

Antimicrobial capacity of experimental chitosan/propolis-based varnishes

Abstract

Background: The present study aimed to evaluate the antimicrobial capacity of experimental chitosan/propolis-based varnishes against the main microorganisms associated with the development of dental caries in the oral cavity, *S. mutans* and *S. sobrinus*.

Materials and methods: Several types of varnishes were made, containing chitosan, nanochitosan, green propolis and red propolis, both in different concentrations. Initially, a 1.5% chitosan (QT) gel was made. Then, we prepared a nanoparticulate chitosan gel (QT-NP), with or without propolis, at 5% and 10%, and added the nanoparticulate gel to the chitosan gel. The following varnishes have been made: V1 - (QT + nanoparticles) + 10% (QT-NP + green propolis); V2 - (QT + nanoparticles) + 10% (QT-NP + red propolis); V3 - (QT + nanoparticles) + 5% (QT-NP + green propolis); V4 - (QT + nanoparticles) + 5% (QT-NP + red propolis); V5 - (QT + nanoparticles) + 10% QT-NP; V6 - (QT + nanoparticles) + 5% QT-NP; V7 - Control - Duraphat® and V8 - Chlorhexidine gel. Subsequently, the analysis was based on the qualitative method of inhibition halo, testing the antimicrobial activity of varnishes against oral microorganisms that cause caries disease. Therefore, the minimum inhibitory concentration (MIC) and the Minimum Bactericidal Concentration (MBC) were established.

Results: Microbial growth inhibition values were obtained for green propolis extract at concentrations up to 12.5% for *S. mutans* and 25% for *S. sobrinus* and for red propolis at concentrations up to 6.25% for both.

Conclusion: It could be concluded that both propolis extracts (green and red) have significant antimicrobial capacity, bactericidal and bacteriostatic effects, highlighting the fact that the antimicrobial action of red propolis is more significant than the action of green propolis, given that at lower concentrations, red propolis has the same capacity to inhibit bacterial growth.

Keywords: antimicrobial, chitosan, propolis, streptococcus, varnish

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Lucas Ladeira-Vieira,¹ Juliana Jendiroba Faraoni,¹ Marina CG Del Arco,² Sérgio L Salvador,² Regina Guenka Palma-Dibb¹

¹Department of Restorative Dentistry, School of Dentistry of Ribeirão Preto, University of São Paulo, Brazil

²Department of Clinical Analyses, School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, Brazil

Correspondence: Regina Guenka Palma-Dibb, DDS, MSc, PhD, Restorative Dentistry Department, Ribeirão Preto School of Dentistry, University of São Paulo, Av. do Café, s/n°, Monte Alegre - 14040-904, Ribeirão Preto, SP - Brazil, Phone: +55 16 3602 4016 Fax: +55 16 3315 4103

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Introduction

Dental caries is a multifactorial disease requiring multiple interventions. This has led the World Health Organization to propose prevention programs associated with educational and public health policies, such as water fluoridation, rather than just carrying out restorative treatment. Furthermore, restorative treatment alone can lead to a repetitive cycle of restorations, as discussed in modern dentistry.¹⁻³ It is important to prevent new carious lesions and use the least invasive treatment possible to ensure the fewest restorations and avoid falling into this cycle, where restorations are doomed to fail due to the chemical and mechanical properties of restorative materials, oral dynamics, chewing, parafunctions, and oral biology, among other variables that influence the durability and quality of a restoration. This results in the loss of more and more healthy tooth structure over time.

Dental caries is a worldwide public health problem affecting all age groups and having a high prevalence and broad social impact. The disease results from the demineralization of acellular hard dental tissues caused by the degradation of the dental structure due to the release of acids produced by acidogenic and acidophilic bacteria adhering to the established dental biofilm.⁴

Dental biofilm is a structure formed by specific, coordinated, complex, metabolically integrated, and spatially organized microbial communities.⁵⁻⁸ Although dental biofilms are composed of several microorganisms, *Streptococcus mutans* plays a key role in the

development of carious lesions due to its ability to attach to tooth structures. This is mainly due to its tolerance of acids and its ability to produce them, which intensifies the demineralization of enamel and dentin by decreasing oral pH through the fermentation of carbohydrates consumed in the diet.⁷⁻¹⁰

In the early stages, demineralization can be reversed through remineralization, whereby the tooth absorbs calcium, phosphate, and fluoride via its natural repair mechanism. This process involves the diffusion of minerals present in saliva into the pores of the demineralized structure.¹¹ To support this process, conservative treatments such as the superficial application of fluorides are advocated to prevent the development of caries lesions and arrest incipient lesions.

