Argentinean Propolis from Zuccagnia punctata Cav. (Caesalpinieae) Exudates: Phytochemical Characterization and Antifungal Activity

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Main Flavonoids, DPPH Activity, and Metal Content Allow Determination of the Geographical Origin of Propolis from the Province of San Juan (Argentina)

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The chemical characterization as well as the assessment of geographical origin of propolis from several areas of the Provincia de San Juan (Argentina) is reported. Chemical characterization of propolis was performed by measuring total phenolic (TP), total flavonoids (FL), free radical scavenging capacity (DPPH bleaching), and metal content in samples of six different districts. Methanolic propolis extracts (MEP) showed TP ranging from 25.7 to 39.3 g of gallic acid equivalents per 100 g of MEP, whereas flavonoids ranged from 6.6 to 13.3 g of quercetin equivalents per 100 g of MEP. Six main flavonoids were isolated and identified from the propolis samples, comprising the flavanones 7-hydroxy-8-methoxyflavanone (**1**), pinocembrin (**2**), and pinobanksin (**3**), the flavones chrysin (**4**) and tectochrysin (**5**), and the flavonol galangin (**6**). Compounds **1**−**6** were quantified by HPLC-PDA. Free radical scavenging activity, measured as percent DPPH bleaching, ranged from 46.6 to 89.5 at 10 μg/mL. Moreover, propolis samples presented high contents of Ca, K, Fe, Na, and Mg, but low amounts of Mn and Zn. Linear discriminant analysis affords eight descriptors, galangin, pinocembrin, pinobanksin, chrysin, tectochrysin, DPPH, K, and Na, allowing a clear distinction with 100% accuracy among different origins within the Provincia de San Juan. A direct relationship of DPPH free radical scavenging activity with TP or with compounds **1**−**6** was not found, showing the need of further evaluation on the origin of free radical activity in propolis samples.

KEYWORDS: Propolis; chemometrics; geographical origin; flavonoids; antioxidant activity; Argentina

INTRODUCTION

Propolis is a resinous material with a complex composition. It is collected by honeybees from sprouts and plant exudates and is modified in the beehive by the addition of salivary secretions and waxes. A single propolis sample can contain more than 300 components, depending on the plant source and its geographical origin (*1*). Moreover, the composition of propolis from diverse origins is quite variable, which causes problems for its medicinal use and standardization (*2, 3*); consequently, the constituents of propolis are strongly dependent on the geographical area of collection.

Resins are major compounds present in propolis. These resins are mainly composed by flavonoids, phenolic acids, and esters, which often form up to 50% of all the ingredients. The composition of propolis is completed by waxes (30%), volatile essential oils (10%), pollen (5%), and other organic and inorganic compounds (5%) (*4*).

Propolis is commonly used in dietary supplements. The main bioactive compounds of propolis are aromatic acids, phenolic compounds, especially flavonoids (flavones, flavonols, and flavanones), and phenolic acids (*3, 5*). For centuries, preparations containing these compounds as the principal physiologically active constituents have been used to treat human diseases (*6*).

The National Food Institute of Argentina (ANMAT) has recognized propolis as a dietary supplement since 1995 (file 2110-003755-4 in the Argentinean Food Code). However, Argentina has no regulations regarding propolis quality. In this

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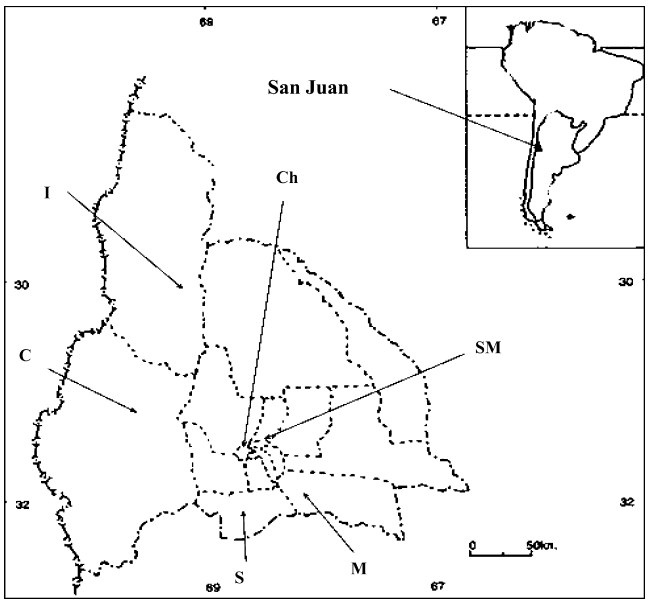


Figure 1. Map of the Provincia de San Juan, Argentina, with the distribution of sampling areas. Districts: **SM**, San Martín; **M**, 25 de Mayo; **C**, Calingasta; **Ch**, Chimbas; **I**, Iglesia; **S**, Sarmiento.

regard, a number of techniques have been compiled involving several important parameters to assess the quality of this natural product (*7*).

Micronutrients constitute a small proportion of food but are essential for the normal functioning of the body and take part in many of the reactions occurring in the human organism. Deficiencies of this type of nutrient may be alleviated by the wide range of new products on the market known as "dietary products". Furthermore, many of the dietary preparations currently available contain propolis as the major component (*8*).

