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ORIGINAL ARTICLE

Antibacterial and Cytotoxic Potential of a Brazilian Red Propolis

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Abstract

Objective: To evaluate *in vitro* the effect of a red propolis ethanolic extract (RPE) in the prevention of growth of a cariogenic biofilm and its cytotoxic potential. **Material and Methods:** Minimum inhibitory and bactericidal concentrations (MIC and MBC) of RPE against *Streptococcus mutans* and *Lactobacillus casei* were determined. The cytotoxic potential of 0.4% RPE in oral fibroblasts was observed after 1, 3 and 5 min of contact. Cellulose membrane disks (13 mm, N=12) were used for biofilm formation (24 h) of *S. mutans* and *L. casei*, which were treated (1 min) with 0.4% RPE or 0.12% Chlorhexidine (CHX). The control group of biofilm formation was not submitted to any treatment. Serial dilutions were then made to evaluate microbial viability. Descriptive data analysis and, for microbial viability, Mann Whitney test were performed ($p \leq 0.05$). **Results:** RPE showed similar MIC and MBC (4.46 mg/mL) against *S. mutans* and, for *L. casei*, they were 8.92 mg/mL (MIC) and 17.85 mg/mL (MBC). CHX presented MIC and MBC <0.00002 mg/mL for *S. mutans* and 0.00047 mg/mL for *L. casei*. After 1, 3 and 5 min, the RPE exhibited, respectively, 69.38%, 43.91% and 40.36% of viable cells. The RPE (6.55) and CHX (6.87) presented similar efficacy to reduce the total number of viable bacteria ($p>0.05$). Regarding the total number of viable bacteria (Log_{10} CFU/mL), the RPE (6.55) and CHX (6.87) presented similar efficacy ($p>0.05$). **Conclusion:** Red propolis extract showed antibacterial activity against the tested strains, exhibited acceptable cytotoxicity and reduced the colonization of *S. mutans* and *L. casei* in a biofilm membrane model.

Keywords: Anti-Bacterial Agents; Complementary Therapies; Phytotherapy.

Introduction

Dental caries results from surface demineralization caused by an organized biofilm exposed to fermentable carbohydrates from the diet [1]. These carbohydrates act as nutrients for biofilm bacteria, especially acidogenic and aciduric species. *Streptococcus mutans* and *Lactobacillus* spp. are the main microorganisms responsible for the onset and progression of caries, respectively [2]. Both are able to proliferate and survive in acidic media, resulting in an elevated potential for caries development [3,4].

In order to prevent the clinical appearance of dental caries and to reduce its progression, several products with antimicrobial activity can be used [5,6]. Natural products have been widely studied due to their diverse biological properties [7-15]. Among these, propolis is a resinous substance originated from botanical compounds and collected by bees, and can be classified into different types (as green, brown and red) according to the chemical composition and geographical origin [12,16,17].

Red propolis presents antibacterial activity [15,18] and may be considered a potential agent to reduce accumulation of cariogenic biofilm and, consequently, to reduce the prevention and onset of dental caries process [15,20]. The incorporation of natural products in several formulations and their use to treat oral diseases have been widely investigated [21]. The main advantages of these products when compared to conventional antimicrobial agents, as chlorhexidine gluconate, for example, is related to lower bacterial tolerance [22], lower toxicity and no gustative change [23].

There are no studies in the update literature in which the cytotoxic and potential antibiofilm effect of a red propolis extract against a combination of *S. mutans* and *L. casei* were evaluated. Therefore, the aim of this study was to evaluate the *in vitro* antibacterial potential of a red propolis ethanolic extract (RPE) against a cariogenic mixed biofilm, as well as its cytotoxic potential.

Material and Methods

Characterization of Red Propolis Extract

Red propolis was collected in December 2015 from Magé marsh vegetation in the state of Rio de Janeiro (latitude 22º 39 '10 "S, longitude 43º 02' 26" W and 5 m altitude) and stored in a desiccator for one week. The extract was produced at the concentration of 30% of solid mass; that is, for each 100 mL, 70 mL of extractive liquid (80% ethanol) and 30 mL of crude propolis, by means of the maceration process for 70 days. Following, it was subjected to filtering. The mixture was heated at 60ºC for 30 min under stirring, then filtered on Watman paper nº 2 and centrifuged at 7500 *g* at 5ºC for 10 min [9].

