Archives of Oral Biology 65 (2016) 77–81

Contents lists available at ScienceDirect

Archives of Oral Biology

journal homepage: www.elsevier.com/locate/aob

Influence of a Brazilian wild green propolis on the enamel mineral loss and *Streptococcus mutans*' count in dental biofilm

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ARTICLE INFO

Article history:
Received 29 May 2015
Received in revised form 19 December 2015
Accepted 1 February 2016

Keywords:
Propolis
Antibacterial activity
Dental biofilm
Streptococcus mutans
Microhardness

ABSTRACT

Objective: This study investigated the anti-demineralizing and antibacterial effects of a propolis ethanolic extract (EEP) against *Streptococcus mutans* dental biofilm.
Design: Blocks of sound bovine enamel ($n = 24$) were fixed on polystyrene plates. *S. mutans* inoculum (ATCC 25175) and culture media were added (48 h–37 °C) to form biofilm. Blocks with biofilm received daily treatment (30 μL/1 min), for 5 days, as following: G1 (EEP 33.3%); G2 (chlorhexidine digluconate 0.12%); G3 (ethanol 80%); and G4 (Milli-Q water). G5 and G6 were blocks without biofilm that received only EEP and Milli-Q water, respectively. Final surface hardness was evaluated and the percentage of hardness loss (%HL) was calculated. The EEP extract pH and total solids were determined. *S. mutans* count was expressed by $\log_{10}$ scale of Colony-Forming Units (CFU/mL). One way ANOVA was used to compare results which differed at a 95% significance level.
Results: G2 presented the lowest average %HL value (68.44% ± 12.98) ($p = 0.010$), while G4 presented the highest (90.49% ± 5.38%HL) ($p = 0.007$). G1 showed %HL (84.41% ± 2.77) similar to G3 (87.80% ± 6.89) ($p = 0.477$). Groups G5 and G6 presented %HL = 16.11% ± 7.92 and 20.55% ± 10.65; respectively ($p = 0.952$). G1 and G4 differed as regards to *S. mutans* count: 7.26 ± 0.08 and 8.29 ± 0.17 CFU/mL, respectively ($p = 0.001$). The lowest bacterial count was observed in chlorhexidine group (G2 = 6.79 ± 0.10 CFU/mL) ($p = 0.043$). There was no difference between *S. mutans* count of G3 and G4 ($p = 0.435$). The EEP showed pH 4.8 and total soluble solids content = 25.9 Brix.
Conclusion: The EEP seems to be a potent antibacterial substance against *S. mutans* dental biofilm, but presented no inhibitory action on the de-remineralization of caries process.

1. Introduction

Dental caries is a condition that depends on the presence of biofilm or dental plaque (Cury & Tenuta, 2009). When the microbial deposits remain adhered to the tooth, episodes of pH drop in the biofilm exposed to sugar occur (Filoche, Wong, & Sissons, 2010) leading to a loss of enamel integrity (Takahashi & Nyvad, 2008). Therefore, in face of a diet rich in sugar and fermentable carbohydrates, the pH of plaque remains low, favoring the cariogenic microbiota growth, which consequently increases the risk for caries disease (Kidd & Fejerskov, 2004).

In this sense, some studies (Bonow, Azevedo, Goettems, & Rodrigues, 2013; Touger-Decker & Van Loveren, 2003) have been conducted with the purpose to determine anticariogenic strategies to reduce the risks posed by sugar and other fermentable carbohydrates, exploring the use of natural products. These products may prevent caries disease without provoke undesirable side effects such as development of bacteria to tolerance, vomiting, diarrhea and others (Chandra Shekar, Nagarajappa, Suma, & Thakur, 2015). Among these products, different types of plants rich in polyphenols, such as coffee, green tea, cocoa and grape, have been investigated with regard to their antibacterial action against caries pathogens (Antonio et al., 2011; Meckelburg et al., 2014; Ooshima et al., 2000; Smullen, Koutsou, Foster, Zumbé, & Storey, 2007; Yano, Kikuchi, Takahashi, Kohama, & Yoshida, 2012).

Propolis, also rich in polyphenols (Koo, Rosalen, Cury, Park, & Bowen, 2002), has aroused scientific interest with regard to its

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http://dx.doi.org/10.1016/j.archoralbio.2016.02.001
0003-9969/

beneficial pharmacological properties to oral health, such as the anti-inflammatory and antimicrobic (Abubakar, Abdullah, Sulaiman, & Ang, 2014; Eick et al., 2014). Additionally, the effective concentrations of mouthrinse containing propolis on oral microorganisms were less cytotoxic on human gingival fibroblasts than Chlorhexidine (Ozan et al., 2007). However, there are still gaps to be elucidated considering its effect on the de-remineralization process of caries. Bearing in mind that the development of new therapies with the use of natural products for prevention and treatment of diseases is relevant in the field of medicine (Walker, 1996), this study aims to investigate the anti-demineralizing and antibacterial effects of a propolis extract against *Streptococcus mutans* biofilm formed on bovine dental enamel.