The intake of dietary flavonoids largely depends on the country. A daily intake of approximately 23 mg could be considered the average consumption, quercetin being the predominant compound with a daily dietary intake of 16 mg (*9*). Argentinean propolis contains high levels of total flavonoid, showing significant correlation between flavonoid content and free radical scavenging activity (*10, 11*).

Ahn et al. (*12*) reported that the biological activity of propolis differs with its provenance. Thus, characterization of propolis from different areas is necessary to assess its pharmacological properties as well as the natural compounds involved in such disease-preventing activity.

In the past 10 years, Argentinean propolis from several locations was studied, mainly from the Provincia de Tucumán. Its potential as an antioxidant, as a free radical scavenger, and for antimicrobial and anticarcinogenic activities has been reported (*10, 11, 13*−*15*). Propolis extract from the Provincia de Tucumán and its isolated compounds pinocembrin and galangin have antifungal effect (*16*). On the other hand, propolis from the Provincia de Santa Fe was investigated as a food preservative (*17*). However, the metal and phenolic profiles as well as the biological activity of propolis produced in the Provincia de San Juan, representing a different bioma with a distinctive biodiversity, have been scarcely studied.

The Provincia de San Juan is located in the central-western part of Argentina, centered on the intersection of 31° S latitude and 69° W longitude to the western Andean slopes (**Figure 1**). The province has a rich tradition in folk medicine including the use of medicinal plants, honey, pollen, and propolis, which may be associated with native or introduced plant species depending on the location (environment) of the hives. The native flora comprises a large number of species including *Capparis atamisquea* (Kuntze), *Larrea divaricata* Cav., *Larrea cuneifolia* Cav., *Prosopis chilensis* (Molina) Stuntz emend. Burkart, *Prosopis flexuosa* DC, *Tessaria absinthioides* Hook. et Arn. DC, and *Zuccagnia punctata* Cav., distributed in different ecosystems characterized by particular edaphic and climatic conditions. Introduced plants considered as propolis sources comprise, among others, *Populus* spp., *Tamarix gallica* L., *Pinus* spp., *Eucalyptus* spp., *Medicago sativa* L., *Vitis vinifera* L., *Olea europaea* L., peach, and plum. Among different tools used to assess the geographical origin of propolis and other foodstuffs, chemometrics allows considering propolis samples from an integrated perspective, evaluating several parameters arising from single samples and achieving conclusions on common features, differences among samples from diverse areas, etc. Chemometric tools also enable verifying the contribution of each variable to the model as well as its capacity to discriminate one category from another (*18, 19*).

The main goal of this study was to characterize propolis samples from different locations of the Provincia de San Juan (Argentina) as well as to evaluate similarities and differences by the application of chemometric tools on their metal content, qualitative and quantitative levels of selected phenolics compounds, flavonoids, and their free radical scavenger capacity. Thus, we look to present a complete picture on the chemical profile characteristic of propolis from this province in association with the levels of health-promoting compounds. Furthermore, we report the isolation and identification of the main flavonoids from the studied propolis.

MATERIALS AND METHODS

Chemicals. All reagents and solvents used were of analytical grade. Ultrapure water (Millipore, Milli-Q system) was used to prepare standard solutions, dilutions, and blanks. Chloroform was purchased from Fisher (Fair Lawn, NJ), and methanol was obtained from J. T. Baker (Phillipsburg, NJ). Acetonitrile from Caledon Laboratory. Ltd. (Canada) and formic acid from Merck (Darmstadt, Germany) were used. TLC analysis was carried out on aluminum-coated silica gel (Aldrich) and cellulose F_{254} plates from Merck. Folin−Ciocalteu phenol reagent, aluminum chloride hexahydrate, sodium carbonate, nitric acid, and sulfuric acid were purchased from Merck. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was acquired from Sigma Chemical Co. (St. Louis, MO). AccuStandard atomic absorption spectrometry standard solutions (1000 mg/L in 1% nitric acid) were used as stock solutions for calibration and in spike recovery studies. Lanthanum oxide solution (Baker, analytical grade) was used as matrix modifier (molecular suppressor). All glassware used was left with sulfonitric solution overnight and then washed with ultrapure water.

Structural Identification of the Compounds. Nuclear magnetic resonance (NMR) spectra were obtained using a Bruker Avance NMR spectrometer (Bruker Elektronik GmbH, Rheinstetten, Germany), operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C. DMSO-d_6, CD_3OD, and $CDCl_3$ were used as solvents. Chemical shifts are presented in parts per million. The UV spectra in MeOH were obtained using a Heλios α V-3.06 UV−vis spectrophotometer. HPLC−photodiode array (PDA) was used for the identification and quantification of the main compounds in the propolis extracts and as a purity criterion of the isolated compounds before NMR measurements. The equipment used was a Merck-Hitachi (LaChrom, Tokyo, Japan) consisting of an L-7100 pump, an L-7455 UV diode array detector, and D-7000 chromatointegrator.

Propolis Samples. Ten raw propolis samples were kindly provided by beekeepers from the Provincia de San Juan (Argentina) belonging to the following districts: San Martín (SMP, three samples); 25 de Mayo (MP, three samples); Calingasta (CP, one sample); Chimbas (ChP, one sample); Iglesia (IP, one sample); and Sarmiento (SP, one sample).

