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Efficacy of red propolis hydro-alcoholic extract in controlling *Streptococcus mutans* biofilm build-up and dental enamel demineralization

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ABSTRACT

Objective: The efficacy of a red propolis hydro-alcoholic extract (RP) in controlling *Streptococcus mutans* biofilm colonization was evaluated. The effect of RP on dental demineralization was also investigated.
Methods: Chemical composition was determined by High Performance Liquid Chromatography (HPLC). Minimum Inhibitory and Bactericidal Concentration (MIC and MBC, respectively) were investigated against *Streptococcus mutans* (ATCC 25175). The cytotoxic potential of 3% RP in oral fibroblasts was observed after 1 and 3 min. Bovine dental enamel blocks (N = 24) were used for *S. mutans* biofilm formation (48 h), simulating 'feast or famine' episodes. Blocks/biofilms were exposed 2×/day, for 3 days, to a cariogenic challenge with sucrose 10% (5 min) and treated (1 min) with: 0.85% saline solution (negative control), 0.12% Chlorhexidine (CHX, positive control for biofilm colonization), 0.05% Sodium Fluoride (NaF, positive control to avoid demineralization) and 3% RP. Biofilms were assessed for viability (CFU/mL), and to observe the concentration of soluble and insoluble extracellular polysaccharides (SEPS and IEPS). Dental demineralization was assessed by the percentage of surface hardness loss (%SHL) and through polarized light microscopy (PLM).
Results: The RP presented 4.0 pH and ºBrix = 4.8. The p-coumaric acid (17.2 µg/mL) and luteolin (15.23 µg/mL) were the largest contents of phenolic acids and flavonoids, respectively. MIC and MBC of RP were 293 µg/mL and 1172 µg/mL, respectively. The 3% RP showed 43% of viably cells after 1 min. Lower number ($p < 0.05$) of viable bacteria (CFU/mL) was observed after CHX (1.8×10^5) followed by RP (1.8×10^7) treatments. The lowest concentration (µg/CFU) of SEPS (12.6) and IEPS (25.9) was observed in CHX ($p < 0.05$) followed by RP (17.1 and 54.3), and both differed from the negative control (34.4 and 63.9) ($p < 0.05$). Considering the %SHL, all groups differed statistically ($p < 0.05$) from the negative control (46.6%); but NaF (13.9%), CHX (20.1%) and RP (20.7%) did not differ among them ($p > 0.05$). After all treatments, suggestive areas of caries lesions were observed by PLM, which were lower for CHX and NaF.
Conclusion: The 3% RP reduced *S. mutans* colonization, decreased concentration of extracellular polysaccharides and reduced dental enamel demineralization.

1. Introduction

Dental caries is a disease resulted from the demineralization of dental surface due to the presence of organic acids released by bacteria that metabolize sugar, especially sucrose (Cury & Tenuta, 2009). The presence of bacteria, mainly streptococci from the mutans group, is a necessary factor to initiate carious lesions, although it is not enough for the disease progression. The bacterial metabolism of carbohydrates like sucrose is a positive determinant factor (Duarte et al., 2006), whilst the presence of antimicrobial agents and fluoride may negatively regulate the evolution of dental caries lesions (Cury & Tenuta, 2009).

The cariogenic potential of *Streptococcus mutans* is determined genetically, being accentuated when sucrose is available. Under these conditions, the metabolic pathways of *S. mutans* favor the reduction of

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pH, the consequent demineralization of the dental substrate, as well as the synthesis of extracellular polysaccharides that acts as an adhesively strategy and energy reserve (Bowen & Koo, 2011). Therefore, *S. mutans* is considered the most relevant bacteria within the cariogenic biofilm (Holbrook & Magnúsdóttir, 2012).

Bacterial biofilm models have been studied to identify environmental factors that could influence the biofilm composition; as well as to develop new antimicrobial strategies (Marsh, 2003). Based on this, products extracted from plants, such as coffee, green tea, cocoa, grape, propolis and other plant derivates have been explored to investigate their preventive potential against cariogenic biofilms (Antonio et al., 2011; Cho, Schiller, & Oh, 2008; Meckelburg et al., 2014; Ooshima et al., 1993; Ooshima et al., 2000; Simonetti, Simonetti, & Villa, 2004; Smullen, Koutsou, Foster, Zumbé, & Storey, 2007).

Propolis is a resinous substance derived from the collection of botanical compounds by bees, which may vary according to geographic location, bee species and season of the year (Alencar et al., 2007; Koo & Park, 1997; Libério et al., 2009). The biological activity of propolis is frequently associated with the presence of phenolic compounds, mainly flavonoids (Park et al., 1998), such as flavone (rutin, luteolin), isoflavone (formononetine, daidzein), dihydroflavonol (pinobanksin, pinobanksin-3-acetate), and others. These compounds are responsible for various biological properties of red propolis (Bueno-Silva et al., 2013; Daugsch, Moraes, Fort, & Park, 2008), including the antiproliferative effect of cancer cells (Kouidhi, Zmantar, & Bakhrouf, 2010) and therapeutic potential against resistant anaerobes found in odontogenic infections and periodontal diseases (Ferreira et al., 2007; Shabbir, Rashid, Tipu, & Propolis, 2016). In addition, this kind of propolis can reduce the colonization of facultative anaerobe microorganisms like *S. mutans*, acting in the prevention of dental caries (Bueno-Silva et al., 2013).

