Green and brown propolis as antioxidant, antimicrobial and inhibitors of matrix metalloproteinases in endodontics

Denise Leda Pedrini de Arruda¹, Adriana Fernandes da Silva², Wellington Luiz de Oliveira da Rosa², Rafael Guerra Lund², Ivana Maria Póvoa Violante³, Orlando Aguirre Guedes⁴, Andreza Maria Fábio Aranha¹*

¹Department of Dental Science, School of Dentistry, University of Cuiabá-UNIC, Cuiabá, MT, Brazil.
²Department of Restorative Dentistry, School of Dentistry, Federal University of Pelotas, Pelotas, Rio Grande do Sul, Brazil.
³School of Biochemical Pharmacy, University of Cuiabá, Cuiabá, Mato Grosso, Brazil.
⁴School of Dentistry, Unievangelica Centro Universitário de Anápolis, Anápolis, Goiás, Brazil.
*Corresponding Author

Abstract—The purpose of this in vitro study was to identify the secondary metabolites and to evaluate the antimicrobial activity, cytotoxicity, antioxidant capacity, and effect on metalloproteinases (MMPs) activity of two Brazilian propolis samples. The extracts of brown (BP) and green (GP) propolis were obtained by rotoevaporation. Phenolic compounds, flavonoids and coumarins were identified by colorimetric methods and determined by spectrophotometry. The minimum inhibitory concentration (MIC) of BP and GP against Candida albicans and Enterococcus faecalis was determined. The cytotoxicity was assessed by MTT assay using L929 mice fibroblast cell line and supplemented DMEM culture medium. The antioxidant capacity was evaluated by DPPH test. The zymography assay was performed to evaluate the activity of BP and GP against MMP-2 and MMP-9. GP had a higher rate of coumarins and flavonoids, whereas BP had a higher content of phenolic compounds. GP and BP extracts showed antimicrobial activity against C. albicans and E. faecalis regardless of concentration, and presented low toxicity, except GP at 2.5 mg/mL, which reduced 40% of fibroblast cell metabolism. GP and PB extracts showed antioxidant capacity against the DPPH free radical at a concentration of 55.489 ± 1.512 μg/mL and 38.378 ± 0.735 μg/mL, respectively. Also, BP and GP showed an inhibitory effect against MMP-2 and MMP-9 from 1 to 5 mg/mL. The green and brown Brazilian propolis showed antimicrobial effect, low cytotoxicity, antioxidant capacity, and ability to inhibit the activity of MMP-2 and MMP-9, having potential to be used in endodontics as root canal irrigant.

Keywords—Anti-infective agents, Citotoxicity, Propolis, Root canal irrigants, Tissue Inhibitor of Metalloproteinases.

1. INTRODUCTION

The success of endodontic therapy is directly related to the elimination of microorganisms and their toxins from the root canal system.¹ For this purpose, a wide variety of antibacterial agents have been developed and tested as endodontic irrigants.²,³ Sodium hypochlorite (NaOCl) is still the endodontic irrigant of choice, since it has good tissue dissolution capacity, antimicrobial activity and acceptable biocompatibility at low concentrations.⁴ However, during contact of NaOCl with the pulp and dentin tissues, organochlorine compounds (chloroform, hexachloroethane, dichloromethylbenzene and benzaldehyde) are formed⁵, which are neurotoxic, highly lipophilic and chemically stable and permanent in nature. Chlorhexidine (CHX) has also been recommended as an endodontic irrigant for its strong disinfectant action, and is related to a broad antimicrobial activity.² However, there is great concern about the use of CHX alone or in combination with calcium hydroxide paste due to its decomposition into a reactive oxygen species (ROS), pCA (4-chloroaniline), human carcinogens.⁶

The known limitations of conventional endodontic irrigants have led to the search for alternative solutions, with increasing interest in agents derived from
natural products and plant extracts. Propolis is a resinous substance produced by bees (Apis mellifera), which is in general composed of 30% wax, 50% resin and vegetable balsam, 10% essential and aromatic oils, 5% pollen, and other substances with the aroma of poplar, honey and vanilla. Flavonoids and phenolic acids present in propolis are among the main responsible for the therapeutic biological properties, such as anti-inflammatory, immunomodulatory, osteoinductive capacity, antioxidant activity and antimicrobial potential.

