

## PROPOLIS ANTIMICROBIAL ACTIVITY AGAINST PERIODONTOPATHIC BACTERIA

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### ABSTRACT

Propolis extract antimicrobial activity against periodontopathic (ATCC) bacteria was investigated "in vitro". Bacterial strains tested were: *Prevotella intermedia*, *Prevotella melaninogenica*, *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans*, *Capnocytophaga gingivalis* and *Fusobacterium nucleatum*. Minimal inhibitory concentration (MIC) for the strains tested was determined using the method of broth dilution with the propolis extract in serial concentrations. Results showed MIC of 1 µg/ml for *Actinobacillus actinomycetemcomitans* and *Capnocytophaga gingivalis*; and 0.25 µg/ml for *Prevotella intermedia*, *Prevotella melaninogenica*, *Porphyromonas gingivalis* and *Fusobacterium nucleatum*. Some superinfectant organisms were also tested: *Candida albicans* susceptibility to propolis ethanolic extract was demonstrated at a concentration of 12 µg/ml. The MIC for *Pseudomonas aeruginosa*, *Escherichia coli* and *Staphylococcus aureus* (wild types) was 14 µg/ml. All periodontal pathogens and superinfectants tested were susceptible to the propolis extract. The positive results suggest that the propolis extract should be further tested as an adjuvant to periodontal therapy.

**Key words:** bacteria, periodontal pathogens, periodontal diseases, minimal inhibitory concentration, propolis

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### INTRODUCTION

Human periodontal disease has been associated with a complex microbiota. The development of destructive periodontitis seems to be the result of a specific infection (46). Gram positive coccoid bacteria have been related to periodontal health, while periodontal disease was associated with Gram negative rods and spirochetes (28). Many authors (5,10,13,46,47,51) suggest that the presence of *Actinobacillus actinomycetemcomitans*, *Bacteroides gingivalis* (*Porphyromonas gingivalis*) and *Bacteroides intermedius* (*Prevotella intermedia*) is related to active periodontal disease. Other species like *Fusobacterium nucleatum* (13,49,51) and *Capnocytophaga* sp. (45,51) were also associated with disease. According to the Consensus report of the World Workshop on Clinical Periodontics (1996), human

periodontitis is initiated and perpetuated by a small group of bacteria that colonize the subgingival region, mainly Gram-negative, anaerobic or microaerophilic bacteria. Furthermore, most cases of human periodontitis are caused by *Porphyromonas gingivalis*, *Bacteroides forsythus* and *Actinobacillus actinomycetemcomitans* (52).

Because periodontitis is an infectious disease, and taking into consideration that some patients do not respond to conventional mechanical therapy, sometimes antimicrobial agents have been prescribed as adjuvants to periodontal treatment (9). However, the emergence of pathogenic bacteria that are resistant to antibiotics, due to inappropriate systemic usage, has become a serious clinical problem. Loesche (29) pointed out that in order not to contribute to a "coming plague", dentists should add the knowledge of an infectious disease specialist to their surgical skills. Gillette (17) suggested that antibiotics should be used only

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when there is a reasonable specific goal, so that the expected benefits will outweigh the risks to the patient and society.

The clinical use of antibiotics and other antimicrobial agents, as adjuvants for the treatment of periodontitis, has been extensively investigated in the past decade (15,20,22,43,44). Recently, special attention has been paid to natural medication, and propolis has been reported to possess certain medicinal properties (30).

Propolis is a natural composite balsam, produced by honey bees (*Apis mellifera*) from the gum of various plants. Bees collect vegetal exudates and form pellets with their mandibles, mixing the exudates with wax and products of their salivary glands. The resulting material is used to strengthen the nest, provide protection from microorganisms, and as an embalming substance to cover the carcass of a hive invader (21). The medicinal properties of propolis have been widely investigated (11,14,15,21,22,23,25,30,31,38,40,41).

Antimicrobial action of propolis has already been shown. Various studies report on antibacterial (11,14,15,21,22,41), antifungic (11) and antiparasitic (21) actions.

