# The Inhibitory Activity of Typified Propolis against *Enterococcus* Species

Bernard J. Moncla[a,b], Peter W. Guevara[b], James A. Wallace[a], Maria C. Marcucci[c], Jacques E. Nor[d], and Walter A. Bretz[e,*]

[a]  School of Dental Medicine, University of Pittsburgh, Departments of Oral Biology and Endodontics, 3501 Terrace Street, Pittsburgh, PA 15260, USA
[b]  School of Medicine, University of Pittsburgh and Magee-Women's Research Institute, 3550 Terrace Street, Pittsburgh, PA 15260, USA
[c]  School of Pharmacy, Universidade Anhanguera-Uniban, Rua Maria Cândida, 1813, São Paulo, SP, Brazil
[d]  University of Michigan, School of Dentistry, Department of Cariology, Endodontics and Restorative Sciences, 1011 N. University Ave, Ann Arbor, MI 48109, USA
[e]  New York University College of Dentistry, Department of Cariology and Comprehensive Care, 345 E. 24th Street, New York, NY 10010, USA.
     Fax: +1-212-9989914. E-mail: wb36@nyu.edu

* Author for correspondence and reprint requests

Propolis, a natural bee product widely used for its antimicrobial activity, was tested against isolates of *Enterococcus* from humans, pig-tailed macaques, isolates of refractory endodontic treatment cases, and isolates from *Lactobacillus*-containing food supplements. Typification of the propolis was performed by high-performance liquid chromatography (HPLC) by which prenylated compounds, cinnamic acid derivatives, and flavonoids were detected as the main constituents. Minimum inhibitory concentrations (MIC) were determined using the agar dilution method. All human and animal *Enterococcus* isolates demonstrated MIC values of 1600 µg/mL. Enterococcal species of human and animal origin were inhibited by propolis. Particularly, human isolates of *E. faecium* and *E. faecalis* of refractory endodontic treatment cases were susceptible to propolis of Brazilian origin.

*Key words:* Propolis, *Enterococcus*, MIC

## Introduction

*Enterococcus faecalis* and *E. faecium* naturally inhabit the gastrointestinal tract and the female genital tract, are often present in various foods, and are found in some natural food supplements by accident or design (Facklam and Washington, 1991; Facklam *et al.*, 1995, 1999; Hughes and Hillier, 1990). These organisms have been of increasing concern in medicine because of their involvement in nosocomial infections, endocarditis, and septicemia (Kirschner *et al.*, 2001; Park and Walsh, 1997). Vancomycin resistance among strains of *Enterococcus* is of concern because the resistance is transmissible, and these organisms may therefore act as a reservoir for resistance (Facklam *et al.*, 1995; Park and Walsh, 1997; Bystrom and Sundqvist, 1981; Heaton *et al.*, 1996).

Treatment of infected root canals is a very successful procedure. However, about 5% of teeth so treated experience treatment failure (Sundqvist *et al.*, 1998; Sjogren *et al.*, 1990). Successful treatment requires the sterilization of the root canal system and complete apical seal (Sundqvist *et al.*, 1998; Sjogren *et al.*, 1990). *E. faecalis, E. faecium*, and other species have been recovered from root canals and periapical tissues of previously endodontically treated teeth and are believed to be involved in treatment failures (Bystrom and Sundqvist, 1981, Sundqvist *et al.*, 1998; Peciuliene *et al.*, 2000; Siren *et al.*, 1997). The possible reasons for treatment failure include: 1) failure to achieve a complete apical seal; 2) incomplete sterilization of the canal system; 3) persistent infection at the time of canal obturation; 4) reinfection or introduction of bacteria during instrumentation; and 5) reinfection through apical dentinal tubules (Siren *et al.*, 1997). The underlying source of the *Enterococcus* species in such failures is unknown but could likely be endogenous, from the diet or from the operator during root canal instrumentation.

Propolis is a resin-like product extracted from plants by honey-bees that mix the resin with salivary secretions and use the resulting mixture to seal and to repair their hives. Propolis use dates back to about 300 BC when it was first used for cosmetics and as a medicine (Dobrowolski *et al.*, 1991; Kujumgiev *et al.*, 1999). The typical chemical composition of propolis is 50% resin and vegetable balsam, 30% wax, 10% essential and aromatic oils, 5% pollen, 5% minerals and flavonoids. The chemical composition is dependent on the vegetation from which the material was collected (Kujumgiev *et al.*, 1999; Marcucci *et al.*, 2000; Moreno *et al.*, 1999; Sforcin *et al.*, 2000). There are several methods that can be used to extract propolis in order to develop preparations and solutions. The most common method that has been used is by ethanol extraction.

