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A new release nanosystem mucoadhesive gel of Brazilian Red Propolis-containing chitosan: *in vitro* Citoxicity and Antimicrobial test. *In vivo* ligature induced periodontitis model- propolis treatment in rats.

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ABSTRACT

Red Propolis has been shown to be a potent antimicrobial, antinflammatory, antitumoral and antioxidant, however, is less studied against microorganisms and periodontal disease. This study aimed to investigate the antimicrobial, citoxicity properties and control of a induced periodontal pocket in rats of a red propolis mucoadhesive gel -containing chitosan. For this, we used two gels with concentrations of 5% (RPG5%) and 10% (RPG10%) of propolis and were compared with chitosan gel base 5% (CHG), propolis extract 5% (FRP5%) and chlorhexidine 0.12% (CHX0,12%). The products were tested against *S. mutans, S. salivarius, S. sanguinis, L. casei, A. actinomycetemcomitans, E. faecalis, P. gingivalis, F. nucleatum, and C. albicans ATCC* standards. The Minimum Inhibitory Concentration, Concentration Minimal Bactericidal and agar diffusion tests were performed according to CLSI standards. Ligature induced periodontitis model were used and the gel product were applied in test and control rats group. The results showed that all microorganisms were sensitive to the gel RPG5% and RPG10%. There was a reduction in insertion loss and alveolar resorption in the animals treated with propolis gel. Chitosan appears to have not affected the antimicrobial activity of propolis and there seems to have been a synergism between the products. On the other hand, new tests and clinical trials studies should be performed to confirm these parameters.

Keywords: Brazilian red propolis, antimicrobial activity, mucoadhesive gel, chitosan, citotoxicity, induced periodontitis,.

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Introduction

Periodontal disease has a great prevalence in the world population and is a major cause of tooth loss in adults. The clinical signs of Chronic Periodontitis are chronic gingivitis caused by dental biofilm or plaque above and subgingival, periodontal pocket, insertion loss concurrently with the resorption of the alveolar bone of tooth support [1].

There is evidence, supported by previous studies that Chronic Periodontitis is the evolution of a chronic untreated gingivitis, associated with biofilm buildup of plaque and tartar and a specific microbiota. Microbiological studies have observed the presence of *Porphyromonas gingivalis, Prevotella intermedia, Prevotella nigrescens, Fusobacterium nucleatum, Eikenella corrodens, Tanerella forsythia, Aggregatibacter actinomycetemcomitans, Enterococcus faecalis*, and in some cases of refractory periodontitis, *Candida albicans* among other less common species[2].The antimicrobial effectiveness of ethanolic extract of propolis (EEP) compared to chlorhexidine gluconate (CHX) on planktonic *Streptococcus mutans*, *Streptococcus sobrinus*, *Lactobacillus acidophilus*, *Lactobacillus salivarius* subsp. *salivarius*, *Aggregatibacter actinomycetemcomitans*, *Prevotella intermedia*, *Porphyromonas gingivalis*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Actinomyces israelii*, *Candida albicans*, and their single-species biofilms by agar dilution and broth microdilution test methods was made for [3]. The authors demonstrated both agents inhibited the growth of all planktonic species. On the other hand, CHX exhibited lower minimum bactericidal concentrations than EEP against biofilms of *A. actinomycetemcomitans*, *S. aureus*, and *E. faecalis* whereas EEP yielded a better result against Lactobacilli and *P. intermedia*. The bactericidal and fungicidal concentrations of both agents were found to be equal against biofilms of *Streptecocci*, *P. gingivalis*, *A. israelii*, and *C. albicans*. The results of this study revealed that propolis was more effective in inhibiting Gram-positive bacteria than the Gram-negative bacteria in their planktonic state and it was suggested that EEP could be as effective as CHX on oral microorganisms in their biofilm state [3].

The presence of the subgingival microbiota generates an inflammatory response involving direct and indirect aggression with the involving neutrophils, macrophages y endothelial cells and chemokine activation of integrins and production of Tumor Necrosis Factor Receptor (TNF-R), Heparan Sulfate proteoglycan (HSP), L-selectin, Integrin Adhesion Molecule-intra-Citoplasm (Integrin -ICAM), PSGL-1 and P-Selectin[2,4].