Gebara *et al.* (15) in 1996 demonstrated propolis antimicrobial activity against *Streptococcus mutans* and *Streptococcus sobrinus*, as well as its action in inhibiting the production of polysaccharides. In 1998, Rosalen *et al.* (40), observed that the application of propolis extract on rat molars reduced the severity of carious lesions in these animals.

Once propolis is known to function as an antibacterial agent against specific organisms, the aim of this study was to investigate the antimicrobial "in vitro" action of a propolis extract (with a previously determined composition) against periodontopathic bacteria, as well as against superinfectants.

## MATERIALS AND METHODS

The propolis ethanolic extract (70% ethanol) used in this study was provided by UNESP - Botucatu - Department of Animal Production- Brazil.

Susceptibility tests were performed using the following ATCC strains: *Prevotella intermedia* (33563), *Prevotella melaninogenica* (25845), *Porphyromonas gingivalis* (33277), *Actinobacillus actinomycetemcomitans* (29523 and 29522), *Capnocytophaga gingivalis* (33624), *Fusobacterium nucleatum* (10953) and *Candida albicans* (10231). Isolates of *Pseudomonas aeruginosa*, *Escherichia coli* and *Staphylococcus aureus* from our own culture collection were also tested.

Aliquots of frozen stocks in 20% glycerol of the different strains were inoculated on agar plates. *P. intermedia*, *P. melaninogenica*, *P. gingivalis*, *A. actinomycetemcomitans*, *C. gingivalis*, *F. nucleatum* were cultured in enriched Brain Heart Infusion agar, for 5 days. *C. albicans* was grown for 2 days in Saboraud dextrose agar, and *P. aeruginosa*, *E. coli* and *S. aureus* were cultured in Brain Heart Infusion agar for 1 day. The resultant cultures were diluted in PBS (phosphate buffer solution), to

reach concentrations equivalent to Mac Farland scale n° 1. *P. intermedia*, *P. gingivalis* and *F. nucleatum* were resuspended to a concentration of 10<sup>5</sup> cfu/ml. *A. actinomycetemcomitans*, *C. gingivalis*, *P. melaninogenica*, were diluted to a concentration of 10<sup>7</sup> cfu/ml. The concentration was 3 x 10<sup>8</sup> cfu/ml for *C. albicans*, *P. aeruginosa*, *E. coli* and *S. aureus*.

Minimal inhibitory concentrations (MIC) for propolis against the tested strains were determined using the propolis extract in serial concentrations: 0 (negative control), 0.0625, 0.125, 0.25, 0.5, 1, 2, 4, 6, 8, 10, 12, 14 and 16 µg/mL. Control plates with serial concentrations of ethanolic alcohol solution were also tested. The strains were inoculated by a Steer apparatus. All tests were performed in quadruplicate.

All strains were grown in Brain Heart Infusion Agar (BHI - Difco) except for *Candida albicans*, which was grown in Saboraud agar (Difco), and incubated at room temperature for 4 days.

*Pseudomonas aeruginosa*, *E. coli* and *S. aureus* inoculated in Brain Heart Infusion agar were incubated aerobically at 37°C for 2 days. Brain Heart Infusion agar enriched with hemin (0.1% - Sigma) and menadione (0.01% - Sigma) was used to grow strains of *P. intermedia*, *P. melaninogenica*, *P. gingivalis*, *A. actinomycetemcomitans*, *C. gingivalis* and *F. nucleatum*. The plates were incubated anaerobically (Gas-Pak-BBL) at 37°C for 7 days.

## RESULTS

Minimum Inhibitory Concentration (MIC) was determined as the lowest concentration of the propolis extract, which inhibited the growth of the tested microorganisms.

The propolis extract showed antimicrobial activity against all tested strains. Table 1 presents the Minimal Inhibitory Concentrations obtained for each strain tested. All control

**Table 1.** Minimum Inhibitory Concentration (MIC) of propolis ethanolic extract\* obtained for each strain tested. Tests in quadruplicates.

