 2020).

Martins et al. (2019) evaluated that red propolis extract showed antibacterial activity against *Streptococcus* spp. and *Lactobacillus casei*, cytotoxicity and antibiofilm like to that obtained with chlorhexidine. In addition, Tiveron et al. (2016) have observed strong antimicrobial activity of organic propolis (from Brazil) against the Gram-positive bacteria—*Streptococcus mutans*, *Streptococcus oralis*, *Streptococcus sobrinus* and *Staphylococcus aureus*, including inhibition of biofilm formation by *S. mutans*.

Based on these considerations, in this study we evaluated the antimicrobial and antioxidant activity of mouthwash produced with hydroalcoholic extracts of bee propolis and/or *Calendula officinalis*.

2 MATERIAL AND METHODS

2.1 EXTRACTS

2.1.1 Cultivation, management, and collection of flowers from *Calendula officinalis*

Calendula seeds (ISLA) from Santa Catarina, Brazil, were sown in January 2020. The cultivation occurred by using sowers, containing mixed substrate for plants (TECNOMAX®). When they reached 6 to 8 cm in height, they were transplanted into soil enriched with organic bovine fertilizer (150g/m²), as recommended by the manufacturer. After flowering, the whole and flowers were collected and transported to the laboratory for processing. The biological material was washed thoroughly under
running water, followed by careful drying in an oven with temperature of 55°C until constant weight, with subsequent grinding in blender (Mondial, 400w). The material obtained was vacuum packed in plastic bags, kept in a dry place, the material obtained was vacuum packed in no lighting and with no ventilation for later use.

2.1.2 Crude propolis sample

Crude propolis was collected from stingless beehives, popularly known as jataí bees (Tetragonisca angustula), on private property located in the state of Santa Catarina, Brazil. The material was transported, fragmented in a blender (Mondial, 400w) to obtain a fine powder. Then, it was frozen and stored for later production of the extracts in a biotechnology laboratory.

2.1.3 Preparation of extracts

Extracts of propolis or C officinalis L. were prepared separately. Thus, for concentration of bioactive compounds, 10 grams of flowers of C. officinalis L. or 10 grams of raw propolis, both dried and ground, were transferred to volumetric balloons containing 150 mL of 70% ethanol solvent (v/v), homogenized at 250 rpm, at 35 °C for 24 hours, by orbital shaking. After this period, the extractive solutions were filtered and the solvents evaporated at 60°C at 80 rpm, under reduced pressure in a rotary evaporator. The crude extracts were transferred to small amber glass vials stored at 4 °C. The extracts were used separately to assess antimicrobial activity or combined in a 1:1 ratio.

2.2 ANTIMICROBIAL ACTIVITY

For all tests were used bacterial strains (N=15) previously isolated (ANSILIERO et al., 2021) from patients with different clinical stages of periodontitis: stage I - Staphylococcus haemolyticus, Klebsiella azaenae, Raoultella sp.; stage II - Staphylococcus aureus, Streptococcus salivarius, K. azaenae, Citrobacter freundii; stage III/IV - Streptococcus pneumoniae, S. aureus, Escherichia coli, Enterobacter aerogenes, C. freundii, Pseudomonas fluorescens and Raoultella sp. In addition, standard reference strains were also used for control: S. aureus ATCC 25923, Streptococcus mutans ATCC 25175, Enterococcus faecalis ATCC 29212, E. coli ATCC 35218; Klebsiella pneumoniae ATCC 700603 and Pseudomonas aeruginosa ATCC 27853. All strains were previously grown under optimal conditions of temperature and culture medium before use in the experiments, observing purity and viability.
2.2.1 Well Diffusion Assay

Antimicrobial activity was evaluated by using well diffusion assay in soybean tryptone agar (TSA - Kasvi, Brazil). Thus, a bacterial suspension was prepared with turbidity adjustment according to the scale 0.5 McFarland (1.5. $10^8$ CFU.mL$^{-1}$) and seeded onto the agar TSA surface using a swab. In wells of approximately 5mm diameter each were added: 100µL of crude calendula extract; 100µL of crude extract of propolis; 50 of crude calendula extract + 50 µL of crude propolis extract. A well containing sterile distilled water (100µL) was used as a negative control, while discs of chloramphenicol 30µg and azithromycin 15µg were used as positive controls. The agar plates were incubated at 37°C for 24 h. The antimicrobial activity was evaluated by measuring the diameter (mm) of the growth inhibition zone. All tests were realized in duplicates.