For remineralization to succeed, minimal support conditions must be present at the injury site, such as the absence of biofilm. The biofilm structure protects bacteria against antimicrobial agents,¹ providing an environment conducive to disease progression.

Varnishes have been widely used in dentistry for years to prevent dental caries, and active principles can be incorporated into these formulations to promote and prolong the anti-caries effect. Examples include fluoride, which has a remineralising action, and chlorhexidine, which has an antimicrobial action.¹² They form a protective film on the tooth surface and promote the release of the active ingredient. More recently, propolis varnish has been introduced due to its low toxicity and antimicrobial properties.⁸

Some natural products contain bioactive principles with antimicrobial potential, particularly against the *S. mutans* group, of which propolis is an example. Propolis exhibits various beneficial biological properties, including anti-inflammatory, antioxidant, antimicrobial, antifungal and antitumor activities.⁸⁻¹² Furthermore, certain types of propolis exhibit greater antimicrobial potential, demonstrating a strong antibacterial effect that aids in the prevention of caries disease by hindering the development and progression of carious lesions.¹³ They also reduce the accumulation of dental plaque on tooth structure.¹⁴ Other substances contained in propolis, such as flavonoids, terpenoids, isoflavones and other phenolic acids, may reduce acid production and tolerance by *S. mutans*.^{8,10,13} A recent clinical study¹⁵ observed that 2.5% red propolis varnish efficiently controlled the formation of *S. mutans* colonies in the oral cavities of children aged 36–71 months.

There is great diversity in the types of propolis and their chemical compositions, consequently affecting their interactions with other substrates.¹⁶ These differences are based on the number of flavonoids and their derivatives, such as cinnamic acid and the phenethyl ester of caffeic acid, as well as 3,5-diprenyl-4-hydroxycinnamic acid (arteppillin C). For example, Brazilian green propolis contains high levels of arteppillin C, which has a variety of biological effects including anti-inflammatory, antiviral and antitumor properties.¹⁷ Commercially available oral care products incorporating propolis, such as toothpastes and mouthwashes, are well-documented in the literature for their antibacterial and bacteriostatic properties.^{18,19} However, propolis-based varnishes remain substantially less common in the market.

Another widely used substance is chitosan, a macromolecule formed by repeating D-glucosamine monomers that is widely used in the development of biomaterials with biomedical and dental applications.^{20,21} It is a naturally abundant compound, notable for its biocompatibility, antibacterial and antifungal properties, mucoadhesion, non-cytotoxicity, and biodegradability. This makes it possible to incorporate it into materials already available, improving their properties.^{22,23} Chitosan can be used in gel or nanoparticle form, with or without other active ingredients. Recently, the use of propolis-loaded chitosan nanoparticles on root canal microorganisms was studied and demonstrated satisfactory antibacterial properties.²⁴ In addition, the use of a propolis-containing chitosan varnish also demonstrated antimicrobial activity with low toxicity.^{8,25} However, studies comparing different types of propolis with chitosan and the effect of concentration on antimicrobial properties are lacking.

In the search for ecologically sustainable solutions, new materials based on natural products are being developed to halt the progression of demineralization and promote remineralization. The present study had the objective of evaluating the antimicrobial capacity of experimental chitosan/propolis-based varnishes in the main microorganisms associated with the development of dental caries present in the oral cavity, *S. mutans* and *S. sobrinus*.

Materials and methods

Experimental solutions were prepared based on modified mineral-loaded chitosan (process BR 10 2021.008804.4), in which different concentrations of propolis extract (5% and 10%) were incorporated with the addition of propolis-loaded chitosan nanoparticles.

Varnish formulation

Chitosan-based gel - The chitosan was dissolved in a 1%vol/vol aqueous solution of acetic acid at a concentration of 1.5% weight/volume and mixed for 24 hours. Then the minerals (Ca and PO₄) were

added into the solution and stirred for 2 hours, and then the propolis extract (green or red) was added at 5% and 10% concentrations.