A number of studies have reported that propolis has antibacterial, anti-inflammatory, antifungal, antiviral, anesthetic, antiulcer, immunostimulant, and wound-healing properties (Kujumgiev *et al.*, 1999; Banskota *et al.*, 2001; Cetinkaya *et al.*, 2000; Koo et al., 2000, 2002; Santos *et al.*, 1999; Yatsuda *et al.*, 2000). The antimicrobial properties of propolis may relate to or be a function of the flavonoids (Banskota *et al.*, 2001; Cetinkaya *et al.*, 2000; Mirzoeva *et al.*, 1997) and of other propolis components such as hydroxyacids, sesquiterpenes or phenolics (Banskota *et al.*, 2001). Accordingly, typification of propolis is an essential requirement in order to characterize its moieties, quantify its main active compounds and their respective biological applications.

Bretz and collaborators (1998) compared the effects of propolis and calcium hydroxide on direct dental pulp exposures in animals. Propolis was at least comparable to calcium hydroxide in exhibiting normal reorganization of the pulp and no increased vascularity, and in maintaining a low inflammatory and microbial cell population.

Because of the suspected importance of *Enterococcus* species in endodontic treatment failure and their increasing importance in nosocomial infections, we have studied a number of human and animal isolates, reference strains, and strains from food supplement sources as to determine their susceptibility to propolis.

## Methods and Materials

### Propolis and vancomycin solutions

Crude propolis [source: alecrim (*Baccharis dracunculifolia*)] was obtained from Piracicaba, São Paulo, Brazil. Propolis was extracted in a Soxhlet extractor with 95% ethanol at 50 °C for 24 h (three 8-h periods). The resulting syrup was dried under vacuum and stored at –80 °C until used. Subsequently, the propolis syrup was washed with 100 mL of cold ethanol. The solution was then filtered and stored at –20 °C until used. Working stock solutions were prepared at a concentration of 160 mg/mL in either 100% ethanol or dimethyl sulfoxide (DMSO). Serial twofold dilutions of the stock solutions were used to give a final concentration of propolis ranging from 50 $\mu$g/mL to 1600 $\mu$g/mL. Vancomycin/HCl (Sigma-Aldrich, St. Louis, MO, USA) dilutions ranging from 0.5 $\mu$g/mL to 16 $\mu$g/mL served as positive controls.

### Bacterial samples

Human isolates of *Enterococcus* species used in this study represented rectal and vaginal isolates from 87 women and animal isolates that were obtained from 3 pig-tailed macaques (Dr. S. L. Hillier, University of Pittsburgh, School of Medicine, Department of Obstetric and Gynecology, Pittsburgh, PA, USA). Two human isolates were attained from clinical cases of endodontic treatment failures (G. Sundqvist, Umeå University, Faculty of Dentistry, Department of Oral Microbiology, Umeå, Sweden). Twenty-four *Enterococcus* isolates were recovered from six *Lactobacillus*-containing health food supplements. Control organisms included *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922, and *E. faecalis* ATCC 29212.

Health food supplements were purchased locally and stored as recommended by the manufacturers. Capsules were aseptically removed, placed into 10 mL of Mueller-Hinton broth (Difco, MA, USA), and incubated at 37 °C as to dissolve the capsules. Samples were dispersed with a vortex mixer, and serial ten-fold dilutions were prepared in the same media, plated on blood agar plates, and incubated overnight at 37 °C. Typical *Enterococcus* colonies were selected, subcultured to establish purity, and identified at the genus level as described previously (Facklam and Washington, 1991; Facklam *et al.*, 1995, 1999).

### Microbiological procedures and minimum inhibitory concentration (MIC)

MIC values of propolis and vancomycin were determined using agar dilution methods in accord-

ance to CLSI standard methods for susceptibility testing (Watts and Shryock, 2005). Briefly, Mueller-Hinton agar (Difco, Dearborn, MI, USA) was used to carry out experiments. Each test solution (at the various concentrations tested) was mixed with the agar to give a final content of the solvent of 2%. Bacterial strains were cultured on blood agar, isolated colonies selected and suspended in saline to a density of 0.5 MacFarland units and then diluted 1:10 in saline. Bacterial samples were placed in 3 different wells of a Steer's replicator (400 $\mu$L) (VWR Corp., Radnor, PA, USA) and inoculated onto agar plates. The bacterial samples were allowed to dry on the agar at room temperature and incubated overnight at 37 °C in air enriched with 5% $CO_2$. Then the bacterial growth was determined. All experiments were conducted in duplicate and were repeated on three separate days.