The periodontal disease has a multifactorial etiology involving besides the microbiota, the oral care of patients, genetic, environmental predisposing factors such as smoking and alcohol, social cultural factors and financial. Although there are several brands on the market of mouthwashes and toothpastes, these products act preemptively acting on the control of dental plaque. However, there is, to date, no product which acts treating inflammation and stimulating the formation of alveolar bone lost during the progression of the disease. Treat the inflammatory process and stimulate new formation of bone, concurrently, continue to be a challenge for scholars and dentists[4].

Propolis has been used for centuries by world population due to its pharmacological properties such as anti-inflammatory, healing, antimicrobial and antioxidant [4,5]. In dentistry, propolis has been used to control the oral microbiota [6,3]. The antibacterial activity of propolis is reported due to flavonoids, aromatic acids, and esters present in resins. Galangin, pinocembrin, and pinostrobin are known as the most effective flavonoids agents against bacteria. Ferulic acid and caffeic acid also contribute to the bactericidal and fungicidal action of propolis. Propolis has plenty of biological and pharmacological properties and its mechanisms of action have been widely investigated in the

last years, using different experimental models in vitro and in vivo [7].

Red propolis, found in Northeast mangrove areas of Brazillian Northeast, is originated from *Dalbergia ecastophyllum* and it is composed for pterocarpans, isoflavonoids, chalcones, prenylated benzophenones and phenyl-propanoids[8]. The red propolis therapeutical effects are imputed to fenolic compounds, in association with fenolic acids, esters, fenolic aldeids[9]. Table 1 shown the chemical markers of red propolis. It act sinergicly and researchers shown that the pool of compounds activity have a more effect than one isolated compound[10]. Studies related clinical uses and biological properties of red propolis as antibacterial, antioxidant, antifungi, anti-inflammatory, healing wounds and that is the reason to investigate propolis role against caries, candidiasis, gengivitis and other oral pathologies[11]. Propolis has an important activity against microorganisms. *In vitro* studies demonstrated its abilities against virus like influenza, herpex virus type 1 and 2 and against a large range of bacteria, including *Staphylococcus aureus* and *Salmonella enteritis,* even resistant strains. It is related the potential of red propolis alone or in synergic combination with certain antibiotics and antifungals[12,13]. Previous reports related the antibacterial activities of isolated compounds of red propolis against *S. aureus*, *Bacillus subtilis* and *Pseudomonas aeruginosa*, evaluated by measuring the Minimum Inhibitory Concentrations (MIC), demonstrated that the main antimicrobial phenolic compounds were found to be vestitol, neovestitol and medicarpin. In particular, medicarpin exhibited the most potent bacterial activity against elected bacteria, indicating that it is the most active antibacterial compound in Brazilian red propolis extracts[7]. There are few studies about the antimicrobial activity of Brazilian red propolis against oral pathogens microorganisms and periodontitis. The therapeutical properties and its low toxicity in low concentrations may enable propolis for many interesting in dentistry use. The development of a gel controlled release system that generates a bioadherence (preventing saliva action) that can act against some of the chronical oral pathologies can have a great value. Table 1 shown the chemical markers of red propolis. So, this study developed a mucoadhesive gel containing Brazilian propolis which was tested *in vitro* against periodontitis envolved microorganisms and *in vivo* animal model of periodontitis.

Material and Methods

Red Propolis Origin

Crude red própolis was obtained at the Ilha do Porto apiary, located in a mangrove área in the city of Marechal Deodoro, Alagoas State, Brazil, lot PAE0111.SR. The 25% propolis etanol extract was prepared and analyzed for botanical origin *Dalbergia ecastophyllum*, identification and bioactive componentes quantification, sensory characteristics and physicochemical control by Néctar Pharmacêutica Ltda, Belo Horizonte, Minas Gerais State, Brazil (Certificate Analyses of the Certified Company ISO 9001-GMP- HACCP, INMETRO/Brazil).

The ethanolic extract was prepared by adding 250g of crude red propolis to 1000mL of absolute etanol. The mixture was stirred and homogenized during 30 minutes at 70oC. After, the mixture was centrifuged and the supernatant discarded. The red propolis gel was prepared at concentrations of 2.5%, 5% and 10% and also without propolis, with the replacement of propolis by an equal amount of absolute alcohol.

Citoxicity assays using ethanolic extract of red propolis

Murine fibroblasts (L929), murine epithelial cells (CHO) and human gingival fibroblasts (FIB) were seeded on 96-well microtiter plates (n=12) at the density of $1,0x10^4$ cells/well and, after 24 hours, exposured to concentrations of ethanolic propolis extract (0,06 to 0,25%). It was incubated at 37ºC in 5% CO_2 for 72 hours. The proliferation index was determined by methyl-thiazolyl-tetrazolium (MTT, Sigma, USA) assay, which is based on the conversion of MTT to formazan crystals by mitochondrial desidrogenases[14,15].