Table 1. Extraction Yield and Total Phenolic (TP) and Flavonoid Contents (FL) of Methanolic Extracts from Studied Propolis (MEPs)

district	propolis extract	extraction yield (%)	TP (g of GA/100 g of MEP)	FL (g of Q/100 g of MEP)	% DPPH fade[a] (10 μg/mL)
San Martín	SMP1	50.35	27.5 ± 0.4	11.3 ± 0.3	61.6 ± 0.4
	SMP2	48.52	25.7 ± 1.6	8.6 ± 0.5	61.6 ± 0.3
	SMP3	72.72	33.2 ± 0.1	10.1 ± 1.9	46.6 ± 0.6
25 de Mayo	MP1	57.49	30.8 ± 2.4	11.2 ± 0.9	82.6 ± 0.2
	MP2	42.13	30.5 ± 0.9	10.8 ± 1.0	89.5 ± 2.5
	MP3	35.50	29.3 ± 2.6	10.6 ± 1.2	73.7 ± 0.3
Calingasta	CP	61.84	39.3 ± 0.9	11.7 ± 0.5	78.9 ± 0.1
Chimbas	ChP	52.41	34.9 ± 3.1	8.8 ± 0.5	80.4 ± 0.2
Iglesia	IP	66.48	29.7 ± 2.6	13.3 ± 0.7	66.6 ± 0.3
Sarmiento	SP	76.26	36.4 ± 0.7	6.6 ± 0.5	79.0 ± 0.5

[a] Total phenolic is expressed as grams of gallic acid (GA) per 100 g of MEP, flavonoid content as grams of quercetin (Q) per 100 g of MEP, and DPPH radical scavenging activity (percent) of MEPs.Values are shown as means ± standard deviations (SD). Reference standards: catechin (88.6 ± 0.5%), quercetin (96.6 ± 0.5%), respectively.

Propolises were collected with propolis traps to minimize their contamination with foreign substances and were frozen to −20 °C. Sampling areas are presented in **Figure 1**.

Preparation of Methanolic Extracts from Propolis (MEP). A representative amount (100 g) from each propolis sample was extracted in triplicate at room temperature with 500 mL of methanol during 24 h. Combined extracts were filtered and concentrated under reduced pressure to afford the corresponding methanolic extracts (MEP); these were stored in the dark at −20 °C until analysis. The extraction yields are shown in **Table 1**.

Total Phenolic and Flavonoid Contents. The total phenolics content of MEPs was determined using the method described by Heldrich (*20*). Briefly, the appropriate extract dilution was oxidized using Folin−Ciocalteu reagent and neutralized with sodium carbonate. After 30 min, the absorbance of the resulting blue solution was measured at 765 nm. Quantification was done by linear regression from a calibration curve constructed from gallic acid. Results are expressed as gallic acid equivalents (percent).

The total flavonoid content was determined on MEPs following the procedure described by Chang et al. (*21*) and using quercetin as reference for the calibration plot. The absorbance of the reaction mixture was measured at 415 nm. Results are expressed as quercetin equivalents (percent). Percentages of total phenolic and flavonoids are expressed as grams per 100 g of MEP. Data are reported as mean ± standard deviation (SD) for at least three replicates.

Free Radical Scavenger Activity on DPPH. Free radical scavenger effects of MEPs and pure isolated compounds were assessed by the fade of a methanolic solution of 1,1-diphenyl-2-picrylhydrazyl radical as previously reported by Tapia et al. (*22*). MEPs were assayed at concentrations of 100, 50, and 10 μg/mL. Scavenging activities were evaluated spectrophotometrically at 517 nm using the absorbance of DPPH radical as reference. Quercetin was used as a reference compound. The loss of color (fade percentage) was calculated as follows:

$$\text{fade percentage} = \left[1 - \left(\frac{\text{absorbance of sample} - \text{absorbance of blank}}{\text{absorbance of DPPH}}\right)\right] \times 100$$

The loss of color indicated the free radical scavenging efficiency of the substances. Values are reported as mean ± SD of three independent determinations.

Extraction and Isolation of Chemical Marker Compounds. A representative sample of methanol extract (50 g) from the 25 de Mayo district (MP1) was applied onto a silica gel (500 g, 0.063−0.2 mesh, Merck 60) column (column length = 70 cm, diameter = 5 cm) and eluted with a petroleum ether (PE)/ethyl acetate (EtOAc) gradient. Twenty-four fractions of 1 L each were obtained. After TLC comparison (silica gel, PE/EtOAc 90:10 and PE/EtOAc 70:30 as the mobile phase), fractions with similar TLC patterns were pooled into eight groups: I (0.11 g), II (0.93 g), III (2.08 g), IV (5.19 g), V (13.996 g), VI (8.70 g), VII (4.04 g), and VIII (14 g). Pools I and II contained mainly waxes and were not further investigated.

