

In vitro* effectiveness of Brazilian brown propolis against *Enterococcus faecalis

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Abstract: The aim of this study was to evaluate the *in vitro* antimicrobial activity of Brazilian brown propolis as an intracanal medication against *Enterococcus faecalis*. Thirty dentin discs prepared from intact freshly extracted bovine maxillary central incisors were infected with *E. faecalis* for 21 days. The specimens were distributed into six groups according to the medicament used as follows: G1- calcium hydroxide paste; G2- Carbowax 400 (control group); G3- 20% brown propolis paste; G4- 40% brown propolis paste; G5- 20% brown propolis paste + calcium hydroxide paste; and G6- 40% brown propolis paste + calcium hydroxide paste. The experimental pastes were placed into the canal lumen and left for 14 days. After each period, irrigation was performed with sterile saline to remove the medicament, and the canals were dried with sterile paper points. The dentin chips were removed from the canals with sequential sterile round burs at low speed and were immediately collected in separate test tubes containing BHI broth. The tubes were incubated at 37°C, and microbial growth was analyzed by spectrophotometry after 15 days. All the experimental medications significantly reduced the number of viable bacteria. The G4 and G5 pastes were more effective than the G1 paste, with 35.8%, 41%, and 21.3% antibacterial activity, respectively. Brazilian brown propolis shows antibacterial capacity against *E. faecalis*.

Declaration of Interests: The authors certify that they have no commercial or associative interest that represents a conflict of interest in connection with the manuscript.

Keywords: Anti-Infective Agents; Propolis; *Enterococcus faecalis*.

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DOI: 10.1590/1807-3107BOR-2015.vol29.0058

Introduction

Approximately 700 species of microorganisms comprise the oral microflora, many of which are closely associated with the development of periapical lesions.¹ Endodontic infections are considered polymicrobial,² and *Enterococcus faecalis* is a gram-positive, facultative anaerobe that is often isolated during root canals with dental pulp necrosis and persistent infections after endodontic treatments.^{2,3} This microorganism is able to penetrate deep into the dentinal tubules and is difficult to eliminate after biomechanical treatment.³ *E. faecalis* is considered a persistent endodontic pathogen,⁴ which makes its control and elimination a challenge for the success of endodontic treatment.

Sanitizing infected root canals through biomechanical treatment and auxiliary agents in order to eliminate bacterial contamination and prevent reinfection has been a challenge for endodontics.⁵ Biomechanical treatment of root canals significantly reduces microbial organisms in the endodontic

Submitted: Sep 14, 2014
Accepted for publication: Jan 16, 2015
Last revision: Apr 08, 2015

microbiota,⁶ but it is unable to completely disinfect some areas due to anatomical complexities, causing persistent microorganisms to survive, multiply, and reinfect the pulp space.⁷ Calcium hydroxide has been the most used intracanal medication due to its antimicrobial properties, promotion of a temporary physical sealing of the root canal, and osteogenic potential.⁸ However, there is evidence of resistance to this medication by *E. faecalis*.³ Because there are limitations to the complete removal of endodontic biofilms by conventional biomechanical treatment techniques, the search for alternative substances that are effective against resistant pathogens, such as *E. faecalis*, is growing.⁴

Propolis is a resinous product containing secretions from bees and plant resins, whose composition depends on the regional climate, flora, and time of year in which it is collected.⁹ There are different types of propolis that are characterized and classified according to their chemical composition.⁹ More than 300 components have been identified, including phenolic compounds, such as flavonoids, phenolic acids, and phenolic acid esters, the main components of propolis from temperate regions.¹⁰ Among tropical countries, Brazil has the highest chemical diversity of propolis types, such as those that contain phenylpropanoids, prenylated phenylpropanoids (e.g., artepillin C), and sesqui- and diterpenoids,¹¹ which confer immunostimulatory, antioxidant, anti-inflammatory, and antimicrobial properties, respectively.¹²

Studies have shown the potential of propolis against resistant microorganisms.^{13,14,15} Thus, this study aimed to investigate the potential antimicrobial activity of brown propolis from the Brazilian Cerrado with or without calcium hydroxide paste as an intracanal medication against *E. faecalis*.