After the exposition, the medium was removed and dye MTT 1 mg/mL was added. Subsequently, the MTT solution was removed and the crystals of formazan was dissolved in dimethylsulfoxide (DMSO, Sigma, USA). Absorbance was measured using a microplate reader (Spectra Max 190, Molecular Devices, USA) at 570 nm.

Antimicrobial Assay

Bacterial sensitivity or resistance to mucoadhesive gel was detected by the agar diffusion assay[16,17]. Aliquots overnight of microorganisms *Streptococcus mutans* (ATCC 25175), *Streptococcus salivarius* (INCQS 00457), *Streptococcus sanguinis* (ATCC 10557), *Lactobacillus casei* (ATCC 393), *Aggregatibacter actinomycetemcomitans* (ATCC 33384), *Enterococcus faecalis* (ATCC 29212), *Porphyromonas gingivalis*(ATCC 33277), *Fusobacterium nucleatum* (ATCC 23726), and *C. albicans* (ATCC 14053) containing 1.0×10^6 Colony Forming Unity (CFU) /mL were subcultured in the proper agar and supplemented for each microorganism. 6mm diameter wells were punched into the agar in triplicate and filled with: 20µL of gel containing chitosan only and red propolis gel 5 and 10%. Ethanol propolis extract 5% (EPE), chlorhexidine 0,12% (CHL,Sigma, USA), 70% ethanol (E, Synth, Brazil)), and sterile distilled water (DW) were dispensed in sterile blanc discs (Laborclin, Brazil) soaked with 20µL of each substance. The bacteria plates were incubated at 37ºC in 5% CO_2 for 24 hours. *Candida albicans* cultured on Sabouraud dextrose agar plates were incubated agar at 37ºC for 24 hours under aerobic atmosphere. The inhibition zone diameter around the well and filter paper formed after 24 and 48 hours at 37ºC were measured and taken medium and standard deviations (M± SD). Any inhibition zone around the filter paper measuring ≤ 7mm was considered a negative result. Minimal Inhibitory Concentration (MIC) test was carried out using microtiter plates (96 wells) containing 100 μL/well BHI. After being transferred to the first well, serial dilutions were performed to obtain concentrations ranging from 75 to 0,1mg/mL. MIC was defined as the lowest concentration of the propolis gel that inhibited microorganism visible growth indicated by resazurin 0.01% (Sigma-Aldrich, St. Louis, MO, USA). To determine minimal bactericidal concentration (MBC), an aliquot of each incubated well with concentrations higher than MIC was subcultured on BHI medium. MBC was defined as the lowest concentration of the propolis gel that allowed no visible growth on the test medium[17].

Ligature induced periodontitis model.[18]

The animal experiment was approved by the Ethics Committee on Animal Use (CEUA / UFMG- protocol number 294/2012). Wistar rats (120-140 g, n=9, divised in 03 groups) were aclimmated to the housing conditions for 5 days. They are allocated into plastic cages with water and food *at libitum* and a 12 hour light/dark were applied. The animals received a intramuscular injection for general anesthesia by association of ketamine (0,4 mL/mg) and xylazine (0,2 mL/mg). The left second molar was selected for the ligadure, using cotton ligadure number 4.0 (Ethicon, J&J). The ligadure was knotted on the buccal side of the tooth resulting in a subgingival position palatal and a supragingival position buccally for 7 days. The right second molar without ligature was used as an internal control of the experiment. Then, the ligadure was removed and the animals were separated in randomized groups and treated with different dosis of propolis gel (2, 5 and 10%) and vehicle chitosan. After 10 days, animals were sacrified. The mandibles were removed and fixed in 10% formalin buffer for 48 hours, then descalcified by EDTA 10% for 10 days and dehydratedin an ascending series of ethanol solution and embedded in paraffin[19]. The 5µm sections were obtained and stained in a hemotoxicilin and eosin (HE). For the histometric evaluation, the lamins were photographed by a microcamera (JVC TK-1270/RGB) coupled on the microscope. The measurement of the alveolar inserction loss were obtained by the difference

of cementum-enamel junction/alveolar bone crest distance of the sides with and without ligature [Figure 1]. The measurement of bone loss were obtained by the difference of dentinoenamel junction/ alveolar bone crest distance. The measurements were expressed in mm using a computer program Image J.1.48. The One- way ANOVA Bonforronipost test was used for histometric comparisons between ligated and unligated groups at significance level of 1% ($p< 0.01$) .

