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RESEARCH NOTE

Effect of Chilean propolis on cariogenic bacteria *Lactobacillus fermentum*

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Abstract

N. Saavedra, L. Barrientos, C.L. Herrera, M. Alvear, G. Montenegro, and L.A. Salazar. 2011. Effect of Chilean propolis on cariogenic bacteria *Lactobacillus fermentum*. Cien. Inv. Agr. 38(1): 117-125. Dental caries is an infectious disease of worldwide public health concern. Among the bacteria involved in this pathology are *Streptococcus mutans, Streptococcus sobrinus* and organisms belonging to the genera *Actinomyces* and *Lactobacillus*. The pharmaceutical industry is focussing on the discovery of new antibacterial products after a greater resistance to those already known. Propolis has been used since ancient times, so their effects against various microorganisms have been already investigated. In our study, we evaluated the antimicrobial effect of 6 commercial ethanolic propolis extracts on the bacterium *Lactobacillus fermentum.* This bacterium was isolated after its identification by Polymerase Chain Reaction using species specific primers, and after growing microbiological samples from cavities of patients diagnosed with dental caries and with indication of tooth extraction. *L. fermentum* was detected in 9 of 40 patients, corresponding to 22%. The susceptibility study, carried out by microplate dilution, found antimicrobial activity in four of the six ethanolic extract of propolis used. These differ in the effective concentration against the microorganism, which can be attributed to factors such as the botanical origin, geographic location and harvest season. Among the results, it was noticed that these polyphenols showed concentrations ranging between 9 ± 0.3 and 85 ± 2.1 mg/mL. The chromatographic analysis allowed the identification of caffeic acid, myricetin, quercetin, kaempherol, apigenin, pinocembrin, galangin and caffeic acid phenethyl ester (CAPE). Our study demonstrates the antimicrobial action of propolis on *L. fermentum,* the patogen related to caries development.

Key words: *Lactobacillus fermentum*, dental caries, propolis, antibacterial activity.

Introduction

Dental caries is one of the most extended infectious diseases worldwide, with more than 90% of individuals infected. In children, for example, it has indexes five times higher than the second most frequent pathology, asthma (Becker *et al*., 2002).

In Chile, the situation is similar. An epidemiological study performed by the Ministry of Health indicates that, in 6 to 8 year-old children, only 15.3% had a dental caries-free record, something similar occurring in 12 year-old children. In regard to adults, it was observed that

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100% of the individuals from 35 to 44 year-old and from 65 to 74 year-old age groups had dental caries (MINSAL, 1997; Soto *et al*., 2007).

Additionally, the World Health Organization (WHO) ranks our country among the highest in DMFT levels for individuals between 35 to 44 year-old (DMFT> 13.9) and moderate for 12 year-old-children (DMFT 2.7 to 4.4) (Pepersen, 2003), through DMFT, which describes the amount of dental caries of an individual, and is used to express prevalence. This is obtained by calculating the sum of decayed, missing and filled teeth.

Among the microorganisms associated with the development of dental caries are mainly *Streptococcus* from the group *mutans* (species *S. mutans, S. sobrinus)*, *Lactobacillus spp*. and *Actinomyces,* among others (Tanzer *et al.,* 2001). The genus *Lactobacillus* is considered a powerful acidogenic that leads to demineralization of the dental surface (Byun *et al*., 2004). However, they only represent a small portion of dental plate microbiota, with a higher presence in more advanced teeth wounds (cavitation). Therefore, they are conferred a role in wound progression more than in the beginning of it (Van Houte, 1994). They colonize preferably in the back of the tongue and are carried in the saliva when the epithelium molts. Their cariogenicity depends on the consumption of a diet rich on carbohydrates by the host (Nishikawara *et al*., 2006).

For some years, the pharmaceutical industry has centered its efforts on the discovery and achievement of new antimicrobial products, in order to solve the continuous problem of bacterial resistance to well-known antibiotics (Normark and Normark, 2002), and the collateral effects observed frequently after their use (Cuhna, 2001), where natural products used for these purposes since ancient times are the main target (Silver, 1990).

Among these natural products, propolis has been considered a good candidate as adjuvant in the treatment and prevention for various infectious diseases. Propolis is relatively non-toxic (Cuesta *et al*., 2005) with a wide range of antimicrobial activity against a varied order of bacteria, fungi, parasites and viruses (Salomáo *et al*., 2005; Orsi *et al*., 2005; Freitas *et al*., 2006).