Total flavonoids were measured according to previous authors [24] by a colorimetric method and the results were expressed as mg of catechin equivalents [25]. The contents of phenolic acids (caffeic, benzoic, ferulic, *p*-coumaric and 5-caffeoylquinic acids) (Merck KGaA, Darmstadt, Germany), and chlorogenic acids (3-caffeoylquinic, 4-caffeoylquinic, 3,4-dicafeoylquinic, 3,5-dicaffeoylquinic, 4,5-dicaffeoylquinic acids, numbered according to IUPAC numbering system, were

investigated by HPLC-DAD-reverse-phase gradient system [26,27]. DAD was set at 325 nm for chlorogenic acids and 280 nm for phenolic acids.

Determination of the Antimicrobial Activity of the Red Propolis Extract

The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were used to evaluate the antimicrobial activity of the red propolis ethanolic extract (RPE), according to the reference protocol of the Clinical and Laboratory Standards Institute [28], with a modification [29].

To evaluate the MIC, 96-well microtiter plates (Alamar Tecno Científica Ltda., Diadema, SP, Brazil) were used. Initially, 100 µL of Brain Heart Infusion (BHI) broth (BD Difco, Franklin Lakes, NJ, USA) was placed into the wells. Then, 100 µL of the RPE at its initial concentration (30%) was placed at the first column of the 96-well plate. The RPE was then serially diluted by transferring 100 µL of the most concentrated well content to the least concentrated. After dilution, 100 µL of the contents of the last column were dispensed to equal the volume of all wells. Finally, 5 µL of the bacterial inoculum (1.0×10^7 CFU/mL) were inserted in each well, resulting in approximately 5×10^5 CFU/mL per well, and RPE presented a final concentration that varied from 142.85 to 0.069 mg/mL.

To validate the methodology used in this study, we used an antimicrobial control (chlorhexidine 0.12%); a growth control (microbial suspension in development, without addition of any antimicrobials); and a sterility control (sterile culture medium, without addition of antimicrobials or suspensions of microorganisms). The prepared plates were incubated for 24 h at 37°C, with 5% CO_2 [9,15]. After this, the viability of the microorganisms was evaluated by using the resazurin salt reduction method, which consists of a visual method [15,30]. The MIC was then considered the lowest concentration at which bacterial growth was not detected.

The Minimal Bactericidal Concentration (MBC) was obtained by subculturing (20 µL) of the dilutions corresponding to MIC and two dilutions immediately preceding MIC (2×MIC and 4×MIC) on BHI agar plates. The MBC was considered the lowest concentration of the substance that prevented the visible growth of the subculture or the formation of up to three Colony Forming Units (CFU) [14]. Concentrations in which more than three CFUs were formed were considered only inhibitory to microbial growth. All tests were performed in triplicate. The results obtained for the RPE were compared with the positive control for biofilm colonization and negative control.

Cytotoxic Potential

The cytotoxic potential of RPE was evaluated by "dye-uptake" technique [31]. Polystyrene 96-well microplates with monolayers of confluent fibroblast cells L929 (ATCC CCL-1 NCTC) was tested against 0.4% RPE. Minimum Eagle Essential Medium – MEM (Cultilab Materiais Cultura Células, Campinas, SP, Brazil) was used as the diluent (cell maintenance medium) and the plates were incubated at 37°C for 1, 3 and 5 minutes with 5% CO_2. The cell viability reading was performed with a spectrophotometer Elx800 (BioTek Instruments, Winooski, USA) at 492 nm. Percentages of viable

cells of each solution tested were obtained by means of optical density values. White cell control (wells with untreated L929 cells) and a reaction control (1% Tween) were also investigated. Assuming the mean of white cell control was equivalent to 100% of viable cells, it was possible to obtain the toxic potential of that solution.