Results and Discussion:

About citotoxity / proliferation detected by MTT method, on that assay, it was shown a statistically significant proliferation using the 0,25% concentration for all lines of cells (L929: 0,407±0,080 %v/v; CHO: 1,44±0,37 % v/v; and FIB:1,43±0,37 % v/v) against the control (0,265±0,04; 0,622±0,080; 0,619±0,56; $p<0,05$, ANOVA, Bonferroni's post test) (Table 2 and Table 3). Other studies found a citotoxity activity of red propolis against tumor cells lines *in vitro* [14,15] and that is the reason why the antitumor activity of propolis has been largely studied. On this report, we found a proliferation of cells caused by red propolis differently of showed on that studies. But the concentrations of propolis used in our assays were lower, comparing to the ones used on that studies cited. Authors found a not citotoxity activity of propolis against osteoblast-like cells, using, as in our report, lower concentrations of propolis[13]. So, the activity of propolis probably is dosage-dependant and it can be specific for certain types of cells. On our studies, it was used cells that are strongly associated to healing process *in vivo*.

Table 1- Chemical markers constituents of Brazilian red propolis(Daugsch et al.,2008; Rufatto et al, 2017).

Number	Compounds contents
01	Rutin
02	Liquiritigenin
03	Daidzein
04	Pinobanksin
05	Quercetin
06	Luteolin
07	Dalbergin
08	Isoliquiritigenin
09	Formononetin
10	Pinobanksin-3-acetate 1.7
11	Biochanin A 0.5
12	Vestitol
13	Neovestitol
14	Guttiferone
15	Xanthochymol
16	Medicarpin
17	Elemicin
18	10-Octadecenoic acid, methyl ester

Table 2- Cytotoxicity test. Number of viable cells after contact with ethanolic extracts of red propolis with human gingival fibroblasts (FIB), murine fibroblasts (L929) and hamster epithelial cells (CHO). Means and Standard Deviations (M ± SD) of three tests.

Cell lineage	RPE 0,06%	RPE 0,12%	RPE 0,25%	Etanol 1,0%	DEMEN (controle)
	(n=11)	(n=12)	(n=12)	(n=12)	(n=12)
CHO	98.4	111.3	229.0	96.8	100
L929	76	92	160	84	100
FIB	103.2	112.9	230.6	101.6	100

Table 3. Cytotoxicity test of ethanolic extracts of red propolis (RPE) on human gingival fibroblasts (FIB), hamster epithelial cells (CHO) and murine fibroblasts (L929). Means and Standard Deviations (M ± SD) of three tests.

Cell lineage	RPE 0,06%	RPE 0,12%	RPE 0,25%	Etanol 1,0%	DEMEN (controle)
CHO	0,61±0.02	0.69±0.04	1.42±o.11	0.60±0.01	0.62±0.02
L929	0.19±0.00	0.23±0.01	0.40±0.02	0.21±0.01	0.25±0.01
FIB	0.64±0.03	0.70±0.04	1.43±0.11	0.63±0.01	0.62±0.02

Table 4 - Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (CBM) of Red Propolis Gel (RPG 5%, RPG 10%) and Red Propolis Extract (RPE 5%) against microorganisms involved in periodontitis.

Microorganisms	MIC (µg)			CBM (µg)		
	RPG 5%	RPG 10%	RPE 5%	RPG 5%	RPG 10%	RPE 5%
A. actinomycetemcomitans	26	54	23	128	244	256
E. faecalis	26	54	23	128	162	250
F. nucleatum	26	54	23	128	244	220
P. gingivalis	26	54	23	128	200	240
P. intermedia	26	54	23	256	244	240
C. albicans	40	50	30	168	400	168

Table 5- Brazilian Red propolis susceptibility antimicrobial diffusion agar test against oral pathogens microorganisms. Inhibition zones medium/ standard deviation(M±SD) results of three experiments.Legend: RPG = red propolis gel ; CHG = Chitosan gel; RPE = red propolis extract ; CHX = chlorhexidine