In addition to the chitosan base, chitosan nanoparticles loaded with propolis to be tested were added. The solution was prepared using the protocol employed by Shi et al.²³ To this end, nanoparticulate chitosan (QT NP) was prepared using the ionic gelation method. Chitosan was dissolved by 1%vol/vol acetic acid at a concentration of 0.5% weight/volume, and the pH was adjusted to 5 with NaOH. Aqueous solution of 5 ml of 0.25% sodium tripolyphosphate (TPP) was also prepared with pH adjusted to 5.5. In the propolis solution, the extract was added in chitosan solution gel under stirring (1000 rpm). Then the TPP solution was dropped drop by drop into the chitosan solution under constant stirring with propolis for nanoparticle formation. After the addition of all the TPP solutions, the solution was stirred for another 30 minutes.

To separate the nanoparticles, the solution was centrifuged for 30 min (at 4000 rpm). At the end of the cycle, the supernatant was discarded and the deposited chitosan nanoparticles were washed intensively with distilled water to remove NaOH residues. After washing, the solution was centrifuged again to remove excess water. The nanoparticles were added to the chitosan base gel in the proportion of 10%p/v, then it was stirred for 1 hour, and at the end we obtained the experimental varnishes.

Evaluation of antimicrobial action

Firstly, bacterial sensitivity or resistance to the varnishes developed was determined using the disk diffusion test, also known as the Kirby-Bauer method. Aliquots of *Streptococcus mutans* and *Streptococcus sobrinus* containing 1.0×10^8 CFU/mL were subcultured on Mueller-Hinton agar (Difco, Trenton, USA), supplemented with 5% dextrose for *Streptococcus* spp. The petri dishes were then divided like a pizza into 8 slices and wells were made using a sterile test tube. Sterile filter papers soaked with 20 µL of each propolis varnish were placed on the agar. The controls will be white varnish/chitosan (V1), Duraphat (V7) and chlorhexidine gel (V8). The diameter of the inhibition zone around the filter paper, formed after 24 and 48 hours at 37°C in a 5% CO₂ atmosphere, will be measured in mm and recorded (M ± SD). This test will also be carried out on propolis extracts at the manufacturer's concentration (Apis Flora).

After the initial test, the minimum inhibitory concentration (MIC) test was carried out using tissue culture microplates (96 wells) containing 100 µL/well of Brain Heart Infusion (BHI) medium. After being transferred to the first well, serial dilutions were made to obtain varying concentrations. Chlorhexidine 0.12% (Sigma-Aldrich, St. Louis, MO, USA) was used as a positive control and BHI as a negative control. The bacterial inoculum (1×10^6 CFU/mL) was added to all the wells, and the plates were incubated at 37°C in 5% CO₂ for 24 hours. The MIC was defined as the lowest concentration of propolis varnish that inhibits the visible growth of the microorganism, indicated by 0.01% resazurin (Sigma-Aldrich, St. Louis, MO, USA).

To determine the minimum bactericidal concentration (MBC), an aliquot of each well incubated at concentrations above the MIC was subcultured in BHI medium. The MBC was defined as the lowest concentration of propolis varnish that would not allow visible growth in the test medium. This test was also carried out with the pure propolis extracts.

Data analysis

The analysis was based on the qualitative inhibition halo method, testing the antimicrobial activity of the varnishes, as well as defining the MIC and MBC.

The data were analyzed for distribution. For the halo of inhibition of *Streptococcus mutans*, the data were not normal, and the Kruskal-Wallis and Tukey tests were carried out; for the halo of *Streptococcus sobrinus*, the data were normal and homogeneous, and the ANOVA and Tukey tests were carried out. Both tests were performed at a 5% significance level.

Results

Considering the analysis of the inhibition capacity (MIC) of the microorganisms *Streptococcus mutans* and the microorganism

Streptococcus sobrinus by the different types of propolis in different concentrations (Table 1), it was observed that both microorganisms were inhibited by red propolis at a concentration of 6.25% or more (considering the dilution of the propolis extract in water), which shows great antimicrobial effect of this type of propolis. On the other hand, green propolis only showed inhibition at concentrations above 12.5% when diluted, which still justifies its use, as it is only slightly less effective than red propolis.