2.2.2 Determination of minimum injunction concentration - IMC

To define the lowest concentration of extracts capable of inhibiting microbial growth, duplicate microdilution tests were performed according to CLSI (2015), using 96 well culture plates. Each well received 100 µL of Mueller-Hinton Infusion broth added of 20 µL of the inoculum of each microorganism (1.5. $10^8$ CFU.mL$^{-1}$), and 100 µL extracts of propolis and calendula, in the concentrations [222,2; 166,5, 111, 55,5 and 11 mg/ mL], in addition to the association of both extracts (1:1) at different concentrations. Distilled water was used as a negative control. The plates were incubated 24h/37ºC and the reading was performed by adding 10µL of 0.01% resazurin solution (7-Hydroxy-3H-phenoxazin-3-one 10-oxide) as realized by Oliveira (2019).

2.2.3 Determination of minimal inhibitory activity

To assess the minimal inhibitory activity 10µL were obtained from each previous broth cultivation and seeded over plate on TSA agar (OLIVEIRA, 2019). The CFU/mL result indicated whether the extract was bactericidal or bacteriostatic.

2.3 PHYTOCHEMICAL ANALYSIS AND ANTIRADICAL ACTIVITY

2.3.1 Determination of total phenols

The total phenol content of the developed extracts was determined by Folin ciocalteau spectrophotometric method, using gallic acid as a standard a, as performed by Singleton, Rossi Jr (1965) anda Andrade (2019), with some modifications. For the determination were used, 0.5 mL of the diluted crude extract 1:1000 [0,0666 mg/ mL].
2.5 mL of Folin 0.2 N (1:10) and 2.0 mL of sodium carbonate at 7.5%. The experiments were kept at room temperature for 2 hours and the optical density measured at 750 nm. The calibration curve was constructed using the standard the lactic acid. The calculation was performed using the straight line equation by calibration curve. The results were expressed in mg of gallic acid/g of equivalent extract (mg EAG/g).

2.3.2 Determination of flavonoids

The method of Rio et al. (1996) was used to determine total flavonoids in the developed extracts, as performed by Andrade (2019), with analysis in triplicates. For this, it was mixed in equal parts 2.0 mL of AlCl₃ and diluted extracts 1:1000. The samples were left in a dark room for 30 minutes. The reading was made by optical density at 425 nm. A calibration curve was constituted by using quercetin as standard, and the results expressed in mg of quercetin equivalent/g of extract (mg EQ/ g).

2.3.3 DPPH

The antioxidant capacity of the extracts was determined using 1,1-diphenyl-2-picrilidrazil (DPPH), according to the methodology performed by Nunes (2019) (initial concentration and dilutions of the 1x e 2x) in test tubes containing 3.0 mL of DPPH ethane solution (0.004%). The analyses were performed in triplicate in a dark room with reading after 30 minutes. The reading was made by optical density at 517 nm. A blank sample was prepared by using ethanol. The radical elimination activity (RSA) was calculated as a percentage of DPPH discoloration by using the equation:

\[
\% \text{ RSA} = \left( \frac{\text{ADPPH} - \text{AS}}{\text{ADPPH}} \right) \cdot 100.
\]

Where, AS represents the absorbance of the solution when the sample extract is added at a certain level, and ADPPH is the absorbance of the solution DPPH (ANDRADE, 2019).

2.4 DEVELOPMENT OF MOUTHWASH

The extracts with the highest antimicrobial and phytochemistry activity were selected for the development of a mouthwash. The formulations elaborated in this work are shown in Table 1.
Table 1. Formulations of mouthwashes developed in the present study.

<table>
<thead>
<tr>
<th>Formulation 1</th>
<th>Formulation 2</th>
<th>Formulation 3</th>
<th>Formulation 4</th>
<th>Formulation 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>3% Sorbitol</td>
<td>3% Sorbitol</td>
<td>3% Sorbitol</td>
<td>3% Sorbitol</td>
<td>3% Sorbitol</td>
</tr>
<tr>
<td>0.02% Nipagin</td>
<td>0.02% Nipagin</td>
<td>0.02% Nipagin</td>
<td>0.02% Nipagin</td>
<td>0.02% Nipagin</td>
</tr>
<tr>
<td>0.05% Mint flavor</td>
<td>0.05% Mint flavor</td>
<td>0.05% Mint flavor</td>
<td>0.05% Mint flavor</td>
<td>0.05% Mint flavor</td>
</tr>
<tr>
<td>Water - enough for 100mL</td>
<td>Water - enough for 100mL</td>
<td>Water - enough for 100mL</td>
<td>Water - enough for 100mL</td>
<td>Water - enough for 100mL</td>
</tr>
<tr>
<td>-</td>
<td>20% Extract</td>
<td>-</td>
<td>20% Extract</td>
<td>0.12% Chlorhexidine digluconate</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>0.05% Sodium fluoride</td>
<td>0.05% Sodium fluoride</td>
<td>-</td>
</tr>
</tbody>
</table>