Pooled Fraction IV. The pooled fraction IV (5 g) was permeated in Sephadex LH-20 (45 cm length, 8 cm i.d.), with PE/$CHCl_3$/MeOH (2:1:1). Twenty-four fractions of 75 mL each were collected and pooled according to their TLC pattern into ten groups: 1 (0.37 g; fractions 1−3); 2 (0.224 g, fraction 4); 3 (0.915 g, fractions 5−6); 4 (1.444 g, fractions 7−9); 5 (0.240 g, fractions 10); 6 (1.040 g, fractions 11−13); 7 (0.017 g, fractions 14−16); 8 (0.149 g, fractions 17−18); 9 (0.226 g, fractions 19−21); 10 (0.021 g, fractions 22−24). After TLC comparison and ^{1}H NMR measurements, fractions containing compounds of interest were further purified. Fractions 19−21 yielded galangin (compound **6**, 226 mg) as yellow needles.

Fraction group 2 (224 mg) was permeated in Sephadex LH-20 (45 cm length, 8 cm i.d.), with PE/$CHCl_3$/MeOH (2:1:1). Fourteen fractions of 75 mL each were collected and pooled according to their TLC pattern into eight groups as follows: 1 (24 mg, fractions 1−2); 2 (31 mg, fractions 3−4); 3 (14 mg, fraction 5); 4 (20.5 mg, fractions 6−7); 5 (42 mg, fraction 8); 6 (18 mg, fractions 9−10); 7 (0.017 g, fractions 11); and 8 (0.149 g, fractions 12−14). Group 4 afforded 20.5 mg of 7-hydroxy-8-methoxyflavanone (compound **1**).

Group 3 (915 mg) was treated with MeOH to afford two groups according to the solubility: a MeOH-soluble (**A**, 492.8 mg) and a MeOH-insoluble fraction (**B**). From the pale yellow MeOH-insoluble fraction was obtained 23.5 mg of tectochrysin (compound **5**) as colorless needles.

Group 6 (1040 mg) was permeated in Sephadex LH-20 (45 cm length, 8 cm i.d.), with MeOH. Twelve fractions of 50 mL each were collected and pooled according to their TLC pattern into seven groups as follows: 1 (fractions 1−2); 2 (fraction 3); 3 (fraction 4); 4 (fractions 5); 5 (fraction 6); 6 (fractions 7); and 7 (fractions 8−12). Group 3 yielded 331.4 mg of pinocembrin (compound **2**).

Pooled Fraction V. Fraction V (13.90 g) was treated with MeOH to afford a MeOH-soluble fraction (**A**, 10 g) and a MeOH-insoluble yellow precipitate (**B**, 3.90 g), which was washed with methanol to afford 3.20 g of chrysin as yellow needles (compound **4**). The MeOH-soluble fraction **A** yielded after successive permeation in a Sephadex LH-20 column 21.6 mg of pinobanksin (compound **3**). All six flavonoids were identified by micromelting point, NMR, UV, MS, and cochromatography with a standard sample.

HPLC Analysis of the Main Flavonoids Identified. Flavonoid quantification was performed according to the method of Sánchez-Rabaneda et al. (*23*) with some modifications, using HPLC-PDA Merck-Hitachi (LaChrom) equipment consisting of an L-7100 pump, an L-7455 UV photodiode array detector, and a D-7000 chromatointegrator. A C18-RP column (Phenomenex 5 μm, 250 mm × 4.60 mm. i.d.) was used. UV−vis spectra were recorded from 200 to 400 nm, with detection at 254 nm. Gradient elution was carried out with water/0.1% formic acid (solvent A) and 20% solvent A in 80% acetonitrile (solvent B) at a constant flow rate of 0.8 mL/min. The linear gradient elution program was as follows: 0−15 min, 40−60% B; 15−30 min, 60% B; 30−40 min, 60−80% B; 40−50 min, 80−100% B; 50−60 min, back to 40% B. Under our experimental conditions, the t_R (minutes) values of the main isolated flavonoids were as follows: compound **1**, 49.1; compound **2**, 32.1; compound **3**, 18.8; compound **4**, 30.4; compound **5**, 49.6; compound **6**, 33.1. Calibration curves were performed to estimate the main compounds content in the samples. The correlation between concentration and peak area was assessed by the ordinary least-squares regression model. The amount of main compounds in the samples was expressed as milligrams per 100 g of methanol extract. Data are reported as mean ± SD for at least three replicates.

Metal Content. Elemental analyses were carried out on a Perkin-Elmer 3110 flame atomic absorption spectrometer (FAAS). An air−acetylene flame and monoelement hallow cathode lamps were used for measuring 11 minerals by atomic absorption spectrometry: Mg, Ca, Fe, Mn, Zn, Cu, Cr, Co, Ni, K, and Na.

Table 2. Standard Conditions and Analytical Parameters for Metal Content in Propolis Samples

metal	wavelength (nm)	% recovery[a]	LOD (mg/L)	LOQ (mg/L)
Ca	422.7	60	0.09	0.25
Mg	285.2	108	0.006	0.02
Zn	213.0	74	0.01	0.04
Cu	324.8	97	0.07	0.2
Cr	357.9	70	0.1	0.3
Fe	248.3	111	0.1	0.3
Mn	279.5	87	0.03	0.1
Ni	232.0	113	0.1	0.4
Co	240.7	97	0.1	0.3
K	766.5	90	0.1	0.3
Na	589.0	98	0.03	0.08

[a] Percentage of recovery from spiking studies. Those elements showing recoveries below 85% (Ca, Cr, and Zn) are reported corrected to 100%.