In endodontics, propolis has already been used as a storage medium for avulsed teeth, pulp capping material, intracanal paste and irrigating solutions. When investigating propolis samples as root canal disinfectant, Kayaoğlu et al. (2011), in a dentin block model, observed the effectiveness of two propolis samples against E. faecalis; however, only one of the propolis samples with the highest level of flavonoids showed efficacy similar to CHX after a period of 7 days. Also, Awadeh et al. (2018) observed similar efficacy levels between propolis, CHX and NaOCl against C. albicansin root canals, and this result was not affected by the presence or absence of the smear layer, suggesting that it could be used as an alternative irrigating solution.

Different types of propolis are characterized and classified according to their chemical composition, whose biological properties are in conformity with their extraction method, as well as with type and botanical origin of the samples. The Brazilian green propolis (GP), whose most important botanical source is Baccharis dracunculifolia DC (Asteraceae), has already showed antimicrobial activity, anti-inflammatory and antioxidant capacity. On the other hand, few studies were found with Brazilian brown propolis (BP) being only one investigation with the BP from Cerrado region, of botanical origin Pterodon emarginatus and Calophyllum brasiliense, in which, the effectiveness of BP-based intracanal paste, associated or not with calcium hydroxide, was observed against E. faecalis.

Few investigations about the effect of propolis samples on matrix metalloproteinase activity (MMPs) have been found. A previous study showed an inhibition of the activity of MMP-2 and MMP-9 by propolis samples. However, no studies were found with Brazilian propolis extracts. MMPs are a family of zinc-dependent proteolytic activity enzymes that are involved in type IV collagen degradation, which are present in large quantities in human dentin, and may also compromise the integrity of the tooth-restoration interface, especially in the contact regions between resinous materials and dentin.

The aim of this study was to identify secondary metabolites and to investigate crucial properties for endodontic irrigants, such as the antimicrobial activity, cytotoxicity, antioxidant capacity and effect on metalloproteinases of two Brazilian propolis samples. The null hypothesis tested was that the type and concentration of propolis samples do not affect the biological activities of interest.

II. METHOD

Ethanolic extracts from propolis samples

For obtaining the crude brown propolis (BP) and green propolis (GP) extracts, BP was collected in the Cerrado region of the state of Mato Grosso, and GP from the state of Minas Gerais state was obtained commercially (BiomendesCosméticos e ProdutosNaturais, Várzea Grande, MT, Brazil). The GP and BP ethanolic extractswere obtained by extraction in 80% cereal alcohol at 60°C and subsequent concentration in a rotavaporator (Rotary evaporator 802, Fisatom, São Paulo, SP, Brazil).

Quantification of secondary metabolites

To quantify the secondary metabolites (phenolic compounds, coumarin and flavonoids) of BP and GP extracts, the assays were performed according to the procedures previously described. To determine the amount of phenolic compounds (milligrams of tannic acid per gram of the extract), the Folin-Ciocalteu colorimetric method was used, using tannic acid as a reference standard, which oxidizes phenolic compounds (phenolates), reducing acids to a blue colored complex. The tannic acid calibration equation was y = 0.005x + 0.0012 (R2 = 0.9946). The absorbance reading was performed by spectrophotometry at a wavelength of 760 nm (Spectrophotometer 800XI, Femto, São Paulo, SP, Brazil). The flavonoid content determination of the brown and green propolis extracts was performed using a spectrophotometer at 415 nm. The solution was prepared using aluminum chloride at 2.0% in methanol in a 1:1 solution. The same procedure was performed using known solutions of quercetin standard to elaborate a standard curve. Furthermore, a blank sample was prepared under the same conditions and the quantity of flavonoid content was expressed as quercetin equivalents (EQ) (mg EQ/g). The amount of coumarin (milligrams of coumarin per gram of the extract) was based on its solubility in polar organic solvents and on the ionization of phenolic hydroxyls in alkaline medium, which causes a chromatic effect at 320 nm, proportional to the coumarin concentration. The calibration equation of coumarin was y = 0.007x + 0.0019 (R2 = 0.9997). In all tests, reagents...
with no samples were used as negative control. The experiments were performed in triplicate.