Although red propolis has demonstrated antimicrobial properties in other studies (Alencar et al., 2007; Bueno-Silva et al., 2013; Daugsch et al., 2008), and has already been evaluated for the potential of reducing the virulence of *S. mutans* biofilms (Bueno-Silva et al., 2013), none of the studies tested the antimicrobial activity through a validated 'feast or famine' regimen in a biofilm model. Also, the dental surface demineralization has not been used yet as a criterion to evaluate anticaries efficacy of red propolis extract. In addition, the efficacy of natural products against cariogenic biofilms should consider other factors than microbial density, such as the biofilm matrix composition. Therefore, the aim of this study was to evaluate the efficacy of a red propolis hydro-alcoholic extract (RP) in controlling *Streptococcus mutans* biofilm colonization and its effect on dental enamel demineralization.

2. Materials and methods

We performed an *in vitro*, randomized, controlled, blind study. The antimicrobial activity of the red propolis extract was defined from the determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) (CLSI, 2012). An experimental biofilm model of *S. mutans* under 'feast or famine' conditions (Ccahuana-Vásquez & Cury, 2010) was also used to evaluate the *in vitro* efficacy of this extract, by evaluating the following parameters: microbial viability (CFU/mL), acidogenicity, concentration of soluble and insoluble extracellular polysaccharides, and demineralization of the dental substrate.

2.1. Red propolis hydro-alcoholic extract

The red propolis hydro-alcoholic extract was supplied by Apiário Edimel, located in the town of João Pessoa - Paraíba, Brazil (Latitude 7° 3′ 25.756″; Longitude O 34° 50′ 55.155″). The propolis was produced by *Apis mellifera* bees from flower buds, new leaves and exudates of several plants, with botanical origin as *Dalbergia ecastophyllum*. The raw propolis material was collected in the spring Brazilian season (September 2015). The product was available at the initial concentration of 30% (300 mg/mL) as a hydro-alcoholic extract, which was produced through the trituration of the crude propolis sample with a pestle. Then 3 g of crushed propolis were mixed with 70 mL of ethanol (cereal alcohol, 98%) and 30 mL of distilled water. Samples were kept at 70 °C for 30 min under constant stirring. After extraction, the mixture was centrifuged, and the supernatant was evaporated under low pressure to produce the hydro-alcoholic extract of propolis (RP) at 3%. The cost of a 30 mL bottle of this product is equivalent to $ 9.16 (or € 7.46).

2.2. pH, total solids

The pH was determined with a pH meter (DM20 Digitized, Santo Amparo, SP, Brazil). Total soluble solids were determined using a digital refractometer (ATAGO®, PAL-1, Japan) and results were expressed in °Brix.

2.3. Determination of total polyphenol content

Total flavonoid and total phenolic contents were measured according to Chlopicka, Pasko, Gorinstein, Jedryas, and Zagrodzki (2012) and Singleton, Orthofer, and Lamuela-Raventos (1999), using quercetin and galic acid as reference standards, respectively. The contents of individual phenolic acids were determined by gradient LC–DAD-ESI-MS reverse-phase systems according to Duarte and Farah (2011). Flavonoids were determined according to Felberg et al. (2015)), with modifications.

The RP was diluted 1:10 in distilled water; then 30 µL of this dilution was mixed with 120 µL of distilled water, 75 µL of Folin-Ciocalteu reagent (Merck, Germany) and 75 µL sodium carbonate 20% (w/v). The absorbance was measured at 280 nm (UV-1800) after 30 min incubation at room temperature. The concentrations were calculated using a calibration curve and were expressed in mg/mL of gallic acid equivalent.

2.4. Bacterial strain and inoculum preparation for evaluation of MIC and MBC

The reference strain of *Streptococcus mutans* (ATCC 25175) was reactivated from its original cultures in Brain Heart Infusion Agar (BHI Agar) medium for 48 h at 37 °C, with 5% CO_2. After this period, a loopfull of bacterial colonies were collected and suspended in 25 mL of BHI Broth medium (Merck, Darmstadt, Germany). The concentration of cells was determined, after incubation at 37 °C for 24 h. The absorbance of 0.18 was set in a spectrophotometer under 625 nm wavelength, obtaining cell density equivalent to 1.0×10^8 colony forming units (CFU) per milliliter (CFU/mL) (Cardoso et al., 2016; Da Cunha et al., 2013).

For the Minimum Inhibitory Concentration (MIC) assay, the inoculum obtained was diluted 1000×, resulting in a final concentration of 1.0×10^5 CFU/mL (CLSI, 2012). For the experiments with biofilm, the inoculum was diluted 10× (1.0×10^7 CFU/mL).

2.5. MIC and MBC determination

The MIC and MBC were used to determine the antimicrobial activity of the red propolis hydro-alcoholic extract (RP), according to the reference protocol of the Clinical and Laboratory Standards Institute (CLSI, 2012).

MIC was assessed using a 96-well microtiter plates (ALAMAR®, Diadema, São Paulo, Brazil), in which 100 µL of BHI broth was inserted into the wells. Then, 100 µL of the RP at its initial concentration (30%) were inserted into the first column of the 96-well plate. The extract was then serially diluted from 75 to 0.036 mg/mL (1:1 v/v) by transferring 100 µL of the most concentrated well content to the less concentrated (Cardoso et al., 2016; Da Cunha et al., 2013). After dilution, 100 µL of the contents of the last column were dispensed to equal the volume of all wells. Finally, 100 µL of the bacterial inoculum (1.0×10^5 CFU/mL)