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Research Paper

Chemical and botanical characterization of Chilean propolis and biological activity on cariogenic bacteria *Streptococcus mutans* and *Streptococcus sobrinus*

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Abstract

Propolis is a non-toxic natural substance with multiple pharmacological properties including anti-cancer, antioxidant, fungicidal, antibacterial, antiviral, and anti-inflammatory among others. The aim of this study was to determine the chemical and botanical characterization of Chilean propolis samples and to evaluate their biological activity against the cariogenic bacteria *Streptococcus mutans* and *Streptococcus sobrinus*. Twenty propolis samples were obtained from beekeeping producers from the central and southern regions of Chile. The botanical profile was determined by palynological analysis. Total phenolic contents were determined using colorimetric assays. Reverse phase HPLC and HPLC-MS were used to determine the chemical composition. The minimum inhibitory concentration (MIC) was determined on *S. mutans* and *S. sobrinus*. All propolis samples were dominated by structures from native plant species. The characterization by HPLC/MS, evidenced the presence of quercetin, myricetin, kaempferol, rutine, pinocembrin, coumaric acid, caffeic acid and caffeic acid phenethyl ester, that have already been described in these propolis with conventional HPLC. Although all propolis samples inhibited the mutans streptococci growth, it was observed a wide spectrum of action (MIC 0.90 to 8.22 μg mL^{-1}). Given that results it becomes increasingly evident the need of standardization procedures, where we combine both the determination of botanical and the chemical characterization of the extracts. Research conducted to date, describes a promising effectiveness of propolis in the prevention of caries and other diseases of the oral cavity, making it necessary to develop studies to identify and understand the therapeutic targets or mechanisms of molecular action of the various compounds present on them.

Key words: propolis, botanical characterization, chemical characterization, *Streptococcus mutans*, *Streptococcus sobrinus*.

Introduction

Among the natural products that have received attention recently, we draw attention on propolis, a resinous substance collected by bees (*Apis mellifera*) from buds, shoots and wounds of various plant species and mixed with mandible secretions for use in construction, maintenance and protection of their hives (Burdock, 1998). It has over 300

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compounds, among which polyphenols (flavonoids, phenolic acids and their esters), terpenoids, steroids, sugars and amino acids have been detected in raw propolis, but its composition is qualitatively and quantitatively variable, depending on the vegetation at the site from which it was collected and the collection season (Koo *et al.*, 1999; Bankova, 2005; Tosi *et al.*, 2007; Valencia *et al.*, 2012). The main constituents of propolis in Europe, China, and North America are flavonoids and phenolic acid esters (Bankova *et al.*, 2000; Chen *et al.*, 2004; Lotti *et al.*, 2010). Brazilian propolis contains, principally, diterpenes, lignans, p-coumaric acid derivatives, sesquiterpenes and acetophenones (Bankova, 2005; Piccinelli *et al.*, 2005).

Propolis is a non-toxic natural substance with multiple pharmacological properties including cytostatic, hepato-protective, antioxidant and anti-inflammatory (Burdock, 1998; Kujumgiev *et al.*, 1999; Borrelli *et al.*, 2002; Russo *et al.*, 2002). It is also considered as an alternative in the treatment and prevention of many infectious diseases, since it displays a wide range of antimicrobial activity against a variety of bacteria, fungi, parasites and virus (Bankova *et al.*, 1995; Kujumgiev *et al.*, 1999; Sforcin *et al.*, 2000; Orsi *et al.*, 2005). Due to this wide range of biological activities, propolis is used in food industry, cosmetology, and complementary medicine products. These observations emphasize the need to extend our knowledge about the chemical and biological characterization of propolis, which would aid with the appropriate use of this natural product in human health (Valencia *et al.*, 2012).

Dental caries are known to be one of the most prevalent and costly oral infectious diseases worldwide (Dye *et al.*, 2007); it is a multifactorial infectious disease in which diet, nutrition, microbial infection, and host response all play important roles. Dental plaque is a typical bacterial biofilm that contains mutans streptococci and other oral bacteria and their products. Among them, *Streptococcus mutans* and *S. sobrinus* have been recognized to be the mayor causative agent of dental caries in humans (Loesche, 1986; Smith, 2002).