Free radical scavenger activity

Antioxidant capacity was determined by the 2,2-diphenyl-1-picyrylhydrazyl (DPPH) assay, as previously described, with some modifications. The BP and GP crude extracts were diluted in methanol at a concentration of 10 mg/mL (stock solution). The antiradical activity of the extracts was evaluated using a dilution series, which involved the mixing of 1.8 mL of DPPH solution (0.208 mM DPPH in 80% methanol) with 0.2 mL of BP and GP extracts (3.125–400 μg/mL). After 30 min, the remaining DPPH radicals were quantified by absorption at 492 nm. The absorbance of each concentration of the BP and GP extracts (only sample with 80% methanol) was subtracted from absorbance of the samples with DPPH solution. Ascorbic acid (0.625–40 μg/mL) was used as reference antioxidant. The tests were performed in duplicate in three independent experiments. DPPH solution without the tested sample was used as a control. The percentage inhibition was calculated from the control with the following equation: Scavenging activity (%) = 100 – [Abs sample/Abs control] X 100

Minimum inhibitory concentration (MIC)

To determine the MIC of the BP and GP extracts, a broth microdilution was performed. The extracts were serially solubilized in dimethyl sulfoxide (DMSO). Four to five 24-hour colonies of Enterococcus faecalis were selected (ATCC 29212) and grown in Muller-Hinton Broth (Difco Laboratories, Mogi das Cruzes, SP, Brazil). Chloramphenicol (64 mg/mL) was used as a standard. Candida albicans (ATCC 90028) were selected in Sabouraud’s medium (Difco® Laboratories, Detroit, MI, USA) and liquid RPMI-1640 (bicarbonate-free, glutamine-phenol red indicator; Cultilab®, Campinas, SP, Brazil) buffered with MOPS buffer [3.9 N-morpholine propanesulfonic acid], at a final concentration of 0.165 mol/L, pH 7.0. Amphotericin B (16mg/mL; Difco® Laboratories, Detroit, MI, USA) was used as standard. Muller-Hinton (MH) broth was used as negative control for both strains. The resazurin technique was performed to assess cell viability. The tests were performed in duplicate in three independent experiments. The data were analyzed descriptively.

Cytotoxicity evaluation

To evaluate the cytotoxic effects, BP and GP ethanolic extracts were tested at 2.5 mg/mL, 1 mg/mL, 0.5 mg/mL and 0.1 mg/mL, which were solubilized in DMSO 1%, and filtered with 0.22 m diameter filter (KASVI, São José dos Pinhais, PR, Brazil). Cells of the mouse fibroblast L929 cell line were plated at an initial density of 20,000 cells in each well of a 96-well dishes (Costar Corp., Cambridge, MA, USA), containing 200 uL of complete DMEM with 10% FBS, supplemented with 100 IU/mL penicillin, 100 g/mL streptomycin and 2 mmol/L glutamine (GIBCO, Grand Island, NY, USA). The cells were allowed to grow for 24 h at 37°C with 5% CO2 and 95% air. Then, the complete culture medium was replaced by 200 uL of different concentrations of BP and GP extracts. DMEM medium was used as negative control, while DMEM + 1% DMSO medium was used as control of propolis extracts. The L-929 cells were kept in contact with the extracts for an additional 24 h in an incubator. The cell metabolic activity was evaluated by succinic dehydrogenase (SDH) activity, which is a measure of the mitochondrial respiration of the cells, using the methyltetrazolium (MTT) assay. The scores obtained from the MTT assay were submitted to the statistical analysis of Kruskal-Wallis complemented by Tukey’s post hoc multiple comparison test, considering the significance level of 5%. Zimography