## Composition of propolis assessed by HPLC

The typified propolis sample (named BRP1) used in this study was analyzed by high-performance liquid chromatography (HPLC) (Merck-Hitachi, Darmstadt, Germany) with L-7100 pumps and an L-7200 auto-sampler. The chromatographic column was a reverse phase column Lichrochart 100 RP-18 (12.5 x 0.4 cm, particle diameter of 5 $\mu$m; Merck). The mobile phase was water/formic acid (95:5, v/v) (solvent A) and methanol (solvent B) at the flow of 1 mL/min using a linear gradient. The time of analysis was 50 min, and the detection was performed at 280 and 340 nm using a diode array (detector L-7450; Merck-Hitachi). The software used for data analysis was that provided by the manufacturer (DAD Manager, Darmstadt, Germany). All compounds were identified by comparison with authentic standards (same retention time and UV spectra) evaluated by diode array.

## Results

MIC values for typified propolis were determined using two different solvents, ethanol and DMSO. Comparable results were observed for both solvents (Table I). The majority of reference strains and isolates from patients refractory to endodontic treatment exhibited MIC values equal to the maximum concentration of propolis tested (Table I), with the exception of *S. aureus* MIC values for both propolis solvents which differed from each other and were of lower concentration. Only one strain of *E. faecium* demonstrated resistance to the maximum concentration of propolis tested when ethanol was used as the carrier for propolis. Similar resistance patterns were found for *E. coli* for both solvents. The reference strains of *S. aureus* and *E. faecalis* remained within control limits of 0.5 – 2 $\mu$g/mL and 1 – 4 $\mu$g/mL, respectively, for the vancomycin assays (data not shown).

Table II presents the percentage of isolates and the corresponding MIC values for propolis with DMSO as a solvent. The 97 human and animal isolates showed susceptibility to propolis at 1600 $\mu$g/mL. In addition, these isolates were sensitive to vancomycin in the concentration range of 0.5 – 8 $\mu$g/mL (data not shown). *Enterococcus* strains isolated from food supplements had MIC

Table I. Propolis MIC values of reference strains and human isolates.

| Organism | MIC in ethanol [$\mu$g/mL] | MIC in DMSO [$\mu$g/mL] |
|---|---|---|
| *E. coli* ATCC 25922 | >1600 | >1600 |
| *S. aureus* ATCC 29213 | 400 | <50 |
| *E. faecalis* ATCC 29212 | 1600 | 1600 |
| *E. faecalis* 3199[a] | 1600 | 1600 |
| *E. faecium* 3266[a] | >1600 | 1600 |

[a] Isolates were recovered from refractory cases of endodontic treatment.

Table II. Propolis MIC values for *Enterococcus* species from various sources.

| Source | Number of isolates | % of isolates | | | |
|---|---|---|---|---|---|
| | | MIC <400[a] $\mu$g/mL | MIC 800 $\mu$g/mL | MIC 1600 $\mu$g/mL | MIC >1600 $\mu$g/mL |
| Human root canal | 2 | | | 100 | |
| Human other sites | 88 | | | 100 | |
| Pig-tailed macaque | 7 | | | 100 | |
| Food supplements | 24 | 19 | 14 | 39 | 28 |

[a] Propolis solutions dissolved in DMSO.

values that ranged from <400 to >1600 µg/mL of propolis.

Fig. 1 shows the HPLC chromatogram for the typified sample of propolis employed in this study. Table III shows the compounds identified by HPLC. The majority of the compounds were derived from cinnamic acid and *p*-coumaric acid. Flavonoids were also detected but to a lesser extent.

## Discussion

Previous reports have demonstrated differences in propolis antibacterial action against Gram-positive and Gram-negative organisms, as well as variations in the chemical composition of propolis material dependent upon the location from where the material was derived and how preparations or solutions were designed (Banskota *et al.,* 2001; Marcucci *et al.*, 2000; Marcucci and Bankova, 1999). Most propolis studies have used ethanol as the solvent and have relied on either agar or disc diffusion methods, or agar dilution methodology to determine the MIC values for various bacterial species.