Microorganisms	Red Propolis Gel (RPG) and Control Products				
	Antimicrobial activity/ Inhibition zones (mm)				
	RPG 5%	RPG 10%	CHG	RPE 5%	CHX 0,12%
Streptococcus mutans	13.0±0.81	18.3±1.24	19.0±0.16	43.6±0.49	22.0±1.55
Streptococcus salivarius	22.6±1.31	18.0±1.41	17.3±0.33	46.6±0.35	20.3±1.47
Streptococcus sanguinis	15.6±0.24	16.0±0.81	15.3±1.24	26.6±0.94	20.0±0.81
Lactobacillus casei	17.0±0.16	18.6±0.81	14.0±1.02	19.0±0.44	21.0±1.08
Aggregatibacter actinomycetemcomitans	25.0±0.00	19.6±0.35	14.0±1.41	19,6±0.47	14.6±0.49
Enterococcus faecalis	16.2±0.77	17.2±0.63	21.5±0.55	14.5±1.13	11.2±0.43
Porphyromonas gingivalis	13.3±1.24	16.6±1.02	20.6±0.86	18.6±1.04	12.3±0.47
Fusobacterium nucleatum	10.3±1.12	16.6±0.02	21.6±1.24	19.0±1.16	11.0±0.81
Candida albicans	15.6±0.09	19.0±0.41	18.3±0.62	23.3±0.94	17.0±2.16

This study shows satisfatory results of ethanolic extract and gel for all the microorganisms, showing that propolis is efficient in both formulation. Table 4 and Table 5 show the antimicrobial properties of red propolis. It retains its antimicrobial properties when associated to chitosan microspheres. Against caries bacteria, propolis gel 5% showed an efficient activity against *Streptococcus salivarius* and *Streptococcus sanguinis* similar to the findings of da Silva et al[12]. Even using ethanolic extract 5%, the results were positive and larger than control, but the deviation was wide, close to chitosan and chlorexidine. These results are according to Bueno-Silva *et al.*[7] that shows a neovestitol-vestitol containing propolis ability of control development of caries growth *in vitro* and *in vivo*, considering as gold standard a fluoride. Better deviations were found on results for *Lactobacillus casei* and *Porphyromonas gingivalis* using propolis gel 10% and *Fusobacterium nucleatum* using gel 5%. Better media were obtained using ethanolic extract 5% and it may be related with alcohol that may reduce the surface tension and solubilize propolis compounds, causing a large difusion. The consequence is a large inhibition zone and deviation[20] performed a difusion in wells instead of a difusion in discs to avoid the interference of alcohol. *Enterococcus faecalis* does not shows signifficant results, but it could not be related to propolis antibacterial activity. Brumfitt et al. [21] related technical problems as low rate of propolis compounds in agar. Gram negative may be less sensitive to ethanolic red propolis extract because of its complex wall cell or its higher fat contents. Righi et al.[5] findings are similar, showing a limited activity against Gram negatives and a major MMC (minimum microbicidal concentration) against *Kleibsiella pneumoniae*, a Gram positive one. Marcucci *et al.*[22] and Sforcin *et al*[23] founded low activity of green propolis against Gram negative bacteria. On this report as in De Luca et al.[14], we found significative results against gram positive and gram negative bacteria. These differences found in propolis activity occurs because according to the variaty of the propolis. Other studies observed that low concentrations of propolis reveals bacteriostatic action instead of bactericidal[5]. This study shows satisfatory results of red propolis gel 5% specially against *Aggregatibacter actinomycetemcomitans* and ethanolic extract 5% specially against *Candida albicans*. These results are similar to those observed by Sokolonski et al. [24,25]. The large number of phenolic compounds (as flavonoids, phenolic acids and their esters [3,22], which have been attributed antibacterial activity, with individual pharmacological activity against microorganisms turns propolis an interesting to act against resistant microorganisms. Reports show that ethanolic extract of red propolis could inhibit even resistant bacteria, as *S. aureus* that is resistant to methicillin. Propolis can act effectively against resistant fungi as *C. glabrata*, specially when used as an adjuvant of fluconazole. Other relates shows the antiinflammatory and immunomodulatory properties that can act combined to the antimicrobial activity and improving it Freires et al., [13]. In addiction, propolis is a natural product reduce orange-complex periodontopathogens [25] and have a good population acceptance used for since ancient times [26].