Some naturals compounds based in tea (Hamilton-Miller, 2001), cranberries (Steinberg *et al.*, 2004), cacao (Osawa *et al.*, 2001), herbal extracts (Limsong *et al.*, 2004) and propolis (Bankova *et al.*, 1995; Koo *et al.*, 2000, 2002a, 2002b; Duarte *et al.*, 2006;), have shown inhibition of biofilm and caries development in some species of mutans streptococci, being propolis and its polyphenolic compounds, the most studied. The biofilm formation is vital for the progression of dental caries and thus, inhibition of this factor is one of the strategies currently used to prevent this disease (Xiao *et al.*, 2007).

In view of the foregoing considerations the aim of the present study was to determine both the chemical and botanical characterization and to determine the biological activity on mutans streptococci of 20 propolis samples obtained in central and southern Chile.

Materials and Methods

Preparation of ethanolic and methanolic extracts of propolis (EEP)

Twenty propolis samples were obtained from various beekeeping producers from the central and southern region (Valparaíso, Metropolitana, Libertador Bernardo O'Higgins and La Araucanía Regions), Chile.

Concentrated ethanol and methanol extracts (EEP) were prepared with 30 g of chopped fresh propolis, which were macerated for 7 days at room temperature, covered with ethanol (70%) in volumetric flask (100 mL) and stirred occasionally, every day, and finally, filtered with Whatman paper Nº 2. The methanolic extracts were centrifuged three times to remove waxes. All the extracts were stored in the dark at -20 °C until analysis.

Botanical analysis of propolis

For this determination was used the methodology described by Montenegro *et al.* (1992). Subsequently, were counted and identified plant structures (pollen grains, trichomes and vessels). The identification was made by comparing the different structures with relevant literature (Heusser, 1971; Montenegro, 1984; Erdtman, 1986), with photographs and permanent preparations available in the Laboratory of Botany (Department of Plant Sciences, Faculty of Agronomy and Forest Engineering, Pontificia Universidad Católica de Chile, Santiago, Chile), and the proportion of each of the total structures counted were estimated.

Determination of total phenolic content

Total phenolic content of propolis extracts was determined by colorimetric assays using the Folin-Ciocalteu method (Singleton *et al.*, 1999), with modifications. Each extract was diluted 1:10 in ethanol 70% and then 1:10 in distilled water; then 40 µL of this dilution was mixed with 560 µL of distilled water, 100 µL of Folin-Ciocalteu reagent (Merck, Germany) and 300 µL sodium carbonate 7.5% (w/v). The absorbance was measured at 760 nm after 2 h incubation at room temperature. The concentrations were calculated from a calibration curve and were expressed in mg/mL equivalent to the standard catechin.

Chemical characterization of a propolis extract

High performance liquid chromatographic (HPLC) analysis was made on an HPLC system (Merck-Hitachi model L-4200) equipped with a pump (model L-6200), a UV-visible detector and a Sphere Column Heater (Phenomenex Terma model TS-130). The separation was made in an RP-18 column (12.5 x 0.4 cm, particle size 5 µm) (Merck, Germany), 149 which separates at 25 °C using a mixture of formic acid 5% in water (A) and methanol (B) as mobile phase. The separation of the compounds was carried out by an isocratic-0 to 10 min-run, with the mixture 70% A

and 30% B, followed by a gradient up to 100% B at 70 min. The compounds were detected at 290 nm, with 0.001 sensitivity; the injection volume was 10 µL. The identification of the phenolic compounds was made using the following standards: myricetin, kaempferol, quercetin, caffeic acid, galangin, pinocembrin, apigenin, caffeic acid phenethyl ester (CAPE) and resveratrol (Sigma, USA).

The analysis of the methanolic extract of propolis was performed on a LC-MS MS system, consisting of a liquid chromatograph (Shimadzu, Japan) connected to MDS Sciex Mass Spectrometer QTRAP 3200 (Applied Biosystems, USA). Chromatographic separation was performed with a RP-C18 Column Inertsil ODS-3 (2.1 x 150 mm, 3 mm). The elution was performed at 35 °C using as solvent A, 0.1% formic acid and solvent B, methanol. Data acquisition was performed using the software Analyst 1.5.1 (Applied Biosystems, USA). Flavonols were studied in both positive and negative polarity. The specific parameters for the MRM experiment (Multiple Reaction Monitoring) in positive and negative polarity were optimized using standards myricetin, quercetin, rutin and kaempferol by direct injection method.

