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Research Article

In Vitro Antimicrobial Activity of Ethanolic Propolis Extracts from Santander against *Enterococcus faecalis*

Laura Viviana Herrera Sandoval[1]*, Marcos Humberto Pisciotti Ortega[2], Otoniel Ramos Monsalve[2], Laura Fernanda Neira Fuentes[3], Julio Roberto Pinzón Joya[4], Adriana Victoria Herrera Becerra[2] and José Antonio Soto Salcedo[2]

[1]Bacteriologist and Clinical Laboratorist, Universidad Industrial de Santander, Master in Basic Biomedical Sciences, Universidad Industrial de Santander, Universidad Santo Tomás, Colombia

[2]Second Year Student, Specialization in Endodontics, Universidad Santo Tomás, Colombia

[3]Bacteriologist and Clinical Laboratorist, Universidad Industrial de Santander, Colombia

[4]PhD. in Organic Chemistry, Universidad Santo Tomás, Colombia

***Corresponding Author:** Laura Viviana Herrera Sandoval, Bacteriologist and Clinical Laboratorist, Universidad Industrial de Santander, Master in Basic Biomedical Sciences, Universidad Industrial de Santander, Universidad Santo Tomás, Colombia.

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Abstract

Objective: To evaluate the antimicrobial activity of an ethanolic propolis extract from Santander province against *Enterococcus faecalis.*

Methods: An experimental *in vitro* study was made. Starting from pristine propolis collected in the city of Lebrija (Santander), an ethanolic extract was obtained using a Soxhlet apparatus. The antimicrobial activity was measured using the macro dilution tube technique by counting the Colony Forming Units (CFU) per mL after 48 hours. Propolis solutions with concentrations in the range from 100 to 0.19 mg/mL were evaluated. Tubes containing dilutions of the antibiotic norfloxacin and solvent (96% ethanol) were used as reference and control.

Results: The propolis solutions within the range from 100 mg/mL to 50 mg/mL inhibited 100% growing of *E. faecalis*. Propolis extract showed an inhibitory concentration 50 (IC_{50}) of 1.19 mg/mL and an inhibitory concentration 90 (IC_{90}) of 7.92 mg/mL respectively. Norfloxacin on the other hand was more effective with IC_{50} and IC_{90} of 0.06 and 2.39 µg/mL respectively. Ethanol showed non-antimicrobial activity at the concentration present within the extracts.

Conclusion: With further studies support it would be possible to suggest the use of propolis from Santander as an alternative of irrigation and medication in endodontics because of its powerful effect against *E. faecalis* considering it is the most frequent pathogen found in persistent infections.

Keywords: *Antimicrobial Activity; Santander; Enterococcus faecalis*

Introduction

The origin of most periapical and endodontic infections is polymicrobial. However, numerous studies establish *Enterococcus faecalis* as the endodontic pathogen most frequently in persistent lesions [1-3] this Gram positive coconut belongs to the intestinal biota of mammals and is associated with multiple infectious processes in man. As a pathogen, it has the ability to adhere to the tissues of the host, including dentin and even penetrate the dentin tubules, forming difficult biofilms that allow it to survive for prolonged periods of time in environments with limited nutrients. This aspect has been considered its main mechanism of pathogenicity in the root canal [4,5].

Nowadays there is a growing interest in the use of natural products or mixtures in the elimination of microorganisms. Propolis is a substance of vegetable origin, made by bees, with biological functions of support and protection of hives; It has been widely used in traditional medicine for infections of the skin, mucous membranes and respiratory system [14]. Its chemical composition varies according to the geographical origin, but in general terms it includes organic compounds mainly of the phenols, esters, flavonoids and terpenes type, among others [15]. Due to the above, propolis from different regions are the object of research in dentistry due to its antimicrobial, anti-inflammatory, anesthetic and healing properties [16,17]. In endodontics it has already been used for irrigation and cleaning of the root canal, its activity is compared with calcium hydroxide, showing similar effects in the improvement of the clinical course of infections and the regeneration of the dental pulp with low toxicity [8,18,19].

The effect of Colombian propolis has been evaluated against some cariogenic agents [20] but there are still no studies on endodontic pathogens. Therefore, the objective of the present investigation was to evaluate the antimicrobial activity of an ethanolic propolis extract of Santander on *Enterococcus faecalis*.