Antimicrobial Assays with Mixed Biofilm of *S. mutans* and *L. casei*

Cellulose membrane disks (13 mm in diameter, N=16) (EMD Millipore, Burlington, MA, USA) were placed on petri dishes with BHI agar and were used for the formation of mixed biofilms of *S. mutans* and *L. casei*. The inoculum of each microorganisms was standardized at a concentration of 1×10^7 CFU/mL (0.1 absorbance under 625 nm wavelength). Microbial mixed suspension (20 µL) composed of *S. mutans* and *L. casei* were placed on 0.22 mm membrane disks over BHI agar plates.

The system was incubated in microaerophilic condition for 24 h at 37ºC in a microbiological oven. After this period, the disks were collected and placed for 1 min in microtubes containing 1 mL of the treatment solutions: 0.4% RPE or 0.12% Chlorhexidine (CHX) - Pharmacological control. Growth control was not treated. Thereafter, the disks were transferred to microtubes containing 1 mL of saline (0.85% NaCl) and stirred for 2 min (60 Hz) in vortex (Biomixer, VTX-2500, Brazil). Serial dilutions (10^{-1} to 10^{-8}) were then performed to evaluate microbial viability (CFU/mL) and, between one dilution and the next, the suspension was homogenized for 30 seconds [32].

The drop technique was then performed in duplicate for counting viable microorganisms in which 60 µL (3 drops of 20 µL) of each dilution were placed on BHI agar (BD Difco, Franklin Lakes, NJ, USA) plates of petri dishes. The number of colonies forming units (CFU) was determined after 48 h incubation, at 37ºC, in microaerophilia. Data from bacterial yields quantification were logarithmically transformed into Log_{10} CFU/mL for better understanding.

Statistical Analysis

Descriptive and statistical analysis of the data were carried out in SPSS software version 21 (IBM, Chicago, IL - License from Cardiff University, UK). The distribution of the data was verified through the Shapiro-Wilk test. The Mann Whitney test was used to compare the microbial viability as a function of the treatments (propolis extract and controls). All tests were performed with a 5% significance level ($p \leq 0.05$).

Results

The red propolis extract presented concentrations of total flavonoids and chlorogenic acids (CGA) equal to 6030.0 µg/mL and 187.93 µg/mL, respectively. Among the phenolic acids identified, *p*-coumaric acid (67.33 µg/mL) presented the highest concentration (Table 1).

MIC and MBC of RPE was 4.66 mg/mL (0.4%) against *S. mutans* and 8.92 mg/mL (0.8%) (MIC) and 17.85 mg/mL (MBC) against *L. casei*. CHX presented MIC and MBC <0.00002 mg/mL for the *S. mutans* and 0.00047 mg/mL for the *L. casei* (Table 2).

Table 1. Contents of phenolic compounds in the red propolis ethanol extract (RP).

Phenolic Compounds	µg/mL (SD)
Caffeic Acid	1.36 ± 0.03
Benzoic Acid	19.70 ± 0.06
Ferulic Acid	26.67 ± 0.04
Sinapic Acid	33.36 ± 0.05
p-Coumaric Acid	67.33 ± 0.07
Chlorogenic Acids	187.93 ± 0.98
Total Flavonoids	6030.0 ± 70.0 (µg quercetin/mL)
Total Phenolic Compounds	6366.35 ± 2091.23 (µg gallic acid/mL)

Results are shown as mean of triplicate analysis, expressed in µg/mL; SD = Standard Deviation.

Table 2. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of Red Propolis Extract and Chlorhexidine solution (CHX) against strains of *S. mutans* and *L. casei*.

Microorganism	Red Propolis Extract				Chlorhexidine			
	MIC		MBC		MIC		MBC	
	mg/mL	%	mg/mL	%	mg/mL	%	mg/mL	%
S. mutans	4.46	0.446	4.46	0.446	<0.00002	<0.000002	0.0009	0.00009
L. casei	8.92	0.892	17.85	1.785	0.00047	0.000047	0.0009	0.00009

After 1, 3 and 5 min, 0.4% RPE exhibited, respectively, 69.38%, 43.91% and 40.36% of viable cells (Table 3).