We investigated the use of DMSO and ethanol in a standard protocol recommended for the measurement of MIC values of *Enterococcus* species (Watts and Shryock, 2005). Agar dilution

plates prepared with propolis dissolved in ethanol were much less homogeneous in appearance, especially at higher propolis concentrations, when compared with plates prepared with propolis dissolved in DMSO. Their comparisons were generally in agreement (Table I) with two exceptions where one strain of *E. faecium* required more than 1600 µg/mL of propolis dissolved in ethanol while complete inhibition was observed on plates prepared with 1600 µg/mL of propolis dissolved in DMSO. *S. aureus* had a MIC value of 400 µg/mL in the former and <50 µg/mL in the latter. These observations may be explained as a result from a more homogeneous suspension of propolis diluted in DMSO than propolis diluted in ethanol. Alternatively, DMSO may facilitate the transport of propolis biologically active compounds into the cells. There are other solvents that can be used for propolis such as Tween 80 and sorbitol that are as equally effective as DMSO (unpublished data). These solvents are safe and would be indicated for clinical use as opposed to DMSO.

Santos and colleagues (2002a) reported that Gram-negative anaerobes and microaerophilic organisms (*Actinobacillus actinomycetemcomitans*, *Fusobacterium* spp., and *Bacteroides fragilis*) were susceptible to concentrations of propolis ethanolic extracts and commercial preparations
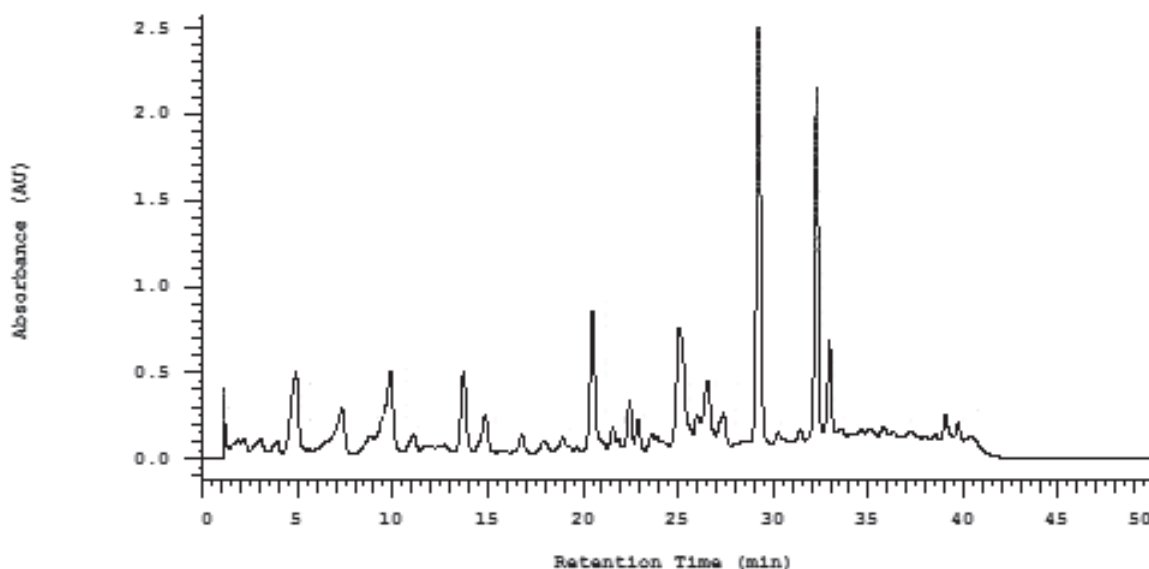


Fig. 1. Retention time (min) at 2.43: caffeic acid; 3.95: *p*-coumaric acid; 4.92: ferulic acid; 20.57: 3-prenyl-4-hydroxycinnamic acid; 22.90: 2,2-dimethyl-6-carboxyethenyl-2*H*-1-benzopyrane; 29.23: 3,5-diprenyl-4-hydroxycinnamic acid; 32.29: compound E; and 32.96: 6-propenoic-2,2-dimethyl-8-prenyl-2*H*-1-benzopyran acid.