Samples were mineralized using a microwave oven (Anton Paar 3000): 0.4 g of raw sample was introduced in quartz vessels, followed by the addition of 8 mL of concentrated nitric acid and 1 mL of 30% hydrogen peroxide. Vessels were cap closed and heated in the microwave oven using the following power sequence: starting 10 min ramp to 200 W, held for 10 min (av $T = 74$ °C; av pressure = 8.9 bar), a second 10 min ramp to 400 W, held for 50 min (av $T = 149$ °C; av pressure = 52 bar); and a final 15 min step disabling power to reach pressure equilibration. Mineralized samples were quantitatively transferred to 25 mL volumetric flasks, and the volume was completed using ultrapure water, followed by filtration using 0.45 μm filters (Millipore, HAWG04756). Samples were prepared in duplicate. A triplicate spiked sample was also prepared to verify recovery percentages of different elements. Therefore, 0.4 g of a sample was introduced into the quartz vessel, followed by the addition of variable amounts of individual atomic absorption spectrometry standard solutions (AccuStandard, 1000 mg/L in 1% nitric acid) to yield twice the starting concentration for each element. The rest of the procedure was the same used for nonspiked samples. Appropriate dilutions and addition of matrix modifiers (CsCl, La_2O_3, $CaCl_2$, and H_2O_2) were performed before individual elements were measured. Repeatability of atomic absorption measurements was usually ≥97%, comparing values obtained from triplicates (three measurements on each duplicate sample or spiked sample). The stability of the equipment was verified by measuring the calibration curve before and after sample measurements. The limit of detection (LOD) was established considering 3 SD of absorption of the blank, whereas the limit of quantification (LOQ) was set considering 10 SD. Analytical parameters, instrumentation conditions, and recovery percentages are summarized in **Table 2**.

Statistical Analysis. *Data Set.* Data matrix was composed by 16 columns, 1 containing the district as the dependent categorical variables and 15 columns with analytical results from each measured variable having positive values (metals below LOD were excluded from the analysis). As some variables present non-normal distribution, we report the median and the interquartile range as a measure of data dispersion.

Chemometrics. Stepwise linear discriminant analysis (LDA) was applied to the data set to identify the variables affording the best discrimination among categories (district) according to F value. Due to the non-normal distribution observed with some variables, we used KNN to verify the rate of classification obtained from LDA. We also used Spearman to evaluate correlations between different parameters analyzed, particularly correlations between concentrations of individual flavonoids, total phenolic, total flavonoids, and DPPH free radical scavenger activity. The software package Statistica 7.1 (StatSoft Inc.) was used for statistical calculations according to our previous experience (*19*).

RESULTS AND DISCUSSION

Total Phenolic Compounds, Flavonoid Content, and Percentage of DPPH Fade. Propolis samples from different districts of the Provincia de San Juan (Argentine) were collected and analyzed. **Table 1** shows the yield of MEPs as well as the corresponding analysis for total phenolic (TP) compounds and flavonoid content (FL). All methanolic extracts showed high contents of TP (25.7−39.3 g/100 g of MEP). Moreover, FL of the studied samples varied from 6.6 to 13.3 g/100 g of MEP. Additionally, 10 MEPs presented high DPPH free radical scavenging activity with values between 82.3 and 97.7% to 50 μg mL^{-1} and between 82 and 96.6% to 100 μg/mL (data not shown). DPPH is widely used for quickly assessing the ability of polyphenols to transfer labile H atoms to radicals, a likely mechanism of antioxidant protection. The reactions of dietary phenols and polyphenols with DPPH radical have been the subject of many investigations. Quantitative kinetic analysis of hydrogen transfer reactions from polyphenols to the DPPH radical has been reported by Pascale et al. (*24*). However, kinetic studies showed that cinnamic acids, their derivatives, and catechols react with DPPH radical in methanol by a fast electron-transfer process from the phenoxide anions to DPPH radical. Therefore, the hydrogen atom abstraction from neutral ArOH by DPPH radical becomes a marginal reaction path because of the strong hydrogen-bond-accepting solvent (*25*).

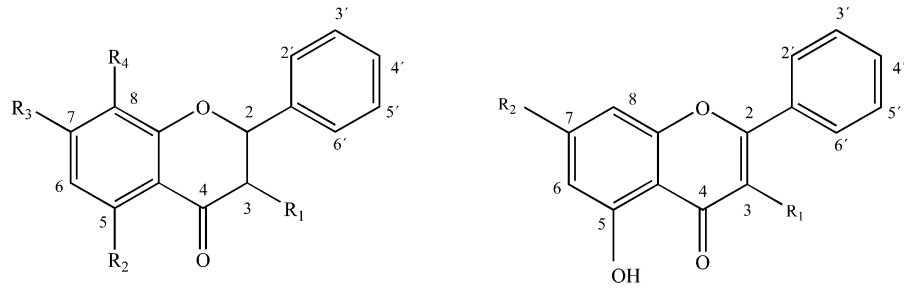
Our current results are in agreement with Nieva Moreno et al. (*11*), indicating that antioxidant activity was closely associated with the free radical scavenger ability (% DPPH fade). These authors also found a significant correlation between high content of FL and free radical scavenging activity in propolis extracts from the Provinces of Tucumán and Santiago del Estero (Argentina).