Biological action of propolis on cariogenic bacteria

S. mutans and *S. sobrinus* were clinical samples obtained from children of La Araucanía Region isolated in a previous study and confirmed by PCR-RFLP (Salazar *et al.*, 2008).

The minimum inhibitory concentration (MIC) was performed as described by the Clinical and Laboratory Standards Institute guidelines (2007) by microdilution methodology, which involves making serial dilutions of the compound to be studied on microplates (96 wells) with tryptic soy broth. The starting inoculum was 5 x 10^5 cfu/mL. The MIC was determined in triplicate for each propolis against bacterial strains isolated, and was evaluated after 48 h of incubation at 37 °C, as the lowest concentration that completely inhibited the formation of visible growth.

Statistical analysis

The analysis of the data was performed using the program GraphPad Prism, version 5.0 (U.S.). When necessary we calculated averages, standard deviations, maximum and minimum values. ANOVA was used for comparisons of the antimicrobial activity of propolis. Comparisons between samples (in triplicate) were performed with Dunnett's Multiple Comparison Test. The statistical significance level considered was $p \leq 0.05$.

Results

Botanical analysis of propolis

The fraction of plant debris in the samples of propolis from the central region (samples P001 to P012), were dominated by structures from native plant species, highlighting the contribution of *Trevoa quinquenervia*, *Aristotelia chilensis* (maqui), *Lithrea caustica* (litre), *Retanilla trinervia* (tebo), *Quillaja saponaria* (quillay), and species of the genus *Escallonia*. Of these species, the leading producers of resins are litre, tevo and species of the genus *Escallonia*. In samples from Southern Chile (samples P013 to P020), the fraction of plant debris was mostly dominated by pollen of the species *Lotus uliginosus* (alfalfa chilota) and structures from native plants, mostly trees or shrubs, such as *Aextoxicon punctatum* (olivillo), *Baccharis linearis* (romerillo) and *Eucryphia cordifolia* (ulmo) (Table 1).

It should be noted, in all samples, the almost total absence of structures from introduced species in the area and that are large producers of resins, such as forest trees of the genera *Eucalyptus* and *Pinus*. There were no structures of the genus *Populus*.

Total phenolic content

Total phenolic content of propolis extracts was determined by colorimetric assays using the Folin-Ciocalteu method. The results obtained indicate the presence of polyphenolic compounds, in varying concentrations, and these results are similar to those described by other studies from our laboratory (Table 2).

Chemical characterization of propolis

The HPLC chromatographic profiles of all propolis samples looked very similar. The main peaks in the chromatograms were identified using standard samples. The different samples of propolis studied show varying concentrations of caffeic acid, myricetin, quercetin, kaempferol, apigenin, pinocembrin, CAPE and galangin (Tables 3 and 4). Further results from the chemical characterization of propolis analyzed by LC-MS using the MRM program in positive and negative polarity, confirm in the propolis samples, the presence of the same compounds and others like rutine (propolis from La Araucanía Region), that were not detected with conventional HPLC. Particularly, this method recognizes the presence or absence of compounds according to retention times made equal to the sample from a standard. Figures 1 and 2 show the flavonols and phenolic acids found in a propolis sample from La Araucanía Region, respectively.

Biological action on cariogenic bacteria

The analysis of results shows that a different propolis sample does not have the same inhibitory activity on bacterial growth, but all of them inhibited the mutans streptococci growth. Also, we can see that this activity has a direct relation with the concentration of polyphenols, as determined by the Folin-Ciocalteau method (Table 2). The EEP P001 and P008 from central Chile showed the lowest antimicrobial activity (MIC 6.67 and 8.22, respectively). Similarly, highlighting the propolis P019 and P020, from

Table 1 - Floral composition of propolis samples studied from central and southern Regions, Chile.