Materials and Methods

An experimental study was carried out *in vitro* in which the capacity of a pro-mole extract of Santander obtained in the laboratory by the Soxhlet method to inhibit the growth of an *E. faecalis* strain was evaluated.

Materials

Propolis: It was obtained from the apiaries of the Santa Teresa farm, located at Km 15 via Cúcu-ta Vereda Agua Blanca, in the department of Santander at 1773.19 meters above sea level. It was collected from the trap traps of the hives of the apiary, which were aseptically made a scraping with spatula that was deposited in sterile bags, then the contents were passed through a sterile sieve. to remove plant remains and large insects that could affect the extraction. The crude propolis was placed in sterile amber glass jars duly labeled [21].

Reference compound: Commercial 400 mg Norfloxacin, obtained from the Colombian Medicines Industry, was used as a reference antimicrobial and was purified by standard laboratory methods for use in the experiments.

Microorganism: A strain of *Enterococcus faecalis* ATCC 29212 was used, which was obtained lyophilized KWIK-STIK from laboratories Microbiologics®, acquired by the basic science laboratory of the University of Santo Tomás. This strain was reconstituted by massive sowing in blood agar (Oxoid®), incubated at 37°C for 48 hours in a CO_2 atmosphere; its growth was verified on *Enterococcus* agar (BD®) through the appearance of black colonies. The cultures used in the experiments of the present investigation corresponded to the recovery pass.

Obtaining of extract: Once the sample was obtained, it was degreased with n-hexane (JT.Baker®) in a Soxhlet equipment. The extracts were obtained by applying the Soxhlet method with anhydrous ethanol (Carlo Erba®). The resulting extract was concentrated by simple distillation until the solvent was completely removed [22].

Activity antimicrobial: It was evaluated through a macrodilution test in tube [23]. For the preparation of the inoculum of the microorganism subcultures of 24 hours were used on blood agar, colonies were taken and a standard solution of *E. faecalis* in trypticase soy broth was prepared (Merck®) with turbi-dez of 3.0 on the McFarland scale.

From the propolis extract, a working solution of 200 mg/mL in 96% ethanol was prepared. Subsequently, serial dilutions 1:2 from 100 to 0.19 mg/mL were made in test tubes. In the case of the reference medicine (Norfloxacin), concentrations from 0.10 to 5.1×10^6 mg/mL were evaluated [24]. 100 µl of the standard solution of *E. faecalis* ATCC 29212 was added to each test tube. groups control one with saline solution as growth control and another with serial dilutions of ethanol from 96% to 0.18% to rule out the possible effect of the solvent. The tubes were incubated at 37°C for 48 hours in a CO_2 atmosphere. Subsequently, the Colony Forming Units (CFU/mL) were

counted, through the plate count technique, and serial dilutions were made in saline solution to 10^6 of the tubes containing the evaluated compound and the microorganism. Then 0.1 mL of each dilution was seeded in Petri dishes with Plate Count Agar (Merck®). Finally, they were incubated for 24 hours at 37°C in a CO_2 atmosphere and counted with the help of a light camera. Antimicrobial activity tests were performed in triplicate in independent experiments.

Statistic analysis

The results of the UFC/mL counts were validated and the univariate analysis for quantitative variables was performed with SPSS software version 15.0 (SPSS Inc®). The UFC/mL counts showed a non-normal distribution according to the Shapiro-Wilk test. The percentage of Inhibition (PI) was calculated for each of the concentrations evaluated through the formula:

$$\frac{\text{(CFU/mL average control - CFU/mL average concentration) x 100}}{\text{(CFU/mL average control)}}$$

XLfit® software (IDBS) was used to determine inhibitory concentration 50 (IC_{50}) and in-hybridity concentration 90 (IC_{90}) of the extract against *E. faecalis*.

Results

The effect of the different concentrations of propolis was established from the antimicrobial activity test by tube dilution. The results are presented in tables, in terms of CFU/mL of *E. faecalis* for each concentration. To facilitate the understanding of the results, the percentages of Inhibition (PI) of each evaluated concentration are shown.