Table III. Quantitative analysis of propolis compounds identified by HPLC.

| No.[a] | Compound | Identification[b] | Content (mg/g)[c] |
|---|---|---|---|
| 1 | (*E*)-3-[4-Hydroxy-3-[(*E*)-4-(2,3)-dihydrocinnamoyloxy-3-methyl-2-butenyl]-5-prenylphenyl-2-propenoic acid | P | 3.67 ± 0.10 |
| 2 | 2,2-Dimethyl-6-carboxyethenyl-2*H*-1-benzopyran | - | 5.05 ± 0.02 |
| 3 | 2,2-Dimethyl-8-prenyl-2*H*-1-benzopyran-6-propenoic acid | - | 13.24 ± 0.23 |
| 4 | 3,4-Dihydroxy-5-prenylcinnamic acid | P | 1.49 ± 0.01 |
| 5 | 3,5-Diprenyl-4-hydroxycinnamic acid[d] (derivative 11) | P | 0.84 ± 0.04 |
| 6 | 3,5-Diprenyl-4-hydroxycinnamic acid[d] (derivative 12) | P | 1.15 ± 0.05 |
| 7 | 3,5-Diprenyl-4-hydroxycinnamic acid[d] (derivative 13) | P | 2.34 ± 0.11 |
| 8 | 3,5-Diprenyl-4-hydroxycinnamic acid[d] (derivative 2) | P | 1.21 ± 0.03 |
| 9 | 3,5-Diprenyl-4-hydroxycinnamic acid[d] (derivative 6) | P | 3.30 ± 0.12 |
| 10 | 3,5-Diprenyl-4-hydroxycinnamic acid (ARTEPILLIN C®) | P | 26.39 ± 1.23 |
| 11 | 3-[4-Hydroxy-3-(oxobutenyl)]-phenylacrylic acid | P | 0.82 ± 0.01 |
| 12 | 3-Prenyl-3(*E*)-(4-hydroxy-3-methyl-2-butenyl)-5-prenylcinnamic acid | P | 1.57 ± 0,05 |
| 13 | 3-Prenyl-4-(2-methylpropionyloxy)-cinnamic acid | P | 0.91 ± 0.04 |
| 14 | 3-Prenyl-4-dihydrocinnamoyloxycinnamic acid | P | 5.09 ± 0.11 |
| 15 | 3-Prenyl-4-hydroxycinnamic acid | P | 5.43 ± 0.20 |
| 16 | 6-Propenoic-2,2-dimethyl-8-prenyl-2*H*-1-benzopyran acid | P | 4.39 ± 0.07 |
| 17 | Betuletol | P | 0.21± 0.02 |
| 18 | Caffeic acid | P | 1.55 ± 0.03 |
| 19 | Caffeoylquinic acid 1 | P | 13.61 ± 0.67 |
| 20 | Caffeoylquinic acid 2 | P | 0.69 ± 0.03 |
| 21 | Caffeoylquinic acid 3 | P | 2.91 ± 0,04 |
| 22 | Cinnamic acid[e] (derivative 2) | P | 2.39 ± 0.02 |
| 23 | Cinnamic acid[e] (derivative 3) | P | 65.68 ± 4.57 |
| 24 | Dihydrokaempferide | P | 2.41 ± 0.13 |
| 25 | Ferulic acid | P | 6.06 ± 0.95 |
| 26 | Kaempferide | P/F | 15.89 ± 1.07 |
| 27 | Kaempferol | P/F | 5.13 ± 0.08 |
| 28 | *p*-Coumaric acid | P | 16.95 ± 1.03 |
| 29 | Pinobanksin | P/F | 30.29 ± 2.95 |
| | Total amount (mg/g) | - | 240.67 ± 14.01 |
| | Total amount (%) (w/w) | - | 24.07 ± 1.40 |

[a] There is no correlation with the retention time.
[b] P, phenol; F, flavonoid.
[c] In milligrams of each compound per gram of crude resin.
[d] Same UV spectra of 3,5-diprenyl-4-hydroxycinnamic acid with different retention times, expressed as 3,5-diprenyl-4-hydroxycinnamic acid derivative.
[e] Same UV spectra of cinnamic acid with different retention time. Expressed as cinnamic acid.

in the range of 50–5000 $\mu$g/mL. The same group of investigators reported MIC values of propolis ethanolic extracts and commercial preparations for *Prevotella intermedia*, *P. nigrescens*, and *Porphyromonas gingivalis* in the range of 64–256 $\mu$g/mL (Santos *et al.*, 2002b). It has been suggested that Gram-positive organisms are more sensitive to propolis than Gram-negative bacteria (Banskota *et al.*, 2001). Our results have demonstrated that *Enterococcus* species required a moderate to high concentration of propolis before inhibition of growth was observed. All human and animal isolates were inhibited by 1600 $\mu$g/mL of propolis dissolved in DMSO (Tables I and II). Interestingly, the *Enterococcus* isolates obtained from food supplements demonstrated greater diversity in the MIC values ranging from less than 400 $\mu$g/mL to greater than 1600 $\mu$g/mL. Differences in MIC profiles for the various pathogens described above could be explained by plant origin of the propolis and by different methodologies in the preparation of propolis solutions.