To analyze pharmacological properties *in vivo*, it was used an animal model of ligature induced periodontitis. Animal models can provide a high quantity of data but it is not possible to determine if it is possible to extrapolate to human form of the disease. The ligature method for periodontitis induction was studied by de Molon et al [18] and Inui et al. [23] and the authors verified a significant increase in the gene expression of pro-inflammatory cytokines, interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α), and proteins involved in osteoclastogenesis, receptor activator of nuclear factor-k B ligand (RANKL) and osteoprotegerin (OPG) was observed in the first week of analysis. In the later periods of evaluation (14-21 days), no significant alterations were noted

with regard to inflammatory processes, bone resorption, and expression of cytokine genes. The ligature-induced PD model resulted in progressive alveolar bone resorption with two different phases: Acute (0-14 days), characterized by inflammation and rapid bone resorption, and chronic (14-21 days) with no significant progression of bone loss. Furthermore, the gene expressions of IL-6, IL-1β, TNF-α, RANKL, and OPG were highly increased during the progress of PD in the early periods [18]. It generates a formation of a pathogenic biofilm, inflammation and bone loss. Rodents are naturally more resistant to periodontitis [27,28,29]. It is possible to observe a tendency of reduction in groups treated with propolis, presenting minor medias for this groups, but it is not significant. Groups tested with propolis gel 5% and 2% showed similar results. These results are related to the bioactive properties of *D. ecastophylum* [30,31]. The Figure 1 showed the periodontal insertion and bone loss (vehicle and treated) compared to the control (without propolis gel).

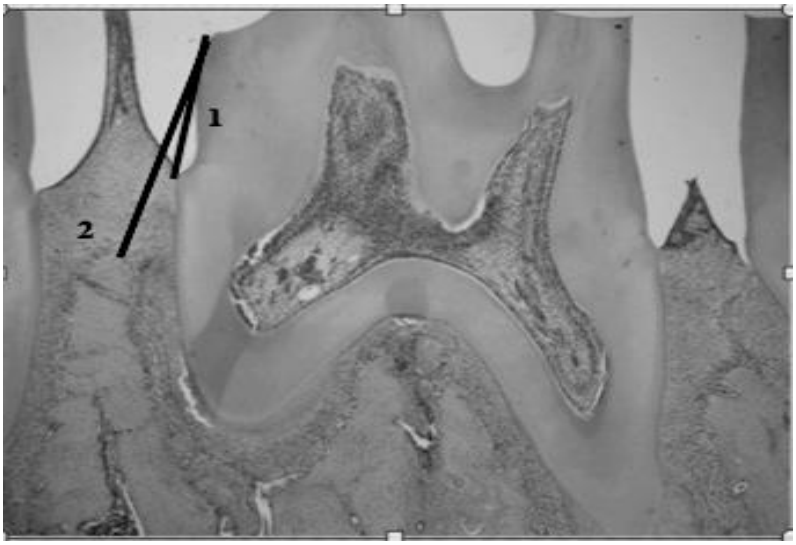

Figure1. Wistar rat Histological cut of molar tooth and periodontalregion of rat (HE 1000 x). Periodontal Disease: Evaluation ofinsertion loss (1) and bone loss (2). Legend: G= Gingiva,P= dental pulp, D= dentin, Pp= Periodontal pocket, AB= alveolarbone, IS= interdental space.

Probably, on that report, it is not shown a significant different between groups because of the low number of animals used (the deviation is high). Other studies with extract ethanolic of green propolis irrigation combinated to periodontal clinic treatment allowed to obtain better results than scaling and root planning by themselves, which results from the assessment of both clinical and microbiological parameters [25,26]. Considering the world's biodiversity, the various types of propolis[27], that microorganisms may become are more sensitive to red propolis and that a mucoadhesive formulation can promote a more contact time with the product, the mucoadhesive red propolis gel can be very useful in periodontitis.

Considering the physical characterists that can facilite the use and the commercialization of the product and the pharmacological activities described, the red propolis gel is an interesting product for oral uses. In spite of the superior microbial activity of ethanolic extract, the gel has the advantage of prolonged controlled liberation. Furthermore, red propolis induced proliferation *in vitro* of some cells related to healing processes. Our data demonstrate that propolis, as an ethanolic extract or as a mucoadherent gel, is a promising agent against dental bacteria and may be useful on the treatment of various oral diseases as caries and periodontitis. However, other complex studies *in vitro* and *in vivo* are necessary to confirm these results.

Conflict of Interests

The authors report no relationships, financial or otherwise, with any entity that may influence the objectivity of this paper.

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