Sample	Region	Predominant species
P 001	Valparaíso	*Trevoa quinquenervia* Gill. et Hook. (37%); *Retanilla trinervia* Gill. et Hook. (18%); *Colliguaja odorifera* Mol. (9%); *Escallonia pulverulenta* Pers. (6%)
P 002	Metropolitana	*Trevoa quinquenervia* Gill. et Hook. (32%); *Brassica rapa* L. (18%); *Melilotus indicus* L. (13%); *Eucalyptus globulus* Labill. (9%)
P 003	Metropolitana	*Cardamine* o *Menonvillea* (11%); *Proustia pyrifolia* DC (10%); *Quillaja saponaria* Mol. (10%); *Eucalytpus globulus* Labill. (9%)
P 004	Metropolitana	*Colletia* or *Discaria* (11%); Tricomas de *Satureja* sp. (11%); *Quillaja saponaria* Mol. (11%); *Cardamine* or *Menonvillea* (8%)
P 005	Metropolitana	*Hypochaeris* o *Taraxacum* (39%); *Plantago* sp. (12%); *Eucalyptus* sp. (11%); *Cissus striata* Ruiz et Pav. (8%)
P 006	Metropolitana	*Aristotelia chilensis* Mol. (9%); *Escallonia* sp. (9%); *Medicago sativa* L. (9%); *Lithrea caustica* Hook. et Arn. (7%).
P 007	Metropolitana	*Brassica* sp. (19%); *Lotus corniculatus* L. (18%); *Azara* sp. (13%); *Escallonia* sp. (12%)
P 008	Metropolitana	*Lithrea caustica* Hook. et Arn. (21%); *Trevoa quinquenervia* Gill. et Hook. (15%); *Haplopappus glutinosus* Cass. (9%); *Aristotelia chilensis* Mol. (9%)
P 009	Metropolitana	*Aristotelis chilensis* Mol. (24%); *Galega officinalis* L. (11%); *Retanilla trinervia* Gill. et Hook. (9%); *Eucalyptus* sp. (5%)
P 010	Metropolitana	*Lithrea caustica* Hook. et Arn. (26%); *Quillaja saponaria* Mol. (15%); *Escallonia* sp. (13%); *Retanilla trinervia* Gill. et Hook. (8%)
P 011	Metropolitana	*Lithrea caustica* Hook. et Arn. (23%); *Trevoa quinquenervia* Gill. et Hook. (16%); *Baccharis linearis* Ruiz et Pav. (9%); *Brassica sp*. (8%)
P 012	Libertador Bernardo O'Higgins	*Lithrea caustica* Hook. et Arn. (19%); *Raphanus sativus* L. (13%); *Galega officinalis* L. (13%); *Retanilla trinervia* Gill. et Hook. (9%)
P 013	La Araucanía	*Lotus uliginosus* Schk. (51%); *Eucryphia cordifolia* Cav. (18%); *Teucrium* sp. (10%); *Sysimbrium* sp. (3%)
P 014	La Araucanía	*Lotus uliginosus* Schk. (36%); *Azara* sp. (8%); *Aristotelia chilensis* Mol. (7%); *Baccharis linearis* Ruiz et Pav. (2%)
P 015	La Araucanía	*Lotus uliginosus* Schk. (84%); *Proustia pyrifolia* DC. (5%); *Caldcluvia* or *Eucryphia* (4%); *Baccharis linearis* Ruiz et Pav. (2%)
P 016	La Araucanía	*Lotus uliginosus* Schk. (64%); *Buddleja globosa* Hope (11%); *Hypochaeris* or *Taraxacum* (5%); *Caldcluvia* or *Eucryphia* (5%)
P 017	La Araucanía	*Lotus uliginosus* Schk. (85%); *Caldcluvia* or *Eucryphia* (4%); *Hypochaeris* or *Taraxacum* (3%); *Amomyrtus luma* Mol. (1%)
P 018	La Araucanía	*Lotus uliginosus* Schk. (81%); *Caldcluvia* or *Eucryphia* (4%); *Proustia pyrifolia* DC (2%); *Lotus corniculatus* L. (2%)
P 019	La Araucanía	*Lotus uliginosus* Schk. (39%); *Aextoxicon punctatum* Ruiz et Pav. (17%); *Eucalyptus* sp. (10%); *Rhamnaceae* (6%).
P 020	La Araucanía	*Lotus uliginosus* Schk. (61%); *Eucryphia* or *Caldcluvia* (23%); *Luma apiculata* (DC.) Burret (5%); *Lotus corniculatus* L. (2%)