Methodology

Obtaining the crude brown propolis extract

Brown propolis was collected in the Cerrado region of the state of Mato Grosso, the second largest biome in Brazil, situated at 19° 40' of south latitude, where the annual average temperature is 24°C. The crude ethanolic extract of propolis was obtained by extraction in 80% cereal alcohol at 60°C and subsequent

concentration in a rotaevaporator (Rotary evaporator 802, Fisatom, São Paulo, Brazil).¹⁶

Minimum inhibitory concentration (MIC)

To determine the MIC of the ethanolic extract of brown propolis, a broth microdilution was performed. The extract was serially solubilized in dimethyl sulfoxide (DMSO)¹⁶ at concentrations of 20 mg/mL, 50 mg/mL, 100 mg/mL, and 200 mg/mL. Four to five 24-hour colonies of *E. faecalis* were selected (ATCC 29212) and grown in Muller-Hinton Broth (Difco Laboratories, Mogi das Cruzes, São Paulo, Brazil). The turbidity of the cell suspension was adjusted to obtain approximately 1.5×10^8 cells/mL and then diluted to obtain the inoculum at a concentration of 10^7 cells/mL. Chloramphenicol was used as a standard. The resazurin technique was conducted to assess cell viability.¹⁶ Each concentration of the extract was assayed in duplicate, and the test was repeated three times. The MIC of brown propolis against *E. faecalis* was 1000 mg/mL.

Intracanal medications

For the preparation of the experimental pastes, the following were used: high-purity calcium hydroxide (Hidroxil, Inodon, Porto Alegre, Brazil), polyethylene glycol (Carbowax 400, Henrifarma, São Paulo, Brazil), 20% brown propolis extract, and 40% brown propolis extract. The intracanal medications, manipulation ratios, and the groups that represent them are described in Table 1. The pH values of the brown propolis and calcium hydroxide pastes, alone and combined, were measured by a digital pH meter.

Preparation of root dentin blocks

The dentin block model used in this study was modified from Gomes *et al.*¹⁷ Briefly, 30 intact freshly extracted bovine maxillary central incisors with complete root formations were selected. After being cleaned, they had their surfaces disinfected and preserved in 0.1% thymol solution under refrigeration until specimen preparation. Root segments with a length of 4 mm were prepared by cutting off the apical 5 mm and coronal two-thirds. Each 18 mm-long specimen with a central pulp space was divided into three blocks. Only the middle segments that had

Table 1. Descriptive analysis of the bacterial growth rate based on the variable intracanal medication.

Intracanal medication*	Number of samples	Mean minimum absorbance (570 nm)	Mean maximum absorbance (630 nm)	Mean bacterial growth rate (%)
G1	24	0.61	0.28	-21.35
G2	30	0.45	0.15	3.66
G3	29	0.61	0.30	-34.59
G4	24	0.67	0.30	-35.82
G5	30	0.71	0.35	-41.05
G6	30	0.50	0.23	-29.83

* G1- base paste; G2- Carbowax 400; G3- 20% brown propolis paste; G3- 40% brown propolis paste; G5- 20% brown propolis paste + base paste; G6- 40% brown propolis paste + base paste.

an external diameter of approximately 6 mm were used. The root cementum was removed, and the root canals were enlarged by using carbide round burs with a 2.1 mm diameter (Dentsply, Petrópolis, Brazil). Organic and inorganic debris and the smear layer were removed. The specimens were individually placed in bijoux bottles containing 3.0 mL BHI and autoclaved for 15 minutes at 121°C. Sterility was checked by incubating the bijoux bottles for 24 hours at 37°C.

Inoculation and disinfection of the dentin specimens

Isolated 24-hour colonies of pure *Enterococcus faecalis* cultures (ATCC 29212) grown on 5% defibrinated sheep blood-BHI agar plates were suspended in 5 mL of sterile Brain Heart Infusion broth (BHI; Difco Laboratories, Mogi das Cruzes, São Paulo, Brazil) and adjusted to approximately 1.5×10^8 CFU mL⁻¹. Two milliliters of sterile BHI was replaced by the bacterial inoculum. The specimens were kept at 37°C for 21 days, and 1 mL of contaminated BHI was replaced by freshly prepared BHI every 2 days to avoid medium saturation. The turbidity of the medium during the incubation period indicated bacterial growth. The purity of the cultures was confirmed by Gram staining and colony morphology on BHI agar blood.

After the infection period, the specimen canals were irrigated with 5 mL of 0.9% sterile saline and dried with sterile paper points. The outer specimen surfaces were covered with nail varnish to prevent contact of the medicament with the external surface. The dentin blocks were fixed at the bottom of the wells in 24-well cell culture plates (Corning, Hexis Científica, Jundiaí, Brazil) with decontaminated sticky wax, which also obliterated the apical surface of the root canal.

The wells were then filled with agar at 46°C until it reached the upper surface of the dentin specimens. The intracanal medications were applied to the canal lumen until the canals were completely full. The 30 dentin blocks were divided into six groups according to the experimental paste used as follows (N = 5/group): G1: Calcium hydroxide paste (high-purity calcium hydroxide + Carbowax 400; 4 g/4.5 mL); G2: Carbowax 400 (negative control); G3: 20% crude brown propolis extract + Carbowax 400 (0.2 g/mL); G4: 40% crude propolis extract + Carbowax 400 (0.4 g/mL); G5: 20% crude propolis extract + calcium hydroxide paste (1:1); and G6: 40% crude propolis extract + calcium hydroxide paste (1:1). Finally, the specimens were incubated at 37°C for 14 days.