In recent years, many researchers have been interested in studying compounds having free radical scavenging activity, because these compounds may have great relevance as antioxidants, which could be used in the prevention of diseases such as atherosclerosis, heart diseases, cancer, and arthritis in which reactive oxygen species or free radicals seem to be implicated (*26*). Flavonoids and phenolic compounds derived from plants have been reported as potent free radical scavengers. Thus, the consumption of foods containing these components is considered to be meaningful for maintaining good health. Usually, these compounds were isolated from medicinal plants, fruits, vegetables, or propolis and included into dietary supplements or formulated as part of medicines aiming to prevent or treat diseases (*27*).

Propolis usually has constituents such as polyphenols (flavonoids, phenolic acids, and esters), terpenoids, steroids, and amino acids; however, its composition varies qualitatively and quantitatively with geographical and botanical origin (*11*). Isla et al. (*15*) reported that propolis from northern Argentina showed a phenolic profile similar to that found in samples from southern Brazil, with a composition close to that of poplar-based propolis. The botanical compositions of South American propolis are, however, quite different from those collected in Europe and other regions, mainly because of differences in plant sources.

Our current results show that all propolis samples presented scavenging activity of DPPH radical. These results could be associated with the presence of phenolic compounds and flavonoids arising from regional flora. There are reports on the antioxidant activity as well as on the composition in phenolics and flavonoids of plants from the Provincia de San Juan, Argentina (*22, 28, 29*). However, we did not find significant Spearman correlations (≥0.5) between DPPH activity and total phenolic content, or with total flavonoids, suggesting the need to explore the flavonoid profile to fully evaluate the real antioxidant capacity of the studied propolis.

Extraction and Isolation of Chemical Markers and HPLC-PDA Analysis. Six main constituents were isolated from



	R_1	R_2	R_3	R_4
1: 7-hydroxy-8-methoxyflavanone	H	H	OH	OCH_3
2: Pinocembrin	H	OH	OH	H
3: Pinobanksin	OH	OH	OH	H

	R_1	R_2
4: Chrysin	H	OH
5: Tectochrysin	H	OCH_3
6: Galangin	OH	OH

Figure 2. Main flavonoids isolated from the propolis samples of the San Juan Province, Argentina.

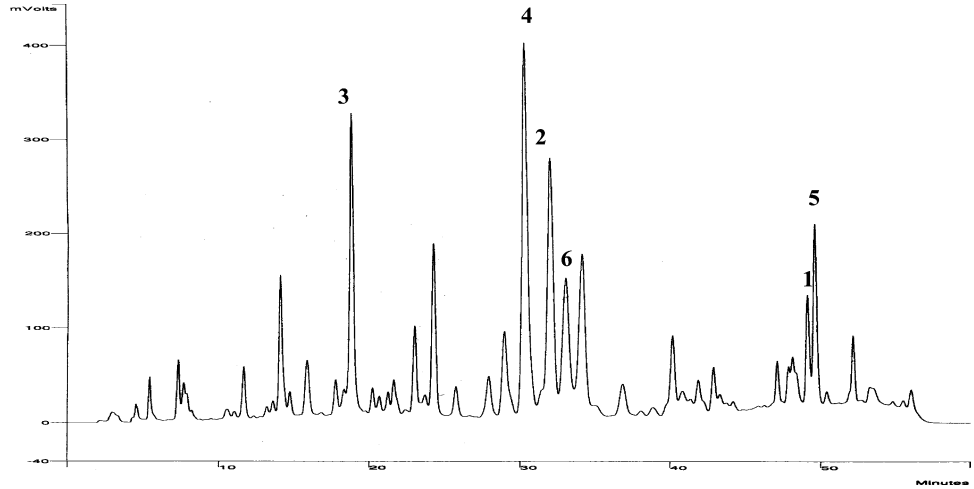


Figure 3. Representative HPLC trace of the methanol-soluble propolis mixture samples from the Province de San Juan, Argentina. Compounds and retention times (t_R, min): **1**, 7-hydroxy-8-methoxylavanone (49.1); **2**, pinocembrin (32.1); **3**, pinobanksin (18.8); **4**, chrysin (30.4); **5**, tectochrysin (49.6); **6**, galangin (33.1).

Table 3. Content of Compounds **1**−**6** in the Methanol-Soluble (MP1) Fraction of Propolis Samples from the Provincia de San Juan, Argentina

		compound content[a] (mg/g of MEP extract)					
propolis extract	total content (mg/g of MEP extract)	**1**	**2**	**3**	**4**	**5**	**6**
SMP-1	149	5.6 ± 0.8	46.0 ± 0.5	18.0 ± 0.7	36 ± 0.9	14.0 ± 0.8	30.0 ± 0.7
SMP-2	92	12.6 ± 0.6	16.7 ± 0.3	11.0 ± 0.6	33.3 ± 0.6	7.4 ± 0.9	11.1 ± 0.7
SMP-3	nq	1.6 ± 0.4	14.0 ± 0.5	0.6 ± 0.1	30.0 ± 0.8	nq	6.0 ± 0.7
MP-1	132	5.4 ± 0.7	32.7 ± 0.6	23.6 ± 0.7	38.2 ± 0.6	10.7 ± 0.6	21.8 ± 0.5
MP-2	110	2.4 ± 0.5	28.0 ± 0.7	8.0 ± 0.7	52.0 ± 0.6	3.4 ± 0.8	16.0 ± 0.9
MP-3	113	4.1 ± 0.7	33.3 ± 0.4	5.9 ± 0.7	51.8 ± 0.4	4.8 ± 0.9	13.0 ± 0.6
CP	157	10.2 ± 0.9	41.5 ± 0.5	18.9 ± 0.6	32.1 ± 0.5	18.3 ± 0.5	35.8 ± 0.8
ChP	69	1.2 ± 0.5	16.7 ± 0.8	3.0 ± 0.9	31.7 ± 0.6	10.0 ± 0.7	6.7 ± 0.5
IP	196	10.2 ± 0.9	64.0 ± 0.8	22.0 ± 0.7	56.0 ± 0.8	11.8 ± 0.5	32.0 ± 0.8
SP	nq	nq	5.2 ± 0.8	nq	12.0 ± 0.7	nq	9.0 ± 0.5