southern Chile showed the highest antimicrobial activity (MIC 1.94 and 0.90, respectively). When comparing the MIC for *S. mutans* and *S. sobrinus* according to the sample origin we observed for both of them that MIC was lowest with southern propolis ($p = 0.011$ and $p = 0.007$, respectively). In addition, the statistical analyses showed that propolis from southern Chile (P013 to P020) have the highest polyphenols contents when compared with other regions of our country (15.288 ± 3.366 *vs.* 10.567 ± 4.764, $p = 0.026$).

Discussion

Among the techniques used to determine the quality of propolis are the botanical identification by microscopic analysis of pollen grains and fragments of leaves or other debris left by the bees during harvesting of plant exudates (Montenegro *et al.*, 2000), and chromatographic tests to confirm the origin of propolis (Burdock, 1998).

The palynological floral origin and composition of propolis depends largely on the plant species present in an area (Peña, 2008). Some investigations suggest common botanical sources and, consequently, similar chemical profiles for large geographical areas. Various authors have concluded that *Populus* spp. and its hybrids are the main sources of the propolis produced in temperate zones (Europe, North America and non-tropical regions of Asia), and that this type of propolis is characterized by a predominance of flavonoids and phenolic acid esters (Greenaway *et al.*, 1990; Bankova *et al.*, 2000, 2005; Lotti *et al.*, 2010).

Table 2 - Total polyphenols contents and antibacterial activity of Chilean propolis against mutans streptococci isolated from human oral cavity.

Sample	Concentration (mg mL^{-1})	*S. mutans* MIC (μg mL^{-1})	*S. sobrinus* MIC (μg mL^{-1})
P 001	10.7	6.67	6.67
P 002	18.7	5.85	2.93
P 003	11.2	3.42	3.42
P 004	7.5	2.45	2.45
P 005	17.0	5.32	5.32
P 006	3.4	2.13	2.13
P 007	14.5	4.52	2.26
P 008	13.2	8.22	8.22
P 009	8.6	2.68	2.68
P 010	10.9	3.41	3.41
P 011	7.0	4.34	4.34
P 012	4.1	2.55	2.55
P 013	17.0	2.66	1.33
P 014	15.5	2.42	1.21
P 015	21.4	3.34	3.34
P 016	13.1	2.05	2.05
P 017	13.2	2.06	1.03
P 018	18.1	2.82	1.41
P 019	12.4	1.94	1.94
P 020	11.6	0.90	0.90

MIC, Minimum Inhibitory Concentration.

Table 4 - Quantification of polyphenolic compounds detected in the propolis from La Araucanía Region.

Compounds	Sample P013
Caffeic acid	12.3
Resveratrol	0
Quercetin	75.2
Apigenin	31.4
Pinocembrin	1006.4
Galangin	75.5
CAPE	532.6

CAPE, Caffeic acid phenetyl ester; Values expressed as mg mL^{-1}.

In the tropics, poplars are seldom cultivated, so alternative plants are sources of propolis resin, as occur in Venezuela with the flowers of *Clusia minor* (Tomas-Barberán *et al.*, 1993) and of *Clusia rosea* in Cuba (Cuesta Rubio *et al.*, 1999). In both cases, flavonoids are minor propolis constituents, and the major compounds are polyprenylated benzophenones. In Brazil, the propolis produced according to botanical origin and chemical composition is from *Hyptis divaricata*, *Baccharis dracunculifolia* and *Populus nigra* (Salatino *et al.*, 2005).

In our study highlights the low penetration of plant structure from introduced plants that are abundant in central and Southern Chile, and recognized producers of resins for the production of propolis, such as *Eucalyptus*, *Pynus*, and *Populus*, the latter absent in all samples.

Table 3 - Polyphenols detected in propolis samples from central and southern Regions of Chile by HPLC analysis.