It was determined that the concentrations of 100 mg/mL and 50 mg/mL of the extract inhibited 100% growth of *E. faecalis* with counts of zero CFU/mL. Likewise, it was evidenced that the minimum inhibitory concentration (MIC) was 50 mg/mL. The PIs obtained showed that all the evaluated concentrations of the propolis extract inhibited the growth of the microorganism of interest with percentages from 100% to 22.26%. On the other hand, the inhibition of 50% of the growth occurred between concentrations as low as 1.56 and 0.78 mg/mL. The lowest percentage of inhibition was 22.26% at a concentration of 0.195 mg/mL (Table 1). It was established that the activity of the diluent (ethanol) of the extracts on *E. faecalis* was not significant in the concentrations evaluated, that is, 48% to 0.09% (data not shown).

Concentration (mg/mL)	CFU/mL (Me)	PI (%)
100	0	100
50	0	100
25	0	99,99
12,50	$1,05 \times 10^6$	99,50
6,25	$2,20 \times 10^7$	71,42
3,12	$2,42 \times 10^7$	86,53
1,56	$7,40 \times 10^7$	60,74
0,78	$3,30 \times 10^8$	33,57
0,39	$14,10 \times 10^8$	27,58
0,19	$7,20 \times 10^9$	22,26
Control	$23,80 \times 10^9$	NA

Table 1: *Activity of ethanolic extract of propolis against E. faecalis. Me: Medium; PI: Percentage of Inhibition; NA: Not applicable..*

Norfloxacin as a reference medicine showed activity at all tested concentrations. The inhibition of 100% of the growth of the microorganism of interest was demonstrated in concentrations as low as 0.01 mg/mL; Likewise, at the minimum concentration evaluated 5.1 × 10^6 mg/mL, norfloxacin inhibited *E. faecalis* in 20.87%. It was possible to establish that the MIC for this drug in the evaluated strain was 0.01 mg/mL (Table 2). When comparing the activities of the evaluated substances, norfloxacin was 1000 times more effective than the natural extract studied.

Concentration (mg/mL)	CFU/mL (Me)	PI (%)
0,10	0	100
0,03	0	100
0,01	0	100
3,70 × 103	0	99,99
1,23 × 103	0	99,99
4,10 × 104	1,1 × 10^6	86,38
1,30 × 104	2,38 × 10^8	48,72
4,50 × 105	3,43 × 10^8	44,57
1,52 × 105	2,84 × 10^8	39,80
5,10 × 106	3,98 × 10^8	20,87
Control	4,82 × 10^8	NA

Table 2: *Activity of Norfloxacin versus E. faecalis.*
Me: Medium; PI: Percentage of Inhibition; NA: Not Applicable.

Given the antimicrobial activity shown by the compounds it was possible to calculate the IC_{50} and IC_{90}. In the case of propolis extract, the activity corresponded to 1.19 mg/mL and 7.92 mg/mL, and norfloxacin showed to be much more effective with IC_{50} and IC_{90} of 6 × 10^5 and 2.39 × 10^3 mg/mL respectively.

Discussion

The main objective of endodontic therapy is to eliminate or reduce the microorganisms present in the root canal system through a chemical-mechanical treatment [11,12]. Nowadays, chemical substances are sought that represent true alternatives for irrigation and treatment. intraconductive medication, given that the most commonly used such as sodium hypochlorite and calcium hydroxide report some toxicity and its effectiveness is reduced against endodontic pathogens [6,10]. The present study demonstrated the *in vitro* antimicrobial effect of an extract of propolis of Santandereana origin against *E. faecalis*, this is a relevant endodontic pathogen, associated with greater frequency in refractory or persistent lesions [4,5,25,26].