Table 3. Cytotoxic potential of 0.4% RPE and control (1% Tween) in oral fibroblasts by the percentage of viable cells.

Incubation Time	% Viable Cells	
	0.4% RPE	1% Tween
1 min	69.38%	18.31%
3 min	43.91%	15.30%
5 min	40.36%	17.50%

The numbers of viable bacteria ($\log_{10}$ CFU/mL) in the RPE (6.55) and CHX group (6.87) were similar ($p>0.05$), but both were better than the growth control to reduce the microorganisms of biofilm ($p<0.05$) (Figure 1).

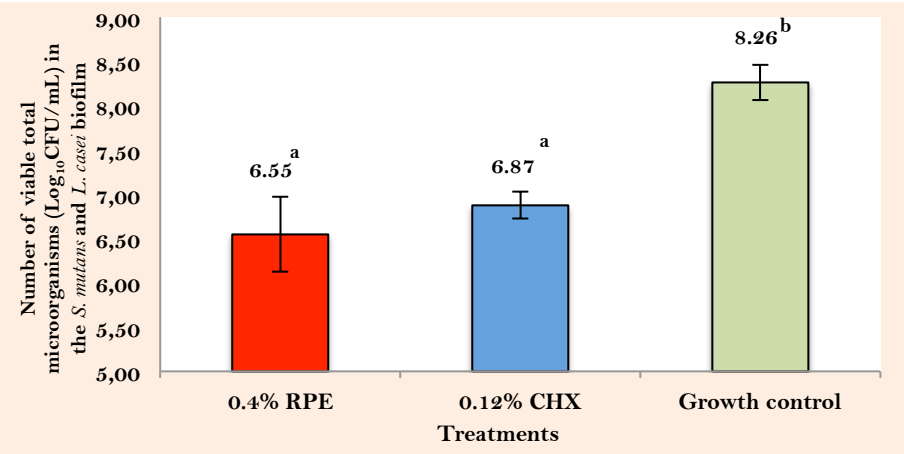

Figure 1. Bacterial viability in biofilms of *S. mutans* and *L. casei* formed during 24 h and submited to a single treatment. Data were logarithmically transformed ($\log_{10}$ CFU/mL). Different letters indicate statistically significant differences between the groups ($p<0.05$, Mann Whitney test).

Discussion

The diversity of natural products and of substances in their composition, gives these products different biological properties, which justifies the development of adjuvant anticaries therapies [34]. Red propolis has previously exhibited antibacterial properties against *S. mutans* [10,15,18], which is the main microorganism associated with the cariogenic biofilm [35].

The phenolic compounds found in the RPE analyzed, as the caffeic acid [36], benzoic acid [36], ferulic acid [18] and *p*-coumaric acid [15], are commonly reported in the literature on red propolis. Different red propolis samples have presented some quantitative variation in their composition, such as the flavonoids and phenolic acids' concentrations, but have similar qualitative profile, which confers various biological properties to the product [36].

RPE showed MIC and MBC of 4.66 mg/mL against *S. mutans* and 8.92 mg/mL (MIC) and 17.85 mg/mL (MBC) against *L. casei*. These values were higher than those exhibited by CHX, that presented good activity against the tested microorganisms [37]. The RPE used in the present study exhibited less antibacterial potential against *S. mutans* when compared with other propolis extracts [10,15,18]. However, it showed similar potential compared to other natural products, as *Melaleuca alternifolia* [14], and better antibacterial activity than *Coffea canephora* [7]. Nevertheless, most of these studies have observed such activity against planktonic cells. Biofilms are much more resistant to antimicrobial agents than planktonic cells [38,39], since bacteria organized in communities and adhered to a substrate are surrounded by an extracellular matrix of polysaccharides, in which there are differentiated phenotypes, metabolism, physiology and genetic transcription, and that acts as a physical barrier against pH changes and antibiotics action, for example [40]. Thus, assays using biofilm models mimic the oral environment more properly.