2. Materials and methods

2.1. Propolis ethanolic extract

Sample: wild green propolis was collected from Apis mellifera beehives in the Atlantic Rain Forest of Bocaina mountains, town of São José do Barreiro, State of São Paulo, Brazil (latitude 22°45′ South, longitude 44°39′ West of Greenwich, 1200 m altitude) and kept in a desiccator for a week. Crude propolis was ground in nitrogen and sieved through a 0.6 mm sieve. The extract was prepared according to Park et al. (1998), modified as follows: 30 mL of 80% ethanol were added to 10 g of ground propolis. The mixture was heated to 60 °C for 30 min, under agitation, filtered through Watman n.2 paper and centrifuged at 7500 × *g* at 5 °C for 10 min. The supernatant (propolis extract—EEP) was directly used for the biofilm assay.

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 polyphenols were measured by colorimetric assays using the Folin–Ciocalteu method (Singleton, Orthofer, & Lamuela-Raventos, 1999) with modifications. Each extract 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 760 nm (UV-1800) after 30 min incubation at room temperature. The concentrations were calculated using a calibration curve and were expressed in mg/mL gallic acid equivalent

2.4. Determination of hydroxycinnamic acids and derivatives

The contents of caffeic, ferulic, *p*-coumaric and 5-caffeoylquinic acids (from Sigma–Aldrich, Germany) and 3-caffeoylquinic, 4-caffeoylquinic,3,4-dicafeoylquinic, 3,5-dicaffeoylquinic, 4,5-dicaffeoylquinic (IUPAC numbering), were investigated by HPLC-DAD-reverse-phase system, with clarification and chromatographic conditions based on Farah, de Paulis, Trugo, and Martin (2005).

2.5. Bacterial strain and inoculum preparation

A bacterial sample of *S. mutans* (ATCC 25175) was used to prepare the inoculum. Initially, the bacterial sample was evaluated to verify the degree of purity. After this, isolated bacterial colonies were selected and transferred to a 0.85% saline solution until an Optical Density (O.D.) of 0.15 at 520 nm (Libra S2 Colorimeter, Biochrom, Cambridge, England) corresponding to approximately 10^8 CFU/mL, was obtained.

2.6. Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The antibacterial activity of EEP was examined inicially, by determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2012) with a modification proposed by da Cunha et al. (2013). MIC was performed in 96-well microplates, inoculated with 5×10^5 CFU/mL, in 100 μL of brain heart infusion medium (BHI, Oxoid Ltd., Hampshire, England). The concentrations of EEP ranged from 266.67 to 0.259 mg/mL. Plain ethanol solution was used as the vehicle control (concentrations ranged from 64 to 0.0625%, (v/v)). Chlorhexidine digluconate 0.05% was used as positive control. Plates were incubated at 37 °C and 5% CO_2 for 24 h and MIC was defined as the lowest concentration of EEP that allowed no visible growth, confirmed by 0.01% resazurin dye (Sigma–Aldrich, St. Louis, MO, USA). The wells with the highest concentration of EEP and visible growth after the incubation were analyzed for purity of the bacterial suspension by surface culture on BHI agar (Oxoid Ltd., Hampshire, England). MBC was determined by subculturing 50 μL aliquots of each incubated well that presented concentration higher than the MIC on BHI agar. Two separate experiments were conducted in triplicate for each concentration of EEP.

2.7. Selection and preparation of bovine tooth samples

Fifty four sound bovine incisors, disinfected in 2% formol (pH 7.0), were selected after confirming absence of caries, stains, cracks or other enamel defects. They were cut using a water-cooled diamond saw (Bühler, Uzwil, Switzerland) to obtain enamel blocks (4 × 4 × 2 mm). These blocks were fixed with wax in an acrylic device to polish the enamel surface: 600- and 1200-grit silicon carbide papers (Extec Corp., Enfield, USA), followed by 1-μm diamond abrasive slurry (Extec Corp., Enfield, USA) and washed ultrasonically in Milli-Q water (Merck Millipore, Darmstadt, Germany). Following, the specimens were stored in a physiological solution for posterior analysis of surface hardness.

2.8. Initial surface hardness evaluation and selection of enamel blocks for test with biofilm

The surface microhardness test was performed using a Microhardness Tester (Micromet 5104; Buehler, Mitutoyo Corporation, Tokyo, Japan) with a Knoop type diamond under a 50 g load for 5 s. Three indentations spaced 100 μm from each other were made at the center of the enamel surface to select the sample (de Mazer Papa et al., 2010). The mean of these three values represented the sample hardness. Twenty four blocks (mean 330.00 ± 31.15 kg/mm^2) were used in the present study. All samples were stored in a humid environment.

2.9. In vitro S. mutans *biofilm formation on bovine tooth fragments*

The experimental protocol was carried out in accordance with the methodology proposed by Soares et al. (2015). Each bovine tooth fragment ($n = 24$) was fixed with wax in a single well in a 24-well polystyrene plate. This plate/fragment system was sterilized with ethylene oxide (Bioxxi, Serviços de Esterilização