To evaluate the effect of BP and GP extracts on metalloproteinases(MMPs), MMP-2 and MMP-9, zymography assay was performed as previously described, with some modifications. MMP-2 and MMP-9 were obtained from stimulated human saliva samples, which were centrifuged for 3 minutes at 1000 RPM, and the supernatant removed to obtain MMPs. Samples were stored at -20°C for later use. This study was approved by the Research Ethical Committee and was carried out in accordance with the principles of the Declaration of Helsinki (CAAE/UFPEL n° 64527316.4.0000.5318). In order to examine the effect of different concentrations of BP and GP extracts on MMPs activity, propolis extracts were solubilized in 2% DMSO at concentrations of 5 mg/mL, 2.5 mg/mL, 1 mg/mL and 0.5 mg/mL. A conditioned medium containing MMP-2 and MMP-9 was loaded onto preparative 0.05% gelatin–containing 10% polyacrylamide gels, mixed with an equal volume of non-reducing sample buffer [2% sodium dodecyl sulfate (SDS); 125 mM Tris–HCl (pH 6.8), 10% glycerol, and 0.001% Bromophenol Blue], and then electrophoresed. After electrophoresis, the gels were washed twice in 2% Triton X-100 for 60 min at room temperature, cut into strips of approximately 1 cm, and then each strip was incubated at 37°C for 24 h in Tris–CaCl2 buffer containing the different concentrations of BP and GP extracts. EDTA (positive control; Reagen, São Paulo, SP, Brazil) was used to inhibit lytic activities caused by MMP-2 and MMP-9, while 0.5 mM N-ethylmaleimide (NEM; negative control) was used to inhibit activities caused by serine proteases.
proteinases. Following incubation, the gels were stained with 0.05% Coomassie Brilliant Blue G-250. The gelatinolytic activity was detected as unstained bands. To quantify the relative inhibition of MMPs by different concentrations of BP and GP extracts, electrophoretic bands were scanned and the transmittance values thus obtained (note that the transmittance values of the zymogen, intermediate and active forms were added) were analyzed using the imagej software (NIH, Bethesda, MD, USA). Inhibition of the enzyme activity was plotted against the BP and GP extracts. Each assay was performed in triplicate and was repeated at least twice. Data were plotted and submitted to linear regression to investigate MMP-2 and MMP-9 inhibition as a function of BP and GP extracts in different concentrations.

### III. RESULTS

The results of the secondary metabolites content in BP and GP extracts and their antioxidant capacity against the DPPH free radical are presented in Table 1. GP presented higher levels of coumarins and flavonoids, while BP, higher levels of phenolic compounds. BP and GP extracts were able to inhibit 50% of free radicals (IC50) at a concentration of 55.4 μg/mL and 38.3 μg/mL, respectively.

**Table 1. Quantification of total phenols, flavonoids and coumarins, and the antioxidant capacity against the DPPH free radical (EC50 ± SD) 50% of ethanolic extracts of green and brown propolis.**

| Ethanol extracts | Quantification of secondary metabolites * | DPPH |
|------------------|------------------------------------------|------|
|                  | Total phenols (mg EAT/g ± SD)             | Flavonoids (mg EQ/g ± SD) | Coumarins (mg EC/g ± SD) | EC50 (µg/mL ± SD) |
| Green propolis   | 19.6 ± 0.17                               | 190.5 ± 0.21               | 238.7 ± 0.11              | 38.378 ± 0.735   |
| Brown propolis   | 41.6 ± 0.12                               | 70.5 ± 0.06                | 103.0 ± 0.26              | 55.489 ± 1.512   |
| Standard substances** | 885.6 ± 0.25                              | 365.5 ± 0.21               | 137.3 ± 0.44              | 4.140 ± 0.613    |

*EAT / g = milligram equivalent of tannic acid per gram of sample; mg EQ/g = milligram equivalent of quercetin per gram of sample; mg EC/g = equivalent milligram of coumarin per gram of sample.

**Total phenols: Tannic acid; Flavonoids: Quercetin; Coumarins: Coumarin; DPPH: Ascorbic acid.

Table 2 shows the analysis of the antimicrobial activity of GP and BP extracts. The BP and GP extracts showed inhibitory activity against *C. albicans* and *E. faecalis*; however, GP showed strong inhibition against *E. faecalis*.