The cytotoxicity percentage of RPE (0.4%) in L929 fibroblasts ranged between 69.38% and 40.36% during the time periods evaluated in the current study. We considered the present extract as having an acceptable cytotoxicity potential, since the results were lower compared to chlorhexidine, the golden commercial standard [23]. It is also known that chlorhexidine is a broad-spectrum antimicrobial agent, which can lead to microbial resistance. In addition, frequent use of chlorhexidine-based solutions results in loss of gustatory sensitivity and tooth pigmentation [23]. In view of these disadvantages, the use of natural products with antimicrobial activity can be considered [15].

Higher concentrations of natural products are generally required to be effective against mature biofilms [38]. However, in the present study, even in the presence of *S. mutans* and *L. casei* biofilms, treatment with 0.4% RPE proved to be effective. Furthermore, a lower concentration of the extract was used compared to other propolis extracts [9,15]. This is an indicator of a higher antibacterial potential of this product, given the greater resistance of microorganisms in biofilms [39]. These findings indicate the potential of this product for prevention of caries lesions, which can be incorporated into different formulations, such as dentifrices or mouthwashes.

The 0.4% RPE exhibited similar efficacy against *S. mutans* compared to a laboratory-manufactured propolis mouthwash (10%) tincture, with a dilution of 1:5 with water, even though the product concentration [41] was higher than the one used in the present study. The vehicle used was also different [41]. In addition, a clinical trial showed that a typified propolis mouthwash (2%) was more effective in suppressing the levels of salivary *S. mutans* at 14 days and 28 days of use, compared with chlorhexidine and also reduced the levels of lactobacilli after 28 days treatment [42]. The cited study showed similar results to those in the present study, because the propolis product was able to reduce the viability of *S. mutans* and lactobacilli.

In the present study, two types of strains were used for the formation of biofilm, representing the species involved in the onset (*S. mutans*) and progression (*L. casei*) of caries lesions. The biofilm was formed during 24 h to mimic the coaggregation of the microorganisms, aiming its application in the prevention of dental caries. However, it is suggested that new studies should be developed with multispecies biofilms after a longer maturation time (48 h), in order to better simulate the conditions that occur in the mouth in the face of critical pH situations and to verify the efficacy of the product in these circumstances, so that the product can be used as caries therapeutic method.

As limitations of this study, the authors can cite the absence of salivary pellicle and the membrane disks substrate usage to form biofilm, which may hinter the proper simulation of the biofilm formed over mineralized tissues and the process of demineralization. However, this may be considered an initial method to evaluate the antimicrobial efficacy of a product. Further studies should be performed to confirm the results found in the present study. In addition, different product concentrations can be tested to find the ideal concentration showing greater benefit (antibacterial potential) and lower risk (lower cytotoxicity).

Conclusion

Red propolis extract showed antibacterial activity against the tested bacterial strains, exhibited acceptable cytotoxicity and reduced colonization of *S. mutans* and *L. casei* in a membrane disk biofilm model similar to chlorhexidine. The extract was effective against biofilm even at a low concentration, which makes it a promising candidate for the development of complementary products for the control of dental caries without presenting a toxic profile.

Authors' Contributions: MLM wrote and reviewed the manuscript, contributed to the intellectual conceptualization of the study and the entire research project, and performed the statistical analysis. ASNM and JCCFF contributed to the intellectual conceptualization of the study, and performed measurements for the assessments and outcome assessment analysis. TIV contributed to the intellectual conceptualization of the study and reviewed the manuscript, and performed measurements for the assessments and outcome assessment analysis. MBCT and AF contributed to the intellectual conceptualization of the study and reviewed the manuscript. MTVR performed measurements for the assessments and outcome assessment analysis. LCM, YWC and AFG reviewed the manuscript, contributed to the intellectual conceptualization of the study and the entire research project, and outcome assessment analysis.

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Conflict of Interest: The authors declare no conflicts of interest.

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