Microorganism	(MIC* - µg/mL)
<i>Actinobacillus actinomycetemcomitans</i> ATCC 29523 and 29522	1
<i>Capnocytophaga gingivalis</i> ATCC 33624	1
<i>Fusobacterium nucleatum</i> ATCC 10953	0.25
<i>Porphyromonas gingivalis</i> ATCC 33277	0.25
<i>Prevotella intermedia</i> ATCC 33563	0.25
<i>Prevotella melaninogenica</i> ATCC 25845	0.25
<i>Staphylococcus aureus</i> (wild type)	14
<i>Pseudomonas aeruginosa</i> (wild type)	14
<i>Candida albicans</i> ATCC 10231, IAL 1611	12
<i>Escherichia coli</i> (wild type)	14

plates, including those with different ethanolic alcohol concentrations and the negative controls, presented regular bacterial growth.

## DISCUSSION

Besides showing antimicrobial activity against periodontopathic bacteria, the propolis extract did not demonstrate selection of superinfectant organisms.

The verification of the antimicrobial action of the propolis extract is not surprising. The primary function of propolis in the hive is to act as a biocide, being active against invasive bacteria, fungi and even invading larvae (16,27,32). There are a number of studies documenting the biocidal functions of propolis, its extracts and constituents. The spectrum of activity is fairly broad, with action against Gram positive and Gram negative rods and cocci, yeast and fungi (6).

The antimicrobial activity of propolis ethanolic extract has been studied by several authors, however, few studies have investigated its activity towards oral pathogens (15,22,38,50).

The present study has shown propolis antimicrobial activity against the following periodontal pathogens: *A. actinomycetemcomitans*, *P. intermedia*, *P. melaninogenica*, *P. gingivalis*, *C. gingivalis* and *F. nucleatum*. Antimicrobial activity against *Candida albicans*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Staphylococcus aureus* was also demonstrated in this study, confirming previous results (3,12,18,22,26,38,50).

Different results were achieved by Nieva *et al.* (35) that reported antimicrobial activity against *Staphylococcus aureus*, but no action against *Pseudomonas aeruginosa* and *Escherichia coli*. A possible explanation for diverse results is the fact that propolis composition is variable depending on the region and season that it is collected (1,19,24,37). Consequently, the active compounds may not be present in sufficient quantities or quality.

One of the limitations to propolis use is the variability in composition and action as a consequence of variations in the flora of the region where it is produced. However, according to Bankova *et al.* (2), antimicrobial action is expected to be always present because of its vital importance as an antimicrobial agent to the bees, independently of the region where the propolis is produced. In a recent study, Sforcin *et al.* (42) did not find a seasonal effect on the antimicrobial activity of the Brazilian propolis.

Propolis mechanism of antimicrobial action, though not completely understood, seems to be complex and may vary according to its composition. The compounds known to have antimicrobial action are mainly the flavonoids and cinamic acids (2).

An aliquot of the same propolis extract tested in this study was sent to Bulgaria, for analysis of constituents by gas chromatography. The flavonoids detected were Kaempferol; 5,6,7 Trihidroxi-3,4 dimetoxiflavone and Aromadendrine-4-metil-eter. Other constituents identified were 9-E-2, 2-dimetil-6-

carboxietenil-8-prenil-2H-1-benzopirano and 9-Z-2,2-dimetil-6-carboxietenil-8-prenil-2H-1-benzopirano (4).

Osawa *et al.* (36) reported that Kaempferol had antimicrobial activity against *Streptococcus mutans* and *Actinomyces viscosus*. Cai and Wu (7) demonstrated that the same constituent inhibited the growth of *P. intermedia* and *P. gingivalis*.

Regarding susceptibility of the tested microorganisms to propolis it seems that they were more susceptible to propolis than to some antibiotics. Carrasco *et al.* (8) showed the MIC for doxycycline, tetracycline, metronidazole, ofloxacin and amoxicillin (8, 4, 8, 6 and 4 µg/mL respectively) against *P. gingivalis*, *P. intermedia* and *F. nucleatum* "in vitro". When their MIC results are compared to ours, it can be observed that frequently used antibiotics had greater MIC than propolis, against *P. gingivalis*, *P. intermedia* and *F. nucleatum*. These periodontal pathogens may be more susceptible to propolis than to the antibiotics shown above.