Table 1 Analysis of the inhibition capacity (MIC) of the *Streptococcus mutans* and *Streptococcus sobrinus* in different concentrations of propolis

Substance	Concentration	Microorganisms	
		<i>S. mutans</i>	<i>S. sobrinus</i>
Green propolis	100%	no	no
	50%	no	no
	37.5%	no	no
	25%	no	no
	12.5%	no	yes
	6.25%	yes	yes
	3.125%	yes	yes
	100%	no	no
	50%	no	no
	37.5%	no	no
Red propolis	25%	no	no
	12.5%	no	no
	6.25%	no	no
	3.125%	yes	yes
	Control	no	no

Table 2 shows the inhibition of halo value of the different microorganisms produced by the different varnishes/solutions studied. In the case of *Streptococcus mutans*, the values were presented as

median and Percentiles, and in the case of *Streptococcus Sobrinus* in mean and standard deviation.

Table 2 Inhibition of halo values, in mm, of the different microorganisms produced by the different varnishes/solutions studied

	<i>S. Mutans</i> *	<i>S. Sobrinus</i> *
Chlorhexidine 0.12%	37.47 (35.85 - 38.80) a	32.22(1.66) a
Green Propolis Varnish 5%	20.24 (19.92 - 20.64) b	16.76 (0.46) b
Green Propolis Varnish 10%	22.60 (22.13 - 23.25) b	16.07 (0.76) b
Red Propolis Varnish 5%	20.12 (19.95 - 20.88) b	15.89 (0.55) b
Red Propolis Varnish 10%	20.47 (19.79 - 20.60) ab	16.36 (0.95) b
Nanochitosan	0 (0) c	0 (0) c
Chitosan	0 (0) c	0 (0) c
Distilled water	0 (0) c	0 (0) c

*Comparison only in column - same letter statistical similarity.

At the same time, in the tests carried out using only pure propolis extracts, in the formulation and concentration produced by the manufacturer itself (Apis Flora), both propolis extracts (green and red) showed antimicrobial activity against the microorganisms evaluated, *Streptococcus mutans* and *Streptococcus sobrinus*, not allowing these microorganisms to grow in the environment, showing the antibacterial capacity of this natural product, which has bactericidal

and bacteriostatic properties, even when associated with the other compounds of a varnish.

Discussion

This study aimed to verify the antimicrobial capacity of the different types of propolis extract, green and red, on the different microorganisms considered to be the main agents related to

the development of caries disease, *Streptococcus mutans* and *Streptococcus sobrinus*.

Therefore, positive results were obtained in terms of inhibiting the microbial growth of the two groups of bacteria used, when using the two propolis extracts (green and red), with the red propolis extract being slightly more relevant, having the ability to inhibit microbial growth even at lower concentrations.^{16,17,26}

When analyzing the results obtained with the research carried out in comparison with other articles already published in the literature, we can see the similarity in the results found,^{16,17} since significant reductions in microbial concentration were already found when using propolis extracts in various forms/products.²⁷⁻²⁹

Some studies presented varnish formulations based on propolis, whether or not associated with fluorides or other natural substances; on the other hand, other studies presented the use of propolis extract for the development of various types of materials/products, dental or otherwise, such as chewing gum, primer, among others, but always intending to reduce the bacterial contingent.⁹⁻¹⁵

Use of the red propolis extract to reduce the periodontopathogenic microorganisms of the orange complex in multi-species biofilms, showed positive results,³⁰ similar results were observed in the present study, with Minimum Inhibitory Concentration with the red propolis extract carried out, and up to 800 micrograms per ml showed a significant reduction in the biofilm's metabolic activity.

In another study, Martins et al.,³¹ evaluated the inhibitory effect of these mouthwashes on the microorganisms *Streptococcus* ssp. and *Lactobacillus casei* of mouthwashes based on red propolis, with the addition of sodium fluoride or not in their composition, and a chlorhexidine-based mouthwash as a control group. Observed results were favorable, with red propolis extract-based mouthwashes showing similar antimicrobial capacity, cytotoxicity, and anti-biofilm effects to chlorhexidine-based mouthwashes, and also concluded that, due to the undesirable effects of using chlorhexidine mouthwashes, red propolis extract-based mouthwashes, combined or not with sodium fluoride, can be used as a natural alternative treatment and antimicrobial agent.