After the experimental period, the medicament was washed with sterile saline. Dentin shavings from the internal surface of the canal were obtained with spherical drills with progressive diameter numbers 6 and 8 (Dentsply, Petrópolis, Brazil) at a low speed, allowing the evaluation of the medication diffusion within the dentin structure. The dentin shavings within each drill bit and the remainder of the specimens were incubated in 3 mL of BHI broth at 37°C for 15 days.¹⁷ The tubes were evaluated daily to verify the turbidity of the broth and to identify indications of bacterial growth. At the end of the experimental period, bacterial growth was analyzed by spectrophotometry (ELISA reader, SpectraMax 190 Absorbance Microplate Reader, Sunnyvale, USA) at wavelengths of 570 nm (reduction) and 630 nm (oxidation). For this purpose, 100 µL from the contents of each tube was transferred in triplicate to 96-well cell culture plates. Thirty microliters of 0.01% resazurin aqueous solution was added, and

the plates were incubated at 37°C for 4 hours. The bacterial growth rate was calculated according to the formula developed by Szliszka *et al.*¹⁶

Statistical analysis

The statistical one-way ANOVA and Tamhane post-hoc tests were performed to compare the different intracanal medications. To evaluate the correlation between the pH values and the bacterial growth rate, the Pearson correlation test was applied. The significance was established at a 5% level ($p < 0.05$).

Results

The average pH values of the medications tested for groups G1 to G6 were 13.1, 9.5, 6.8, 5.8, 11.8, and 12.6, respectively, which showed no correlation with bacterial growth ($p = 0.758$).

Bacterial growth was only associated with the intracanal medication ($p = 0.000$) and not with the diffusion of the pastes within the dentin ($p = 0.174$), evaluated by the different bur sizes or the combination of both ($p = 0.705$). All the experimental pastes showed bacterial growth inhibition, with the exception of the negative control group (G2), confirming the efficacy of the methodology (Table 1). However, the 20% brown propolis paste associated with calcium hydroxide and the 40% brown propolis paste exhibited better results against *E. faecalis*, compared with the calcium hydroxide paste (Figure 1). Moreover, the calcium hydroxide paste showed similar results to the 20% brown propolis paste

and the 40% brown propolis paste associated with calcium hydroxide (Figure 1).

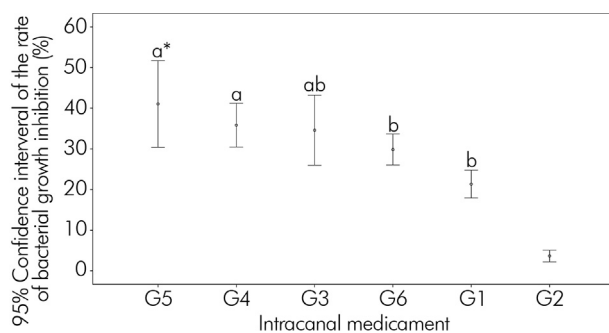
Discussion

Several methods have been proposed to evaluate the potential antimicrobial activity of intracanal medications for treating endodontic infections, such as the agar diffusion test,⁵ the broth microdilution test^{13,18} and the dentin infection and disinfection tests.^{14,15,17,18,19}

In this study, broth microdilution was conducted to determine the MIC of brown propolis against *E. faecalis*, which was 1000 mg/mL. By contrast, lower MIC values against *E. faecalis* were observed for other types of propolis, such as 4 mg/mL of the Turkey TB propolis,²⁰ 340 mg/mL of the Iran propolis,¹⁹ and higher values of 6,425 mg/mL for the propolis from southern Brazil.²¹ In addition, different MIC values were observed for Brazilian green propolis against several microorganisms, including *Peptostreptococcus anaerobius* (20.8 mg/mL), *Porphyromonas gingivalis* (294.4 mg/mL), *Fusobacterium nucleatum* (256 mg/mL), and *Prevotella melaninogenica* (204.8 mg/mL).²² There is evidence that the differences in antimicrobial activity depend on the bacterial strains involved and the composition of the propolis extracts,²³ which our group is already researching.