2.4.1 Determination of antimicrobial activity of oral rinses in vitro

As previously described, the antimicrobial activity of mouthwash was carried out as performed for the extracts. All tests were realized in duplicates. After incubation at 37°C for 24 h the antimicrobial activity was evaluated by measuring the diameter of the inhibition zone.

To verify whether the mouthwashes maintained their antimicrobial activity during storage at 6ºC or 25ºC, the samples were tested on day 1 of production (1st evaluation), after 60 days (2nd evaluation) and at 120 days (3rd evaluation).

2.5 STATISTICAL ANALYSIS

The statist analysis was performed to evaluate whether there was a statistical difference between the results obtained. The data were submitted to variance analysis, Tukey test (p < 0.05) and student T test, selected according to the characteristic of each data group, using the GraphPad Prism version 8 (GraphPad Software).

3 RESULTS AND DISCUSSION

3.1 ANTIMICROBIAL ACTIVITY

Propolis and calendula extracts with concentration of 222 mg/mL (22.2%), showed activity against seven (n=7; 35%) strains from patients with periodontitis and against only one (n=01; 5%) microorganism from standard strain (Table 2).
Table 2. Antimicrobial activity of própolis and/or calendula extracts (Against microorganisms associated with periodontitis).

<table>
<thead>
<tr>
<th>Microbial strain (clinical periodontal stage)</th>
<th>Crude extract.</th>
<th></th>
<th></th>
<th>Chloramphenicol 30µg</th>
<th>Azithromycin 15µg</th>
<th>H2O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propolis</td>
<td>13.25±0.00A</td>
<td>0.00±0.00B</td>
<td>11.50±0.70A</td>
<td>29.75±1.76C</td>
<td>9.75±0.35A</td>
<td>-</td>
</tr>
<tr>
<td>Calendula</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Propolis/Calendula</td>
<td>17.25±2.47A</td>
<td>16.50±2.12A</td>
<td>16.00±0.00A</td>
<td>40.75±1.06B</td>
<td>32.00±1.42D</td>
<td>-</td>
</tr>
<tr>
<td>Chloramphenicol 30µg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Azithromycin 15µg</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>H2O</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Staphylococcus aureus (II)</td>
<td>15.25±0.35A</td>
<td>0.00±0.00B</td>
<td>14.75±0.35A</td>
<td>35.50±0.70C</td>
<td>8.00±0.00D</td>
<td>-</td>
</tr>
<tr>
<td>Streptococcus salivarius (II)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Staphylococcus pneumoniae (III/IV)</td>
<td>16.10±1.10A</td>
<td>0.00±0.00B</td>
<td>14.75±0.35A</td>
<td>39.00±1.41C</td>
<td>24.50±3.53D</td>
<td>-</td>
</tr>
<tr>
<td>Staphylococcus aureus (III/IV)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Streptococcus mutans ATCC 25175</td>
<td>13.25±0.35A</td>
<td>0.00±0.00B</td>
<td>12.50±0.70A</td>
<td>31.00±1.41C</td>
<td>25.00±0.35D</td>
<td>-</td>
</tr>
<tr>
<td>Enterococcus faecalis ATCC 29212</td>
<td>12.00±1.41A</td>
<td>0.00±0.00B</td>
<td>11.25±0.35A</td>
<td>29.50±0.70C</td>
<td>24.50±2.12D</td>
<td>-</td>
</tr>
</tbody>
</table>

Values displayed in average ± standard deviation. Different letters, horizontally differ from each other by the Tukey test (p < 0.05).

In short, the propolis extract was more efficient than calendula extract and the combined use of both did not potentiate antimicrobial action, as evaluated by the variance analysis test and Tukey test (p<0.05). On the other hand, the above-mentioned extracts had a similar action against the S. salivarius.