More than 160 propolis components have been identified, commonly consisting on waxes, resins, water, inorganic compounds, phenolic compounds and essential oils (Mohammadzadeh and Shariatpanahi, 2007), where most of the biological activity is attributed to flavonoids (Santos and Bastos, 2002). These compounds are present in cells carrying out photosynthesis and that may be found in fruits, legumes, nuts, stems and flowers, as well as tea, wine, and obviously in apicultural products like honey and propolis (Cushnie and Lamb, 2005).

In regard to the above, the present study was aimed to chemically characterize six Chilean commercial propolis products and evaluate their antimicrobial action on the cariogenic bacteria *Lactobacillus fermentum*, isolated from patients with dental caries.

Materials and methods

Patients

A total of 40 individuals participated in this study with an age range varying between 6 and 78 years old. All presented diagnosis of dental pieces extraction due to deep dentine caries (D3). After signing an informed consent form, the piece destined to extraction was anaesthetized. Then, with a sterile excavator, the existing caries damage was scraped off. After the sample was obtained, it was impregnated in a sterile cotton swab and introduced in a Stuart medium.

Microbiological culture

In the laboratory, the sample was immediately sown on a plate with a Difco culture medium, Lactobacilli MRS agar (Winkler Ltda., Santiago, Chile), selective for Lactobacillus, streaking it after the medium swab was rubbed in the superior face of the plate. Then, it was incubated in an oven (Thermo HEPA CLASS100) at 37º C in 5% CO_2 atmosphere for 24 hours.

Identification of Lactobacillus fermentum by PCR

The colonies to be identified were diluted adding a colony of medium size in 500 µL of sterile distilled water; dilution from which the amplification technique was made directly with the technique of Polymerase Chain Reaction (PCR). A specific fragment of the subunit 16S of RNAr (334 bp) was amplified using and conditions described by Dickson *et al*. (2005).

The amplification by PCR was made in a total volume of 50 µL, containing 2 µL of colonies dilution, and 48 µL of the reaction mixture including 1x Buffer [75mM Tris-HCl, 2.2 mM $(NH_4)_2SO_4$, 0.01% Tween 20], 0.2 mM of dNTPs, 200 nM from each primer, 2.0 mM of $MgCl_2$ and 1 unit of *Taq* DNA polymerase (Fermentas, Lithuania). The reaction mixture was prepared in a laminar flow chamber (ESCO, Singapur), that was previously decontaminated during 20 minutes with UV radiation.

The amplification reaction was made in a Thermal Cycler MyCycler (BIORAD, EE.UU). It consisted of an initial denaturation at 98 °C for 3 minutes, followed by 35 cycles with denaturation at 94 °C for 1 minute, hybridization at 50 °C for 1 minute and extension at 72 °C for 1 minute, and ending with a 10-minute-final extension at 72 °C. For the visualization of amplification products obtained by PCR, an electrophoresis in agarose gel at 2% in buffer TBE 0.5x at 100V was made, during 35 minutes, using 100 bp-commercial Ladder as standard of molecular size. Subsequently, the gel was dyed with ethidium bromide (0.5 µg/ml), and visualized in a digital photodocumentation system E-Box 1000 (Vilber Lourmat, France).

Commercial propolis extracts

Six commercial Ethanolic Extracts of Propolis (EEP) were used, which were diluted in distilled water 1:4 and, subsequently, filtered in Wathman Nº 2 paper to discard the waxes. Afterwords, a new filtered process was applied with a 0.2 µm cellulose acetate filter in order to sterilize the solution.

Determination of minimum inhibitory and bactericidal concentrations

For the determination of the minimum inhibitory concentration-MIC (lowest antimicrobial concentration inhibiting the visible growth of a microorganism after the incubation period allowing its growth) and the minimum bactericidal concentration – MBC (lower antimicrobial concentration that hinders a microorganism growth, after a subcultivation in an antimicrobial substance-free-medium) (Andrews, 2001), the following took place:

A 1 x 10^5 UFC x mL inoculation was used for the MIC study, obtained by dilutions made from a tube with an inoculation equivalent to 0.5 McFarland (1.5 x 10^8 UFC x mL), according to Andrews (2001). Trypticase Soy Agar contained in sterile microplates was used as a culture medium. A negative control (sterility control, culture medium and EEP), a positive control (culture medium and inoculation) and six dilutions for each EEP (1/8, 1/16, 1/32, 1/64, 1/128, 1/256) were considered. Subsequently, the colony developments were observed against the light.