The infection and disinfection tests of bovine dentin were conducted using *E. faecalis* (ATCC 29212) to evaluate the antimicrobial potential of brown propolis; this bacterium has been extensively investigated^{14,15,21} because it is often isolated in root canals with persistent infections.¹⁸ There is evidence that the presence of *E. faecalis* in teeth with filled canals, cases of chronic apical periodontitis,²⁴ and infections after endodontic treatments are due to its high resistance to antimicrobials,^{3,21} its survival for long periods in environments deprived of nutrients and oxygen,²⁴ and its high virulence. These characteristics of *E. faecalis* are likely a result of its production of aggregation substances, surface adhesins, extracellular superoxide, lytic enzyme gelatinase, and hyaluronidase.²⁵ In the present study, the period of bovine dentin contamination was 21 days, which was confirmed previously by electron scanning microscopy.¹³

Bovine teeth were used in this research because they are easy to obtain and handle.¹⁷ Additionally, the



*Identical letters represent statistically equal values.

Figure 1. Graphical representation of the 95% confidence intervals for growth rate inhibition according to the tested intracanal medications.

structure of the bovine dentin resembles the human dentin with respect to quantity, size, shape, diameter, and density of the dentinal tubules, which enables the use of bovine dentin in methods evaluating antimicrobial agents.¹⁸

To overcome the difficulty of controlling endodontic biofilms with biomechanical preparations of the root canal system, irrigation agents and intracanal medications should be chosen based on their biocompatibility with periapical tissues and their efficacy against resistant pathogens. The use of propolis has been encouraged due to its therapeutic properties, including antimicrobial activity against endodontic pathogens.^{13,14,15,19} The biological activities of propolis are mainly related to the presence of phenols and polyphenols, which are aromatic substances that derive flavones, flavonoids, and flavonols and are active against the bacterial cell wall.²⁵

There is no evidence of the optimal concentration of brown propolis against *E. faecalis*. Therefore, based on MIC results and the fact that experimental pastes that may work as a barrier for microorganisms have been applied within bovine dentin,^{17,18} we decided to study the antimicrobial activity of 20% and 40% brown propolis extracts.

In the present study, the bacterial growth of *E. faecalis* was inhibited by all the experimental medications. However, the 40% brown propolis extract (G4) and the 20% brown propolis extract associated with calcium hydroxide paste (G5) were more effective than calcium hydroxide paste (G1), with bacterial growth inhibitions of 35.8%, 41%, and 21.3%, respectively. Although no previous studies have evaluated the antibacterial activity of brown propolis, the greater effectiveness of propolis over calcium hydroxide has been observed in previous studies.^{13,14,15,19}

Calcium hydroxide paste has strong biological and antimicrobial properties due to its highly alkaline pH⁸ and is considered the gold standard intracanal medication for treating endodontic infections. However, calcium hydroxide has disadvantages, such as its low potential against *E. faecalis*,^{3,26} which can survive in dentin tubules for long periods in the presence of the antimicrobial agent.^{18,26} Thus, in this study, the brown propolis extracts were mixed with

calcium hydroxide to observe a possible improvement of the calcium hydroxide paste against *E. faecalis*.

All medications based on the tested brown propolis showed antimicrobial activity against *E. faecalis*, which is in agreement with previous studies^{13,14,19,27} and with the fact that propolis exhibits significant antimicrobial activity against the more resistant, gram-positive facultative and strictly anaerobic species.²⁷ The 20% crude propolis extract associated with calcium hydroxide paste exhibited an antimicrobial activity similar to the 40% crude propolis extract associated with calcium hydroxide paste, but it performed better than the isolated calcium hydroxide. Because there are no previous studies regarding the association between calcium hydroxide paste and crude propolis extracts at different concentrations, this result indicates the need for further investigation into the isolation and identification of brown propolis components. Furthermore, future studies will help to explain the synergy between the calcium hydroxide components and the propolis components, which is of fundamental importance for determining the antimicrobial potential of propolis.¹⁴

In this investigation, the average pH of the 20% and 40% propolis pastes was 6.8 and 5.8, respectively. Most bacteria present in endodontic infections show growth at a pH between 6.5 and 7.5, and only a few microorganisms thrive at higher pH values,¹⁸ such as *E. faecalis*, which is capable of growing in an alkaline pH exceeding 11.5.⁴ In agreement with a previous study,²⁸ the association of the 20% and 40% propolis pastes with calcium hydroxide resulted in a pH increase from 6.8 and 5.8 to 11.8 and 12.6, respectively. However, in this study, the pH did not affect the antimicrobial activity of the intracanal medications studied.

Finally, further investigations are necessary to evaluate biocompatibility with periapical tissues, to isolate and identify the components of brown propolis, and to analyze the synergism between the components of propolis and calcium hydroxide.

Conclusion

The present study demonstrated that, although medications based on brown propolis with or without calcium hydroxide have limitations inherent to an *in vitro* study, they are effective against *E. faecalis*.

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