From a different perspective, Tulsani et al.,³² carried out a clinical trial study in which they developed chewing gums based on propolis and xylitol to evaluate the reduction in the number of *Streptococcus mutans* (bacterial contingent) in schoolchildren. Saliva samples were collected, and a regimen of time of use and intervals between chewing gums was established for the different groups. The microorganism count was compared to the initial count in the children's saliva, and in all groups, there was a significant reduction in the bacterial count when compared to the initial values, without the propolis-based chewing gum regimen. This shows the antimicrobial capacity of propolis, similar to what was observed in this study.

Another study, by Wang et al.,²⁷ used propolis in a different formulation to evaluate its ability to inhibit the bacteria that cause caries; in this study, a propolis essential oil microemulsion (PEOME) was used, and the study showed the significant antibacterial and anti-biofilm capacity of this formulation called PEOME (propolis essential oil microemulsion); therefore, this study obtained similar results to the present study in relation to the action on microorganisms.

On the other hand, similar to the study presented in relation to the material studied, in the *in vivo* study by Imani et al.,²⁸ propolis nanoparticles were also used in different concentrations to compare the minimum inhibitory concentration in an orthodontic primer

developed to evaluate the antimicrobial activity of propolis in the primer for orthodontic purposes. The different primers contained propolis nanoparticles at concentrations of 0, 1, 5 and 10%; the concentrations of 1, 5, and 10% showed significant inhibition of the number of *Streptococcus mutans* colonies in 24 hours of incubation compared to the control group (0% propolis). Thus, primers containing propolis nanoparticles showed a marked reduction in the count of cariogenic bacteria in the oral cavity of rats.

The antimicrobial capacity of propolis-based dental varnishes stands out, which justifies their use in dentistry, given that they have shown satisfactory and proven antimicrobial activities against the main microorganisms that act in the development and maintenance of caries disease, as analyzed in the study by De Luca et al.,³³ which has used other microorganisms associated with caries in addition to *Streptococcus mutans*, such as *S. salivarius*, *S. sanguinis*, *Lactobacillus casei*. Furthermore, the use of propolis-based varnishes proved to be efficient and safe as they showed low cytotoxicity being biocompatible, which ensures their safe and efficient use in dentistry.

Also, the use of wild green propolis as a mouthwash solution on dental biofilm was evaluated by Cardoso et al.¹⁸ In their study, they observed an antimicrobial action against *S. mutans*, however, this type of propolis has not been evaluated in the form of dental varnish, that's what we made. For endodontics, Predrinha et al.,³⁴ investigated the effects of antimicrobial compounds like propolis and copaiba oil-resin associated with hydrocortisone in periodontal ligament fibroblasts, and they compared their impact to that of Otosporin, founding positives effects. Observed the potential effects on inflammatory responses and periodontal ligament fibroblast proliferation.

The use of propolis in dental varnishes is therefore justified, as it has antimicrobial capacity against the main microorganisms that cause caries, *Streptococcus mutans* and *Streptococcus sobrinus*, even when associated with the other substances to develop a varnish. That way, it could be an excellent natural compound (an excellent biomaterial) in preventive treatment options, having in view its low toxicity, which makes it more biocompatible (in addition, its low toxicity makes it more biocompatible) than many substances currently used in dentistry, such as chlorhexidine and essential oils, among others, in addition to being a renewable resource, thus justifying the study and development of new dental materials with natural extracts.

Conclusion

It can be concluded that both propolis extracts (green and red) have significant antimicrobial capacity (more specifically against the microorganisms *Streptococcus mutans* and *Streptococcus sobrinus*, which were the microorganisms cultivated and used in the microbial inhibition capacity and minimum inhibitory concentration tests in this study). Propolis extracts have both bactericidal and bacteriostatic effect, highlighting the fact that the antimicrobial action of red propolis is more significant than the action of green propolis, given that at lower concentrations, red propolis has the same capacity to inhibit bacterial growth, when diluted in water, red propolis continues to have the capacity to inhibit microbial growth, even at low concentrations (equivalent to 6.25% in the dilution).

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None

Conflicts of interest

The authors declare that there are no conflicts of interest.

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