For the MBC determination, the wells that resulted negative to growth and, therefore, showing EEP antimicrobial activity were subject to subcultivations in EEP-free media, sowing again on agar plates, incubated at 37 °C in 5% CO_2 atmosphere for 24 hours. All determinations were made in triplicate.

Determination of total polyphenols

For the determination of the total polyphenols present in the evaluated extracts, the method Folin-Ciocalteu was used (Singleton *et al*., 1999). Therefore, each extract 1:10 was diluted in ethanol 70% and then 1:10 in distilled water; subsequently 40 µL of this dilution was mixed with 560 µL of distilled water, 100 µL of the reactive Folin-Ciocalteu (Merck, Germany) and 300 µL of sodium carbonate at 7.5% (p/v). The absorbance was measured at 760 nm after 2 hours of incubation at room temperature. The concentra-

tions were calculated from a calibration curve and expressed in mg/mL equivalent to the mixture of the pinocembrin/galangin standards in a 2:1 proportion (Popova *et al*., 2007).

Chromatographic analysis

The analysis was performed in a High Pressure Liquid Chromatography (HPLC) Merck-Hitachi, equipped with an L-6200 model pump, a UV-visible detector, model L-4200 and a column heater Phenomenex Terma Sphere, model TS-130. The separation was made in a RP-18 column (12.5 x 0.4 cm, particle size 5 µm) (Merck, Germany), which was eluted at 25 ºC using the mixture of 5% formic acid in water (A) and Methanol (B) as mobile phase. The compound separation was made through an isocratic run from 0 to 10 minutes, with the mixture A 70% and B 30%, followed by a gradient until 100% B at 70 minutes. The compounds were detected at a 290 nm wave length, with a sensitivity of 0.001; the injection volume was 10 µL. The identification of phenolic compounds was made by the use of the standards myricetin, kaempherol, quercetin, caffeic acid, galangin, pinocembrin, apigenin, caffeic acid phenethyl ester (CAPE) and resveratrol (Sigma, USA).

Results

Microbiological culture

The 40 samples obtained from patients with diagnosis of deep dentine caries and indication of tooth extraction, were sown in appropriate culture media, obtaining bacterial development in all of them, at the end of the incubation period.

Colonies identification by PCR

Amplification by PCR was made to all the types of colonies developed, resulting 9 positive colonies (22.5%) for *Lactobacillus fermentum* at the end of the analysis.

Lactobacillus fermentum isolation

Nine *Lactobacillus fermentum* strains from the samples cultures of deep dentine caries were isolated from patients with indication of dental extraction, which were subject to a sensitivity test in triplicate.

Determination of the minimum inhibitory concentration

Only EEPs 2, 3, 4 and 5 showed antimicrobial activity, as they inhibited the visible growth of *Lactobacillus fermentum* (Figure 1). In this case, the minimum inhibiting concentrations from each propolis showing activity were: EEP2, 2.5% and EEP3, 2.0%. In the case of EEP4 and EEP5, we may only mention that the growth inhibition was observed in the initial dilution 1:4 for EEP4 and 1:16 for EEP5, as the initial concentration initial of these propolis extracts was not available. Similar results were observed for the 9 isolations analyzed. The wells content showing negativiness was transferred to an EEP-free medium. Those subcultures did not show development after they were observed when the incubation time had finished. This means that both the inhibiting and bactericidal concentrations matched.

Determination of total polyphenols

The propolis extracts evaluated showed large differences of total polyphenol concentrations, where the values found varied between 9 and 85 mg/mL. Additionally, there was no relation between the concentrations specified and the propolis percentages declared by the manufacturers. All the determinations were made in triplicate and the results obtained are shown in Table 1.

Chromatographic analysis

The chromatographic analysis of the propolis studied, in the conditions mentioned before, indicated that they present caffeic acid, myricetin, quercetin, kaempherol, apigenin, pinocembrin, galangin and CAPE (Table 2). The dissenting re-

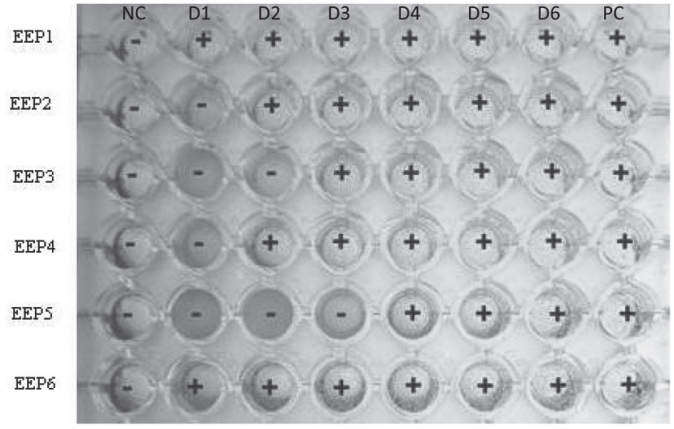

Figure 1. Determination of minimum inhibitory concentration (MIC) in microplates. EEP1 to EEP6, ethanolic extracts of propolis; D1-D6, dilutions of EEP; NC, negative control; PC, positive control; +/- indicates presence/absence of bacterial growth.