Sample	Caffeic acid	Resveratrol	Myricetin	Quercetin	Kaempferol	Apigenin	Pinocembrin	CAPE	Galangin
P 001	+	n.d.	n.d.	+	+	+	+	+	+
P 002	+	n.d.	+	+	+	+	+	+	+
P 003	+	+	+	+	+	+	+	+	+
P 004	n.d.	n.d.	+	+	+	+	+	+	+
P 005	+	n.d.	+	n.d.	+	+	+	n.d.	+
P 006	+	n.d.	+	+	+	+	+	+	+
P 007	+	n.d.	+	n.d.	n.d.	+	+	+	+
P 008	+	+	+	+	+	+	+	+	+
P 009	+	n.d.	+	n.d.	+	n.d.	+	+	+
P 010	+	+	+	+	+	+	+	+	+
P 011	+	+	+	+	+	+	+	+	+
P 012	+	n.d.	+	n.d.	+	+	+	+	+
P 013	+	n.d.	+	+	+	+	+	+	+
P 014	+	n.d.	+	n.d.	+	+	+	+	+
P 015	+	n.d.	+	+	+	+	+	+	+
P 016	+	n.d.	+	+	+	+	+	+	+
P 017	+	n.d.	+	+	+	+	+	+	+
P 018	+	n.d.	+	+	+	+	+	+	+
P 019	+	n.d.	+	+	n.d.	+	+	n.d.	+
P 020	+	n.d.	n.d.	+	n.d.	+	+	+	+

CAPE, Caffeic acid phenetyl ester; +, indicates presence; n.d., not detected.

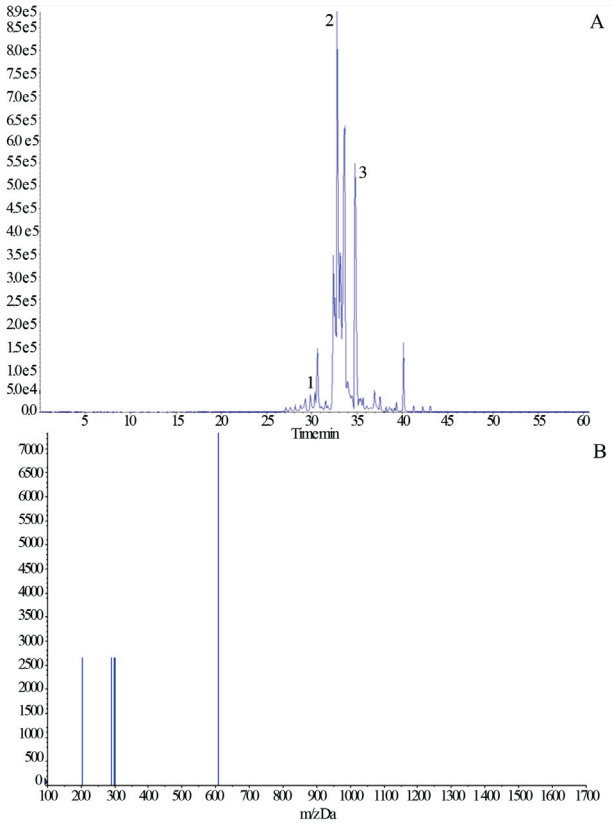

Figure 1 - A. Chromatogram of flavonols from a propolis sample: 1) Rutin (29.25), 2) myricetin (32.82) and quercetin (32.89), 3) kaempferol (34.79). B. Negative ions HPLC-MS mass spectra of rutin identified in propolis sample P013.