Similarly, Campos et al. (2014) also showed antimicrobial action of calendula against strains of S. salivarius, using for both tinctures obtained in a compounding pharmacy, with inhibition halos of 10mm.

According to Okinczyc et al. (2020), propolis extracts are potent antibacterial agents. Moreover, the results obtained in the present study were similar to that observed by Almeida et al. (2017) who evaluated the antimicrobial potential of tinctures and extracts of red propolis obtained in northeastern Brazil, they were shown to be bioactive against S. aureus with inhibition halos of 8 and 17 mm at the concentration of 200 µg/mL, respectively. From this perspective, Roh and Kim (2018), of Korean propolis extract (50mg/mL), observed inhibition halos from 8.07±0.21 to 9.97±0.65 mm against oral pathogenic microorganisms.

The hydroalcoholic extracts produced here showed antimicrobial action only for the strains of gram-positive microorganisms. This result is consistent with previous studies developed by Torres et al. (2018), which demonstrated antimicrobial activity, mainly against Gram-positive bacteria. In fact, there are differences in the cell wall of gram-negative and gram-positive cells. Although the cell wall of Gram-negative bacteria
is of low thickness, it houses an additional outer membrane with high content of lipopolysaccharides and lipoproteins, which gives these cells greater resistance to antimicrobial substances (VADILLO-RODRIGUEZ et al., 2021).

Based on the results of the well diffusion assay, the propolis extract showed superior antimicrobial action against *Streptococcus pneumoniae* isolated from patients with periodontitis (levels III/IV) compared to the control antibiotic azithromycin (Figure 1). Regarding the tests with *Staphylococcus aureus* isolated from patients with level II periodontitis, no statistical differences were observed by Tukey (p<0.05). Thus, the extract presents itself as an alternative substance with the potential to inhibit pathogens that pass through the oral cavity and are implicated in periodontitis.

Figure 1. *Streptococcus pneumoniae* inhibition by hydroalcoholic extract of propolis in well diffusion assay.

Caption. 1: Crude extract of propolis; 2: Azithromycin (15µg).

Microbial resistance to antimicrobials of clinical use generates interest in the search for natural substances with biological activity, as is the case of propolis, which has been gaining prominence due to its biological potential (AFROUZAN et al., 2018).

The results obtained regarding the minimum inhibitive concentration (IMC) of extracts can be observed in Table 3. It was verified that the IMC of the propolis extract varied according to the target microorganism, with values from 111 to 166.5 mg/mL. The concentration of 111 mg/mL has been shown to be effective in controlling *Streptococcus*
pneumoniae (from a patient with stage II/IV periodontitis), *Staphylococcus aureus* (from a patient with stage II/IV periodontitis) and *Enterococcus faecalis* ATCC 29212.

<table>
<thead>
<tr>
<th>Strains (clinical periodontitis stage)</th>
<th>H₂O</th>
<th>Propolis</th>
<th>Calendula</th>
<th>Propolis/Calendula (1:1)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus aureus</em> (II)</td>
<td>-</td>
<td>166.5</td>
<td>-</td>
<td>166.5</td>
</tr>
<tr>
<td><em>Streptococcus salivarius</em> (II)</td>
<td>-</td>
<td>166.5</td>
<td>55.5</td>
<td>166.5</td>
</tr>
<tr>
<td><em>Streptococcus pneumoniae</em> (III/IV)</td>
<td>-</td>
<td>111</td>
<td>-</td>
<td>166.5</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> (III/IV)</td>
<td>-</td>
<td>111</td>
<td>-</td>
<td>166.5</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> ATCC 25923</td>
<td>-</td>
<td>166.5</td>
<td>-</td>
<td>166.5</td>
</tr>
<tr>
<td><em>Streptococcus mutans</em> ATCC 25175</td>
<td>-</td>
<td>166.5</td>
<td>-</td>
<td>166.5</td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em> ATCC 29212</td>
<td>-</td>
<td>111</td>
<td>-</td>
<td>111</td>
</tr>
</tbody>
</table>

Almeida et al. (2017) evaluated the antimicrobial potential of tinctures and chloroform extracts of red propolis obtained in northeastern Brazil and observing IMC values of 135.87 - 271.74 µg / mL using Gram-positive strains and 271.74 - 543.48 µg / mL for Gram-negative strains. In the same meantime, regarding the use of alcoholic extract of propolis from Iran, IMC of 150, 300, 300 µg/mL was evidenced for *Staphylococcus aureus*, *Streptococcus mutans* and *Enterococcus faecalis*, respectively (NAZERI; GHAIOUR; ABBASI, 2019). Thus, has been observed that the propolis has bactericidal action, however, its action is strongly related to the species of the microorganism used and its origin (FALÇÃO, 2014).