**Table 2. Minimum Inhibitory Concentration (MIC; mg/mL) of GP and BP extracts against *E. faecalis* and *C. albicans*.**

| Ethanol extracts | *C. albicans* (mg/mL ± SD) | *E. faecalis* (mg/mL ± SD) |
|------------------|---------------------------|---------------------------|
| Green propolis   | 5.00 ± 0.17               | 2.50 ± 0.11               |
| Brown propolis   | 5.00 ± 0.12               | 5.00 ± 0.12               |
| Standard substances* | 2.0 ± 0.06                | 0.25 ± 0.11               |

* *C. albicans*: Amphotericin B; *E. faecalis*: Chloramphenicol.

The cytotoxicity of BP and GP extracts, regardless of concentration, was statistically equal to the control group (DMEM), except for GP at the 2.5mg/mL, which caused about 40% reduction in fibroblast cell metabolism (Fig. 1).
Fig. 1. Graphical representation of the cytotoxicity assessment (MTT test) of BP and GP extracts at different concentrations (Kruskal Wallis/Tukey test; p<0.05).

*Statistically different group (p = 0.006).
** GP-Green Propolis; BP-Brown Propolis.

The evaluation of the effect of BP and GP extracts on MMP-2 and MMP-9 was showed in Fig. 2. Four major bands were detected in the zymographic assays. The strongest intensity ranges corresponded to an approximate molecular mass of 66 kDa (Act-MMP-2) and 72 kDa (pro-MMP-2). Two other bands of weaker intensity corresponded to approximate values molecular mass 77 kDa (Act-MMP-9) and 92 kDa (pro-MMP-9). Both BP and GP extracts, after 24 h of incubation, showed inhibitory effect against MMP-2 and MMP-9 from 1 to 5 mg/mL, being equivalent to the positive control (0.5% EDTA).

Fig. 2. Zymography for evaluation of the effect of BP and GP extracts at different concentrations against matrix metalloproteinase-2 (MMP-2) and -9 (MMP-9) expression.

Control: Tris–CaCl$_2$ buffer only; EDTA (positive control); N-ethyl-maleimide (NEM/negative controle).

IV. DISCUSSION

The use of propolis extracts as endodontic irrigants might be of interest to patients and endodontists as part of the growing trend to seek natural medications as part of dental treatment. 27 The null hypothesis tested in this study was rejected, since the quantification of the secondary metabolites and the biological characteristics investigated were affected by both the type and concentration of propolis samples.

Propolis samples have been classified and characterized according to its chemical composition, which depends on the extraction methods$^{11,12}$ and phytogeographic characteristics, such as the climate of the region, the type of vegetation, the season and the existing...
environmental conditions near the hive, or even the genetic variability of queen bees. In this study, ethanolic extracts of propolis samples were used, since the extraction method that uses alcohol as solvent has shown superior therapeutic results than the supercritical extraction method, as well as greater release and better purification of flavonoids, active components, which may be justified by the high solubility of propolis in ethyl alcohol. Also, the evaporation method for obtaining the BP and GP ethanolic extracts used in the present is a simple and fast technique that allows a high yield of propolis extracts.

The brown propolis investigated in this study originates from the Cerrado of the Pantanal Mato-Grossense, an area rich in guanandi trees (Calophyllumbrasiliense), whose stem and leaf extracts have been shown to be active against Gram-positive bacteria and some types of fungi. However, studies with Brazilian brown propolis from different regions have shown less biological activity. Zaccaria et al. (2017) observed that a sample of European brown propolis was more active against oxidative stress and inflammation than a Brazilian green propolis, which reinforces the importance of the chemical composition of the samples that determines their biological functions through different mechanisms of action.

In this study, BP showed a higher level of phenolic compounds when compared to GP, which is in agreement with previous study; while GP had higher levels of coumarins and flavonoids, which is consistent with an earlier study. Phenolic compounds, especially flavonoids, have been reported to be responsible for the antimicrobial activity of propolis. 