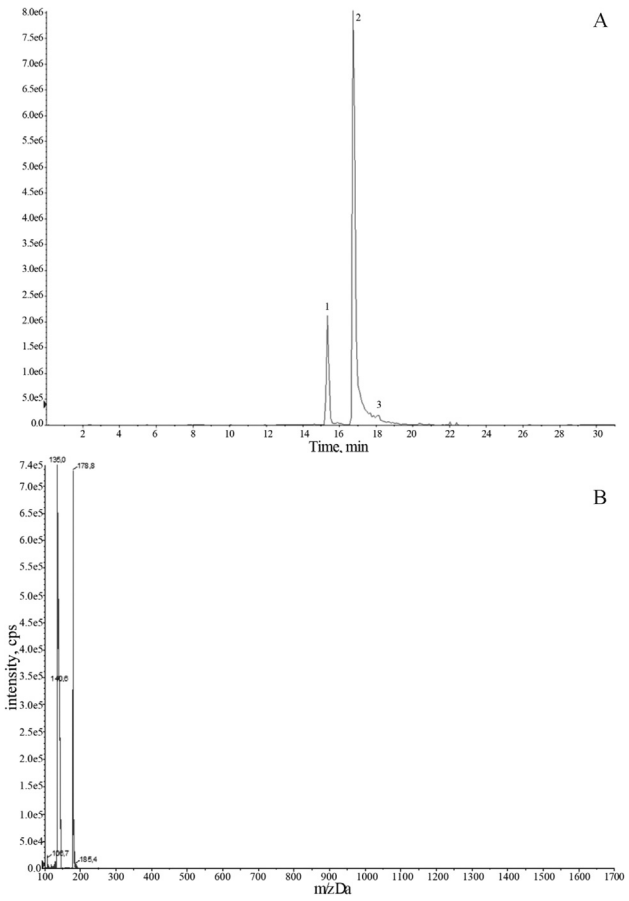

Figure 2 - A. Chromatogram of phenolic acids from a propolis sample: 1) Caffeic acid (15.45), 2) coumaric acid (16.78); 3) and 3) coumaric acid derivatives (20.345). B. Negative ions HPLC-MS mass spectra of caffeic acid identified in propolis sample P013.

Koenig (1995) and Montenegro *et al.* (2001a) described *Salix humboldtiana* species and *E. globulus* between endemic and introduced plants, respectively, as the most frequent, found in an apiary network of central Chile.

The botanical origin of propolis from central Chile has been determined previously by micro-morphological analysis of pollen and epidermal attachments (Montenegro *et al.*, 2001b). However, spectrophotometer methods, including the Folin Ciocalteu are among the most widely used because there are some authors who consider inaccurate this identification system, applied to propolis, because the pollen is produced in plant anatomical areas different from where are extracted propolis resins, such as buds and wounds of plants and trees.

Studies of the biological activities of propolis should thus be complemented by information about chemical composition and botanical source of the sample, or at least mention it geographical origin, so that these biological activities can be linked to the specific type of propolis (Koo *et al.*, 1999).

The results of chemical characterization of propolis samples by HPLC showed varying concentrations of caffeic acid, myricetin, quercetin, kaempferol, apigenin, pinocembrin, CAPE and galangin. The chemical characterization by HPLC/MS, which was carried out with a screening of flavonols: quercetin, myricetin, kaempferol and rutine, evidenced the presence of the first three compounds that have already been described in these propolis with conventional HPLC (Herrera *et al.*, 2009; Saavedra *et al.*, 2011). In addition, it was possible to detect the presence of coumaric acid, caffeic acid and caffeic acid phenyl ester.

Polyphenols detected in propolis samples from central and southern Regions of Chile by HPLC analysis demonstrated the presence, in all samples, of pinocembrin and galangin. The quantification of polyphenols showed that pinocembrin is the main compound present in Chilean propolis, being the propolis from southern Chile which contained in greater proportion this compound. These results are similar to that obtained for Agüero *et al.* (2010) who observed that galangin and pinocembrin are the main compounds of an Argentinean propolis. Also, Gardana *et al.* (2007) demonstrated that pinocembrin was one of the most abundant in propolis samples from several countries, including seven samples from Chile. They also highlight the higher proportion of total flavonoids and phenolic acid

content in raw samples of propolis from different geographic areas, similarly to our results.

In other report Koru *et al.* (2007) evaluated the chemical composition of propolis samples from different geographic origins using chromatographic techniques and mass spectrometry, and showed that the main components were the flavonoids pinobanksin, quercetin, naringenin, galangin, chrysin and aromatic acids such as caffeic acid.

Among other research, Castro *et al.* (2009) identified a new bioactive compound of propolis, which would be responsible for the antimicrobial activity of propolis called type 6, originally from the state of Bahia (Brazil). The compound was identified by HPLC-MS, as belonging to the group of benzophenones (aromatic ketones). In addition, the authors showed that this compound has antibacterial action on several strains of cariogenic streptococci. They evaluated fractions of propolis type 6, and found that only certain portions of it showed antimicrobial activity. It was noted that the fraction containing fatty acid has antibacterial action. In contrast, the fraction containing benzophenone has antibacterial effect on *Staphylococcus aureus* and *Streptococcus mutans*.