Through the method of direct contact and macro-dilution in tube, the antimicrobial activity of propolis extract from Santander was demonstrated in concentrations of 100 mg/mL to 0.195 mg/mL against *E. faecalis*. These findings coincide with previous *in vitro* studies by Cortés., *et al.* (2010) who showed through the agar diffusion method that a propolis extract from Cundinamarca had activity against *E. faecalis* up to concentrations of 3.1 mg/mL [27]. Likewise, Ferreira and collaborators (2007) evaluated the effect of an ethanolic extract of propolis at 10% (100 mg/mL) by the method of macrodilution in tube and compared it with intra-conductive medications such as calcium hydroxide, camphor-para-chlorophenol and form-cresol; in this way, they found a similar effectiveness of all substances against endodontic pathogens such as *Prevotella nigrescens, Fusobacterium nucleatum, Actinomyces israelii, Clostridium perfringens* and with less activity towards *Enterococcus faecalis* [28]. However, Lima and collaborators (2007) reported conflicting results when evaluating a Chilean extract

of 50% propolis (50 mg/mL) and establishing that it did not possess significant *in vitro* activity against *E. faecalis* among other microorganisms compared to chlorhexidine and calcium hydroxide [29].

The ethanol extract of propolis from Santander in-hybriated the growth of *E. faecalis* from 100% to 22.6%. The ability of this natural preparation to affect the growth of bacteria demonstrated in this research, could be related to aspects such as: the methodology used to be the macrodilution method a technique that allows a better direct contact of the microorganism and the substance, compared with the agar diffusion technique, the latter is the one most commonly used in previous studies [23]. On the other hand, the literature attributes the antimicrobial properties to its high content of flavonoids, lactones, saponins, phenols, triterpenes, these. of caffeic acid and a flavone known as galangin; additionally, it has been shown that these components probably affect bacterial DNA through RNA polymerase [21,30,31]. However, the results of propolis research must be carefully interpreted since these mixtures present high variability in their composition according to geographical origin and times of collection [20].

In this study it was shown that pro-pollens extract was less effective against *E. faecalis* compared to norfloxacin as a reference medicine. These differences can be interpreted as a result not only of the nature of the compounds, but also of the complex chemical interactions involved in the biological effects of a natural product and that can occur in a mixture of substances such as propolis [32]. Norfloxacin is a drug of known chemical structure whose mechanism of action has been extensively studied and which was purified for use in this investigation [24].

The experimental evidences present the possibility of application and use of propolis in diverse dentistry specialties and coincide in highlighting the wide benefits that its therapeutic use could have in the oral cavity. All this is supported by the fact that it is a natural substance to which antimicrobial, analgesic, anti-inflammatory and healing effects are attributed [17]. It is worth noting the rapid advance of studies on propolis in endodontic models during the last years. Kayaoglu., *et al.* (2011) and Jahromi and collaborators (2012) determined that as intraconductive medication and after seven days, propolis extracts showed activity similar to calcium hydroxide in the elimination of *E. faecalis* from infected dentinal tubules [26,33]. Similarly, Awawdeh., *et al.* (2009) demonstrated in models of infected dentin discs that a commercial purpose (3000 mg/mL) was more effective than calcium hydroxide in total elimination of *E. faecalis* with only 24 or 48 hours of medication [25]. Recently, Madhubala., *et al.* (2011) reported that a commercial propolis was as effective as tri-antibiotic paste (ciprofloxacin, minocycline and metronidazole) in the elimination of *E. faecalis* in tooth models, after only 48 hours of medication [25]. In this way, it is possible to propose this natural product as an antimicrobial agent of potent intra-root canal use.

The results of this research work, the first in the region, propose santandereano propolis as an alternative for specific use in the area of endodontics, given the potent effect against *E. faecalis* which is one of the pathogens. We are more resistant to disinfectants and intraconductive medications used in endodontic therapy. In a complementary way, propolis has advantages over the substances that are currently used in irrigation and medication, which are based on antibacterial, immunostimulant, healing and low toxicity properties [17]. However, it is necessary to carry out complementary studies on the chemical composition of the propolis studied as well as establishing the chemical route to identify the leading compounds present in this mixture.

From the *in vitro* findings presented, it is pertinent to continue with studies in *in vitro* tooth models and later clinical trials that demonstrate the advantages of propolis compared with other irrigating substances or intra-conductive medications in patients. Simultaneously, it is necessary to investigate with different methodologies the surface tension of the preparations of the proposal to suggest that they can have high penetration capacity in the sites of difficult or impossible access in the system of root canals, dentinal tubules, itsmos and sacs among others.

Conclusion

With further studies support it would be possible to suggest the use of propolis from Santander as an alternative of irrigation and medication in endodontics because of its powerful effect against *E. faecalis* considering it is the most frequent pathogen found in persistent infections.

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