Residual microorganisms may lead to treatment failure in endodontic therapy. E. faecalis and C. albicans have been selected due to their presence in persistent endodontic infections and their use in previous studies examining the effectiveness of disinfecting agents in endodontics. In this investigation, BP and GP showed activity against E. faecalis and C. albicans, in agreement with previous findings. However, twice the dose of BP (5mg/mL) in relation to GP (2.5mg/mL) was necessary to inhibit E. faecalis, which is consistent with previous investigations that found that antimicrobial activity is chemical composition and dose dependent. Pimenta et al. (2015) observed MIC for BP of 10 mg/mL, which was 2 times higher than our results, although the methodology was the same. Also, the authors investigating different intracanal pastes with BP samples, showed that 40% BP paste and 20% BP associated with calcium hydroxide paste were more effective than calcium hydroxide paste against E. faecalis in an in vitro dentin model. The antibacterial and fungicidal activity of coumarin was previously observed, which could be a possible explanation for the action of BP and GP in this study against E. faecalis and C. albicans.

Although the chemical composition of propolis samples is extremely important for its standardization, its distinct pharmacological activities may also stem from the synergism that occurs between the many components, since the biological potential of propolis does not occur solely by the presence of a particular substance, but is resulting from a complex action of various compounds. It has been claimed that biocompatibility assessment through primary cell culture are appealing, because these extracts as endodontic irrigants or any endodontic biomaterials could interact with such kind of cells after in vivo contact. However, in the present study, the biological properties of propolis extracts were evaluated in L929 mice fibroblast cells. Fibroblasts are the major constituents of connective tissue, the predominant cell type of periodontal ligament and are the most important collagen producers in this tissue. Moreover, fibroblasts secrete MMPs that are capable of initiating the degradation of extracellular matrix macromolecules, and this seems to be a key event for the progression of the inflammatory process. In this study, both BP and GP extracts, after 24 h of incubation, showed inhibitory effect against MMP-2 and MMP-9 whose expression may induce an extracellular matrix proteolysis, and it seems to be a key initiating event for the progression of the inflammatory process.

Although the root canal irrigant should be contained within the root canal space during irrigation procedures, unintentional extrusion through the apical constriction may occur. This might cause irritation, inflammation, and possible delay in wound healing after endodontic procedures. Matrix metalloproteinases (MMPs) play an important role in physiological and pathological matrix degradation. Flavonoids, at physiologically relevant concentrations, inhibit MMP-2 and -9. Flavonoids with increasing number of hydroxy groups and other modifications were compared for their capacity to inhibit recombinant catalytic domains of MMP-2 and -9. Furthermore, particular plants are an excellent yielder of the flavonoids luteolin, apigenin, and their respective glycoside derivatives (7-O-rutinoside, 7-O-glucoside, and 7-O-glucuronide). The inhibitory activity of these flavonoids and their respective glycoside derivatives on the metalloproteases MMP-1, MMP-3, MMP-13, MMP-8, and MMP-9 was assessed and...
rationalized correlating in vitro target-oriented screening and in silico docking. Additionally, coumarins are heterocyclic organic compounds widely distributed in the plant kingdom and they exhibit important biological properties including antioxidant, anticancer, vasorelaxant, antiviral and anti-inflammatory activities. They have also been shown to exhibit an inhibitory effect on the activity of matrix metalloproteinases. In our study, the GP ethanolic extract presented higher levels of antienzymatic potential to inhibit collagenases and matrix metalloproteinases. Therefore, GB and BP ethanolic extracts are promising irrigant solutions that promote significant bacterial and confirm the optimal cycompatibility and anti-enzymatic potential. However, to establish protocols for their clinical application, further studies are necessary to evaluate their antimicrobial potential against other bacteria, animal models as well as to assess the possibility of dentin staining when using these ethanolic extracts.

V. CONCLUSION

The results of the present study demonstrated the antimicrobial and anti-enzymatic activities as well as the optimal biocompatibility potential of both Brazilian propolis extracts.

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