The use of agents that reduce the viability but also control the colonization on the tooth surface by inhibiting the biofilm formation could be a promising approach for the prevention of dental caries. In this context, we examined the potential activity of propolis extracts against *S. mutans* and *S. sobrinus*. In this study, we observed differences in the action demonstrated by each of the propolis evaluated. This difference in the minimum concentration that inhibits visible growth of bacteria could be due to differences in chemical composition of this propolis, which depends on several factors including geographic location, botanical origin and the season time of collection, according to the results obtained by Sonmez *et al.* (2005).

Koru *et al.* (2007) evaluated the effect of propolis on certain oral pathogens as *Prevotella oralis*, *Lactobacillus acidophilus, Veillonella parvula, Actinomyces naeslundii*, among others. They showed that propolis was most effective on Gram-positive anaerobic bacteria than Gram-negative bacteria. This result is important, considering that mutans streptococci (main microorganisms associated with the development of caries) are Gram-positive. The authors conclude that because of increasing antimicrobial resistance, propolis could be considered in the treatment of diseases affecting the oral cavity.

Also, Velázquez *et al.* (2007) showed that Mexican propolis have antibacterial and antioxidant activity. The authors attribute this to the presence of flavonoids, especially the presence of CAPE (caffeic acid phenethyl ester), the second compound in abundance present in the Chilean propolis samples evaluated.

Previous reports with Chilean propolis have highlighted the inhibition of *Candida* spp isolated from the oral cavity of removable dentures users, and the cariogenic bacteria *Lactobacillus fermentum* (Herrera *et al.*, 2009; Saavedra *et al.*, 2011), and also the inhibition of mutans streptococci group with honeys from Southern Chile (Salazar *et al.*, 2009).

Chaillou and Nazareno (2009) demonstrated that the bioactivity of Argentinean propolis sample from Santiago del Estero is assigned to pinocembrin, present in high concentration in all the samples studied. Also, they found a good correlation between the antimicrobial activity and pinocembrin content for *S. aureus*.

While it is well recognized the antimicrobial action of propolis, the mechanisms by which it exerts its antimicrobial effect remain unclear. It has been reported that some components present in extracts of propolis such as flavonoids (quercetin, galangin, pinocembrin) and caffeic acid, benzoic acid, and cinnamic acid probably act at a site on the membrane or cell wall, causing structural and functional damage (Kosalec *et al.*, 2005; Scazzocchio *et al.*, 2006). Others suggest that the β ring structure of flavonoids may play a role in integration or hydrogen bonding of the bases, which would explain the action on the synthesis of DNA and RNA. Also, it is proposed the inhibition of DNA gyrase and ATPase by compounds found in propolis. Likewise, it has been shown a decrease in bacterial membrane fluidity, increased permeability and membrane potential dissipation (Cushnie and Lamb, 2005). A recent study showed that the EEP completely abolished virulence factor coagulase enzyme of *Staphylococcus aureus* and demonstrated a preventive effect dose-dependent on biofilm formation (Scazzocchio *et al.*, 2006).

Conclusion

The botanical analysis and chemical composition of 20 propolis samples has been determined by HPLC and HPLC-Ms analysis on the basis of 9 standards of phenolic acids and flavonoids. Different propolis sample does not have the same inhibitory activity on bacterial growth, but all of them inhibited the mutans streptococci growth. Also, we can see that this activity has a direct relation with the concentration of polyphenols, and propolis from southern Chile have the highest polyphenols content when compared with other regions of our country. The higher concentrations of pinocembrin suggest that this flavonoid could be responsible by the bioactivity against cariogenic bacteria studied.

However, given the wide range of biological activity exhibited by propolis and the high variability and complexity of their chemical composition, it becomes increasingly evident the need of standardization procedures, where we combine both the determination of botanical and geographical origin, as the chemical characterization of the extracts. Research conducted to date, describes a promising effectiveness of propolis in the prevention of caries and other diseases of the oral cavity. Nevertheless, it is necessary to

develop studies to identify and understand the therapeutic targets or mechanisms of molecular action of the various compounds present on them.

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