sults on antibacterial activity might be explained by the different content and/or presence of these ploy phenols.

Discussion

Dental caries is defined as a multifactorial pathology that begins after dental eruption, softening the hard tooth tissue and evolving to cavity formation (WHO, 1987). Additionally, it is categorized as a transmissible disease, induced by diet, where the main etiological factors responsible are cariogenic bacteria, fermentable carbohydrates and the host susceptibility (Harris *et al*., 2004).

Among the microorganisms associated to dental caries are mainly *Streptococcus* from the *mutans* group (*S. mutans, S. sobrinus*), *Lactobacillus spp.* and *Actinomyces,* among others (Tanzer *et al*., 2001; Cchour *et al*., 2005). Our study proposes the use of propolis as an alternative to the treatment of this pathology, due to a proven antimicrobial effect as previously mentioned by Hayacibara and Koo (2005), Sonmez *et al*. (2005), Olmez and Erdem (2004), Castaldo and Capasso (2002), Pietta *et al*. (2002) and Santos and Bastos (2002).

In this study, *Lactobacillus fermentum* was detected by PCR in 9 out of 40 patients, that is, in 22.5% of the samples analyzed, which is coherent with the results by other authors, where 65 carious dentin samples taken from extracted dental pieces were analyzed, showing a prevalence of *Lactobacillus fermentum* (22%) (Byun and Madkarni, 2004). These results show the already proven capacity of molecular biology techniques for microorganism's identification, which are less troublesome than identification by morphology and biochemical tests.

Table 1. Content of total polyphenols in extracts of commercial Chilean propolis determined by Folin-Ciocalteu method.

Propolis extract	Propolis concentration[1], %	Total polyphenols[2], mg mL^{-1}
EEP 1	20	85 ± 2.1
EEP 2	10	9 ± 0.3
EEP 3	20	41 ± 0.4
EEP 4	ND	16 ± 0.2
EEP 5	ND	19 ± 0.9
EEP 6	20	16 ± 0.4

[1]Propolis concentration declared by manufacturers; [2]Values expressed as mean ± standard deviation.ND: Not declared.

Table 2. Composition of flavonoids of six commercial Chilean propolis determined by HPLC.

Propolis	Caffeic acid	Myricetin	Quercetin	Kaempherol	Apigenin	Pinocembrin	Galangin	CAPE
EEP 1	+	nd	nd	+	+	+	+	+
EEP 2	nd	nd	+	+	+	+	+	+
EEP 3	+	+	+	+	+	+	+	+
EEP 4	nd	nd	+	+	+	+	+	+
EEP 5	+	nd	+	nd	+	+	+	nd
EEP 6	nd	+	+	nd	+	+	+	+

CAPE: Caffeic acid phenethyl ester; + indicates presence; nd: no detected.

With the antimicrobial effect of the propolis selected for this study, differences in the action shown by each were observed. EEP 2, 3, 4 and 5 showed antimicrobial activity, obtaining minimum inhibitory concentration in the dilutions 1:8, 1:16, 1:8 and 1:32, respectively. These variations in the minimum inhibitory concentrations may be due to differences in the chemical composition of propolis, which depends on different factors like collection place, botanical origin and collection season (Sonmez *et al*., 2005).

The inhibitory effect to the concentrations used in the study was not detected only in the cases of the EEP 1 and 6, which may be due to differences in the concentrations of the detected polyphenols. In a future study, it would be also interesting to characterize the selected propolis in sensitivity tests from a botanical point of view, and so, propolis with good antimicrobial quality may be associated with their original species.

The determination of the minimum inhibitory and bactericidal concentrations was complicated by the lack of information on the propolis concentration contained in commercial products; therefore, this is necessary in order to know clearly which concentrations show antimicrobial efficiency, which may then be applied in future studies.