3.2 PHYTOCHEMICAL ANALYSIS AND ANTIRADICAL ACTIVITY

The extracts of propolis and calendula here produced showed no statistically significant difference, according to the t-test, due the concentration of total phenols and free radical elimination activity. However, a higher flavonoid content was verify for propolis extract when compared to calendula extract (Table 4; p<0.005). Thus, the flavonoid content can interfere with the bioactivity of the extracts, considering that it depends on their chemical compositions (AFROUZAN et al., 2018).
Table 4. Phytochemical compounds and anti-radical activity of hydroalcoholic extracts of propolis and *Calendula officinalis*.

<table>
<thead>
<tr>
<th>Phytochemical compounds and anti-radical activity-RSA</th>
<th>Propolis (average ± Standard deviation)</th>
<th>Calendula (average ± Standard deviation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total phenols (mg EAG/g)</td>
<td>6.806 ± 0.199&lt;sup&gt;A&lt;/sup&gt;</td>
<td>6.345 ± 0.345&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>Flavonoids (mg EQ/g)</td>
<td>0.288 ± 0.038&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.146 ± 0.034&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td>RSA (%)</td>
<td>86.946 ± 0.636&lt;sup&gt;A&lt;/sup&gt;</td>
<td>86.375 ± 0.070&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Caption. Different letters differ statistically from each other (p < 0.05), by the t-test.

Polyphenols and flavonoids are natural antioxidant products with action varying according to concentration and chemical structure (SYTAR et al., 2018). Antioxidants of plant origin act by reducing and/or eliminating the deleterious effects of oxidative processes, thus being used *in natura* or in the food, cosmetic and pharmacological industry (ANDRADE, 2019).

The results obtained regarding the content of phenolic compounds with the propolis extract were different in relation to the results found by Martins et al. (2018). These authors obtained only 2.73±0.01 (mg gallic acid/100 mL) of phenolic. However, the flavonoid content was lower than that observed by the authors, who obtained values of 2.46±0.31 (mg quercetin/100mL). About RSA, Almeida et al. (2017) observed that tinctures and microcapsules loaded with Brazilian red propolis extract obtained presented high antioxidant activity, with values from 77.12% to 98.06%. Ristivojević et al. (2020) evaluated the antioxidant potential of propolis samples from different geographical locations and showed that propolis from Serbia (P3) presented the highest antioxidant potential, with RSA values of 53.21%.

It is estimated that the antimicrobial activity of propolis is related to phenolic and flavonoid compounds of its composition, and polarities (ABDULLAH et al., 2020). In this way, Sytar et al. (2018) evaluated the total phenolic content, total flavonoids, and antioxidant activity of methanol extracts of leaves of *Calendula officinalis* L. whose values obtained were of 6.5 ± 0.004 (mg EQ/g), 1.125 ± 0.153 (mg EQ/g) and 92.56 ± 0.35%, respectively.

### 3.3 ANTIMICROBIAL ACTIVITY OF MOUTHRINSES

Among the five formulations (F1 to F5) of mouthwashes, only F1 and F3 did not present antimicrobial activity against the evaluated microorganisms (Table 5).

No active antimicrobial from either propolis or calendula was present in these two formulations (F1/F3), except that, as in the other formulations, nipagin (methylparaben),...
a pharmaceutical preservative, was added. This reinforces that it did not interfere with the antimicrobial action of the extracts in relation to the target microorganisms evaluated. On the other hand, the formulations containing propolis extract (formulations 2 and 4) and chlorhexidine (formulation 5) showed biological activity, with action varying according to microbial lineage. Thus, these results reinforces that the antimicrobial action in formulations containing calendula or propolis

Table 5. Antimicrobial action of mouthwashes formulations to pathogenic isolates from patients with different clinical stages of periodontal disease.