Previous study on the susceptibility of *A. actinomycetemcomitans* to selected antimicrobial agents indicated that MIC90 to penicillin varied from 1.0 to 6.25 µg/ml, to amoxicillin from 1.0 to 2.0 µg/ml, to tetracycline from 0.5 to 8.0 µg/ml, to doxycycline from 1.0 to 3.1 µg/ml, and to metronidazole from 12.5 to 32 µg/ml (34). In the present study, the MIC of this pathogen to propolis was 1 µg/ml.

Susceptibility tests of *P. gingivalis* have shown that MIC90 to penicillin varied from 0.016 to 0.29 µg/ml, amoxicillin from 0.023 to < 1.0 µg/ml, metronidazole from 0.023 to 2.1 µg/ml (34). Interestingly, in present study MIC to propolis was 0.25 µg/ml.

In 1990, Rams *et al.* (39) noted that some strains of *S. aureus* isolated from the periodontal pocket were resistant to tetracycline, penicillin, metronidazole and erythromycin. Additionally, when the antimicrobial activity of 18 antibiotics was tested against *Enterobacteriaceae* and *Pseudomonadaceae*, only ciprofloxacin was able to eliminate these microorganisms from the periodontal pocket (48).

Our results showed that propolis extract presented "in vitro" antimicrobial activity, not only against some periodontopathic bacteria (*F. nucleatum*, *P. gingivalis*, *P. intermedia*, *P. melaninogenica*, *A. actinomycetemcomitans* and *C. gingivalis*) but also against some organisms able to cause superinfection (*S. aureus*, *P. aeruginosa*, *E. coli* and *Candida albicans*).

It is important to remember that "in vitro" tests do not reflect the real conditions found in periodontal pockets. They do not take into account biofilm formation. In addition, determination of MIC values depends on technical details that may vary between laboratories (33).

The antimicrobial action observed for the propolis extract suggest its usage as an adjuvant to periodontal therapy. A step further should be given to verify if a dose sufficient to kill the target microorganisms can be reached within the subgingival environment, without causing major local or systemic adverse effects.

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## RESUMO

### Atividade antimicrobiana da própolis contra bactérias periodontopatogênicas

A atividade antimicrobiana da própolis contra bactérias periodontopatogênicas (ATCC) foi investigada através de testes “in vitro”. As cepas bacterianas testadas foram: *Prevotella intermedia*, *Prevotella melaninogenica*, *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans*, *Capnocytophaga gingivalis* e *Fusobacterium nucleatum*. A concentração inibitória mínima (CIM) foi determinada usando-se o método de diluição do extrato de própolis no meio de cultura em diferentes concentrações. Os resultados demonstraram CIM de 1 µg/ml para *Actinobacillus actinomycetemcomitans* e *Capnocytophaga gingivalis*; e 0,25 µg/ml para *Prevotella intermedia*, *Prevotella melaninogenica*, *Porphyromonas gingivalis* e *Fusobacterium nucleatum*. Alguns microrganismos que desempenham “in vivo” papel de superinfectantes também foram testados: a susceptibilidade de *Candida albicans* ao extrato etanólico de própolis foi observada na concentração de 12 µg/ml. A CIM para *Pseudomonas aeruginosa*, *Escherichia coli* e *Staphylococcus aureus* (tipo selvagem) foi de 14 µg/ml. Todos os patógenos periodontais e microrganismos superinfectantes testados foram sensíveis ao extrato de própolis testado. Os resultados obtidos encorajam a realização de novos estudos com esse extrato de própolis, para avaliar sua utilização como coadjuvante ao tratamento periodontal.

**Palavras-chave:** bactéria, patógeno periodontal, doença periodontal, concentração inibitória mínima, própolis.

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