The presence and identification of caffeic acid, myricetin, quercetin, kaempherol, apigenin, pinocembrin, galangin and caffeic acid phenethyl ester (CAPE) was determined through the chromatographic analysis, which is coherent to the results by other authors (Chaillou and Nazareno, 2009; Kalogeropoulos *et al*., 2009; Popova *et al*., 2005; Uzel *et al*., 2005), who analyzed propolis samples from other countries. Regardless the differences in the number of compounds detected among the samples analyzed, we may indicate that the chromatographic patterns presented wide similarities, and their differences would correspond mainly to differences in the concentration of each compound.

Although the antimicrobial action of propolis is well known, the mechanisms of how this effect works are still unknown. Some components present in the propolis extracts have been described, like flavonoids (quercetin, galangin, pinocembrin) and caffeic, benzoic, and cinnamic acids. These probably act somewhere on the membrane or the bacterial wall, causing functional and structural damage (Scazzocchio *et al*., 2006; Kosalec *et al*., 2005). Other authors suggest that the ring B of the flavonoids structure may play a role in hydrogen integration or union of the bases, which might explain an action on DNA and RNA synthesis. It has also been proposed that the DNA gyrase and ATPase are inhibited from the components found in propolis. Likewise, bacterial membrane fluidity decrease, permeability increase and membrane potential dissipation have been also proven (Cushnie and Lamb, 2005). A recent study showed that EEP completely suppressed the virulence factor of the enzyme coagulase in *Staphylococcus aureus* and had a preventive effect on the formation of dose dependent biofilm (Scazzocchio *et al*., 2006).

Therefore, we may indicate that, once the antimicrobial action has been proven, it is important

to find out about the metabolic processes of the microorganism in order to detect which are altered by the EEP action, and then clarify the effect from this substance on the different microorganisms. Additionally, due to the wide range of biological activity exhibited by the propolis, the high variability and complexity of their chemical composition, and the variability existing among the concentration of total polyphenols present in commercial extracts, the need of regulation becomes more evident with both the determination of the botanical-geographical origin and the chemical characterization of the extracts. Therefore, it would be possible to standardize the particularities of this product and to know their composition clearly and thus, to develop biotechnological products for caries control and other infectious diseases and clinical syndromes.

In summary, we may indicate that Chilean propolis has the capacity of inhibiting the development of cariogenic bacteria *L. fermentum.* However, this activity is variable and it depends on the chemical composition of the propolis used.

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Resumen

N. Saavedra, L. Barrientos, C.L. Herrera, M. Alvear, G. Montenegro y L.A. Salazar. 2011. Efecto de propóleos chilenos sobre la bacteria cariogénica *Lactobacillus fermentum.* Cien. Inv. Agr. 38(1): 117-125. La caries dental es una de las enfermedades infecciosas más prevalentes en el mundo. Entre las bacterias involucradas en esta patología se encuentran *Streptococcus mutans, Streptococcus sobrinus*, *Actinomyces* spp. y *Lactobacillus* spp. La industria farmacéutica ha volcado sus esfuerzos al descubrimiento de nuevos productos antibacterianos ante el aumento de resistencia a los ya conocidos. El propóleos se ha utilizado como tal, desde tiempos antiguos, por lo que se ha investigado su efecto contra variados microorganismos. En este estudio se evaluó el efecto antimicrobiano de seis extractos etanólicos comerciales de propóleos, sobre la bacteria *Lactobacillus fermentum.* Ésta fue aislada luego de su identificación mediante PCR con el uso de primers especie específicos, posterior al cultivo microbiológico de muestras de caries de pacientes con indicación de extracción de pieza dental, y se detectó en 9 de 40 pacientes, correspondiendo a un 22%. El estudio de susceptibilidad se realizó mediante dilución en microplacas y se comprobó la actividad antimicrobiana en cuatro de los seis extractos etanólicos de propóleos utilizados, difiriendo en la concentración efectiva contra el microorganismo, lo que puede ser atribuido a factores como el origen botánico, el lugar geográfico y la estación de recolección. Los propóleos mostraron concentraciones de polifenoles que variaron entre 9 ± 0,3 y 85 ± 2,1 mg/mL. El análisis cromatográfico permitió detectar la presencia de ácido cafeico, miricetina, quercetina, kaempferol, apigenina, pinocembrina, galangina y ácido cafeico fenil éster (CAPE). Nuestro estudio demuestra la acción antimicrobiana del propóleos sobre *L. fermentum*, patógeno relacionado al desarrollo de caries.

Palabras clave: *Lactobacillus fermentum*, caries dental, propóleos, actividad antibacteriana.

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