[a] Values are expressed as mean ± standard desviation (SD) of triplicate analyses for each sample. Compounds: **1**, 7-hydroxy-8-methoxyflavanone; **2**, pinocembrin; **3**, pinobanksin; **4**, chrysin; **5**, tectochrysin; **6**, galangin. nq, not quantified.

representative propolis samples from the Provincia de San Juan (Argentine). The compounds were identified as the flavanones 7-hydroxy-8-methoxyflavanone (**1**), pinocembrin (**2**), and pinobanksin (**3**), the flavones chrysin (**4**) and tectochrysin (**5**), and the flavonol galangin (**6**). Compounds **1**−**6** were identified by their spectroscopic data, which are in agreement with those previously reported in the literature (*30, 31*). The chemical structures of these compounds are presented in **Figure 2**. The flavanones **1**−**3**, the flavones **4** and **5**, and the flavonol **6** present an unsubstituted B ring. **Figure 3** shows a representative HPLC trace of MEP samples. The identified peaks are indicated by corresponding compound numbers (**1**−**6**). Each compound was quantitatively analyzed by a calibration curve, constructed from HPLC chromatograms of authentic compounds. Quantitative results are shown in **Table 3**.

Compounds **1**−**6** were detected in all MEPs, pinocembrin (*2*) and chrysin (*4*) being the main flavonoids isolated and identified in 10 MEPs analyzed. Particularly, propolis from district Iglesia contained the largest amounts of pinocembrin and chrysin, at 64 and 56 mg/g of MEP, respectively. Galangin (*6*) was detected as the predominant compound in sample CP (district Calingasta), at 35.8 mg/g MEP. Our present results

Table 4. Concentration of Metals Measured in Propolis Samples by FAAS

		element[a] (mg/kg)													
		Ca[b]		K		Fe		Na		Mg		Zn		Mn	
district	sample	median	range	median	range	median	range	median	range	median	range	median	range	median	range
San Martin ($n = 9$)	SMP-1	1650	1609−1691	473	453−494	473	453−494	855	791−922	237	228−244	61	53−68	14	8−19
	SMP-2	1365	1345−1386	577	540−614	577	540−614	925	921−939	589	551−633	72	69−75	20.7	20.2−22.0
	SMP-3	3981	3847−4138	1454	1453−1462	1454	1453−1462	480	479−483	1007	981−1031	134	131−137	32.9	31.6−33.6
25 de Mayo ($n = 9$)	MP-1	2282	1998−2565	470	453−488	470	453−488	807	791−826	148	115−177	46	39−46	11	10−12
	MP-2	1201	1121−1280	572	101−572	572	101−572	497	489−503	205	183−228	133	89−147	16	15−16
	MP-3	3264	3236−3294	1668	1658−1697	1668	1658−1697	662	658−668	769	756−781	82	81−84	24.2	24.1−24.2
Calingasta ($n = 6$)	CP	1837	1634−2888	608	601−621	608	601−621	108	108−110	643	518−750	37.8	37.8−38.2	11	7−13
Chimbas ($n = 6$)	ChP	3397	3259−3581	1192	1148−1274	1192	1148−1274	370	339−402	875	819−944	80	71−87	22	20−25
Iglesia ($n = 6$)	IP	1475	911−1661	1053	1047−1075	1053	1047−1075	213	207−215	412	310−524	33.1	33.1−33.5	14	13−16
Sarmiento ($n = 6$)	SP	42	39−71	174	169−197	174	169−197	368	344−390	395	337−465	68	60−72	17	15−18

[a] Cu, Co, Cr, and Ni were below LOD. LOD (mg/kg): Cu (0.07); Co (0.1); Cr (0.1); Ni (0.1). [b] Concentrations corrected to 100% recovery.