There is evidence in the literature suggesting that propolis ethanolic extracts can inhibit the growth of *Streptococcus mutans*, *E. faecalis*, and *S. aureus* (Koo *et al.*, 2000) indicating that our re-

sults are consistent with these reports. In fact, recent reports corroborate our results where it has been shown that propolis has significant antimicrobial activity against *E. faecalis* (Kandaswamy *et al.*, 2010; Kayaoglu *et al.*, 2011; Arslan *et al.*, 2011).

The assessment of antimicrobials that are not water-soluble is difficult at best. Propolis is not soluble in water and requires an organic solvent as a carrier such as ethanol or DMSO. The chemical composition of propolis may be crucial for its solubility. For example, the typified Brazilian propolis includes numerous phenolic acid compounds derived from cinnamic acid (Marcucci and Bankova, 1999), which have different solubilities in ethanol and water resulting in variations that could affect MIC values. Most studies looking at MIC values for propolis have used agar diffusion from filter paper discs or wells cut into the agar to assess the antimicrobial properties of propolis. In such systems one cannot expect the propolis to easily diffuse out. In this respect, the agar dilution method may be more appropriate for determination of the action of water-insoluble antimicrobials. Accordingly, if the use of propolis for the prevention of refractory endodontic treatment is to be foreseen, a gel or paste vehicle for propolis may be more appropriate for intracanal sealing.

In a review of the antimicrobial effects of propolis, Banskota and collaborators (2001) cited other studies that demonstrated that a minimum of 60–80 µg/mL of propolis is required to inhibit *S. aureus* and *Bacillus subtilis* while a minimum concentration of 600–800 µg/mL is required to kill *E. coli*. Bankova and collaborators (2000) have demonstrated that polar phenolic compounds are responsible for the antimicrobial effects of propolis. Our results have shown that *S. aureus* was inhibited by 400 µg/mL when ethanol was used as a carrier, suggesting that our particular lot of propolis had a lower content of polar phenolics. When DMSO was used as a carrier, the propolis had an at least eightfold greater activity against *S. aureus* (Table I) suggesting some synergistic effect of propolis with DMSO. The cinnamic acid and flavonoid derivatives have been

shown to uncouple energy transduction across the cytoplasmic membranes of *E. coli* and *B. subtilis* (Mirzoeva *et al.*, 1997). Other components of propolis have been isolated which are active against other organisms (Koo *et al.*, 2002; Marcucci *et al.*, 2001; Bankova *et al.*, 2000). Fractionation of a propolis aqueous ethanol extract revealed that these fractions exhibited antimicrobial activity against periodontal pathogens. The propolis extract, however, was more active than were the individual fractions suggesting a synergistic effect of the different propolis compounds (Santos *et al.*, 2002a). The propolis used in our study was classified as BRP1 (Brazilian propolis with the highest content of prenylated compounds) as previously described by Miorin and colleagues (2003). The compounds found in our propolis sample confirm previous studies that have examined compounds found in Brazilian propolis (Marcucci *et al.*, 2000, 2001). The main compounds identified in these studies were derivatives of caffeic acid and of *p*-coumaric acid, 3-prenyl-4-hydroxycinnamic acid (PHCA), 3,5-diprenyl-4-hydroxycinnamic acid (DHCA), 2,2-dimethyl-8-prenyl-2*H*-1-benzopyran-6-propenoic acid (DCBEN), and 2,2-dimethyl-6-carboxyethenyl-2*H*-1-benzopyran (DPB). The compounds DHCA and DPB were inhibitory against *E. coli*, *S. aureus*, and *S. faecalis*.

In summary, enterococcal species of human and animal origin were found to be susceptible to propolis with moderate to high MIC values (1600 µg/mL). Particularly, human isolates of *E. faecium* and *E. faecalis* of refractory endodontic treatment cases were susceptible to typified propolis of Brazilian origin at these concentrations. These findings would warrant future investigations on the clinical applications of typified propolis against organisms that are associated with endodontic treatment failure.

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