<table>
<thead>
<tr>
<th>Strains (clinical stage of periodontitis)</th>
<th>Antimicrobial action of Mouthwash (well diffusion assay)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Formulation 1</td>
</tr>
<tr>
<td>Staphylococcus aureus (II)</td>
<td>0.00±0.00A</td>
</tr>
<tr>
<td>Streptococcus salivarius (II)</td>
<td>0.00±0.00A</td>
</tr>
<tr>
<td>Streptococcus pneumoniae (III/IV)</td>
<td>0.00±0.00A</td>
</tr>
<tr>
<td>Staphylococcus aureus (III/IV)</td>
<td>0.00±0.00A</td>
</tr>
<tr>
<td>Staphylococcus aureus ATCC 25923</td>
<td>0.00±0.00A</td>
</tr>
<tr>
<td>Streptococcus mutans ATCC 25175</td>
<td>0.00±0.00A</td>
</tr>
<tr>
<td>Enterococcus faecalis ATCC 29212</td>
<td>0.00±0.00A</td>
</tr>
</tbody>
</table>

Caption. Values displayed in average ± standard deviation. Different letters, horizontally, differ from each other by tukey test (p<0.05).

*Streptococcus salivarius* was the most sensitive isolate against formulation 2 and *Streptococcus pneumoniae* against formulation 4.

Bapat et al. (2021) showed that the oral rinses of propolis, led to a decline of the microorganism *S. mutans* in saliva samples from patients and, in addition, propolis has been shown to be as efficient as chlorhexidine in reducing plaque pathogens, gingivitis and dental caries.

Moreover, the use of sodium fluoride (0.05%) in mouth was not potentiated by their antimicrobial action, according to the Tukey test (p<0.05).

Formulation 5 containing chlorhexidine (0.12%) showed better antimicrobial action compared to the other (Figure 2). The drug is considered the gold standard in the control of oral microorganisms, however due to the side effects triggered by the same, such as smears and irritation in the oral mucosa, its prolonged use should be avoided (GÜRGAN et al., 2006; BRAGA et al., 2019).
Figure 2. Antimicrobial potential of mouthphering oral formulations developed against *Staphylococcus aureus* ATCC 25923.

Caption. 1: Mouthwash oral formulation 01; 2: mouthwash formulation 02; 3: mouthwash formulation 03; 4: mouthwash formulation 04; 5: mouthwash formulation 05.

The formulation 5 containing chlorhexidine was more efficient in the control of oral pathogens, followed by formulations 2 and 4 (containing propolis extract), which showed no statistical difference between them, according to the Tukey test (p<0.005).

Porwal et al. (2018) showed that chlorhexidine gluconate (0.2%) was more effective in reducing dental plaque in patients when compared to crude propolis extract. However, propolis was more effective in reducing gingival inflammation, suggesting that it can be used as an alternative to rinsing with chlorhexidine.

We evidenced that formulations 2, 4, 5 and crude propolis extract decreased their biological activity over the course of days (Figure 3). However, the crude propolis extract showed a greater viability decline in relation to the formulations of rinses. After 120 days the biological activity was lost (zero inhibition). The samples stored at 25°C showed lower inhibition halos, when compared with the samples stored at 6°C, but this difference was not significant (p<0.005).
4 CONCLUSIONS

The propolis extract produced in this study showed antimicrobial activity against the pathogens *Staphylococcus aureus*, *Streptococcus salivarius*, *Streptococcus pneumoniae*. Standard strains *Staphylococcus aureus* ATCC 25923, *Streptococcus mutans* ATCC 25175 and *Enterococcus faecalis* ATCC 29212 were also inhibited. Propolis extract was more efficient than calendula extract or its combined use. The hydroalcoholic extracts showed antimicrobial action only to Gram-positive microorganisms, not being efficient in the control of gram-negative microorganisms here evaluated. In addition, we can emphasize that propolis extract produced under the conditions of this study had antimicrobial action to azithromycin compared to *S. pneumoniae*, isolated from patients with periodontitis stage III/IV, but with similar action of this same antibiotic for *Staphylococcus aureus* isolated from patients with clinical periodontal stage II.
Although the extracts did not differ regarding the content of phenolic compounds and RSA, the propolis extract had a higher content of flavonoids than the marigold extract, which may explain its bioactivity.

In this perspective, the propolis extract inhibited pathogens implicated in causing periodontitis. Considering that it presented not only antimicrobial activity, but also antioxidant activity, its use in pharmaceutical formulas, such as the one presented here, could be especially useful in oral hygiene, contributing to the prevention of periodontitis.

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