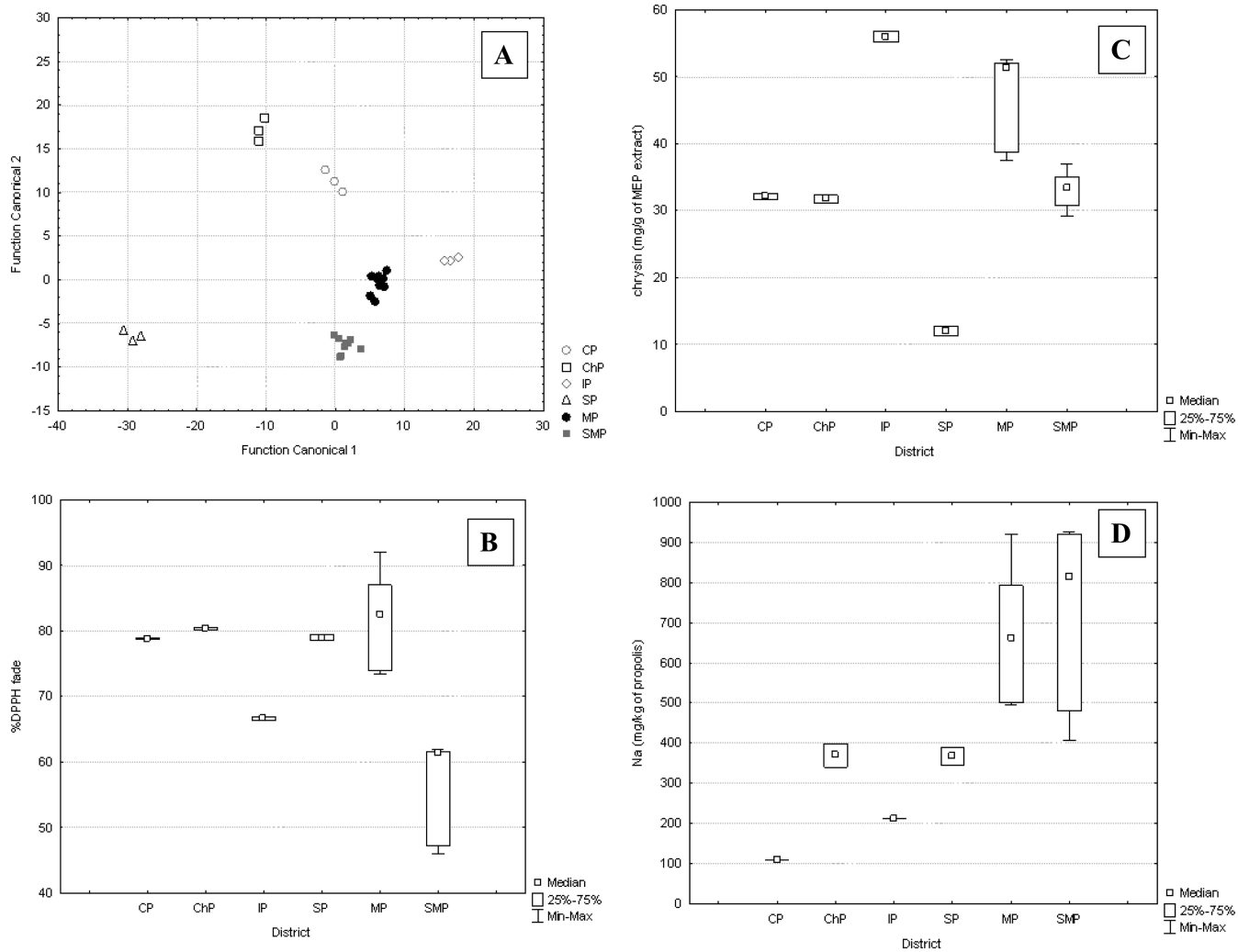


Figure 4. Distribution of propolis in the plane defined by the first two canonical functions of LDA.

agree with those reported by Kumazawa et al. (*32*), who described the antioxidant activity of ethanolic extracts of propolis (EEP) from various geographic regions, including Argentina. This research group found propolis containing large amounts of chrysin (68.5 mg/g of EEP), cinnamylideneacetic acid (30.4 mg/g of EEP), galangin (32.5 mg/g), pinobanksin (22.5 mg/g of EEP), pinobanksin 3-acetate (56.3 mg/g of EEP), pinocembrin (68.7 mg/g of EEP), and tectochrysin (31.4 mg/g of EEP).

Additionally, several recent studies on Argentinean propolis have been published. Volpi and Bergonzini (*33*), in an analysis of flavonoids from propolis by online HPLC−electrospray mass spectrometry, reported that ethanolic extracts of propolis from Argentina, Italy, and Spain had great amounts of pinocembrin (approximately 49, 48, and 39% of the total identified flavonoids, respectively). Recently, Gardana et al. (*34*) have developed a reverse phase LC-PDA-MS method for the quantification of phenolics and flavonoids in raw propolis from different geo-

graphical areas, including Argentina. These authors suggest that European, Chinese, and Argentinean propolises are characterized by the presence of phenolic acids and flavonoids, chrysin (2−4%), pinocembrin (2−4%), pinobanksin acetate (1.6−3%), and galangin (1−2%) being the most abundant; also, they suggest that, considering the total flavonoid content as quality index, propolis with a total flavonoid content of <11% should be considered of low quality, whereas propolis with a content of 11−14, 14−17, or >17% should be classified as propolis of acceptable, good, and high quality, respectively. However, in these papers, they did not indicate the origin of the Argentinean propolis samples.

Metal Content. The median contents of analyzed metals and the range found are presented in **Table 4**. Ca and K presented the higher concentrations in analyzed samples, whereas the other elements analyzed decrease in the order Fe > Na >Mg. The median contents of minor and trace elements decrease in the order Zn > Mn, whereas Cu, Cr, Co, and Ni were below the detection limit in all analyzed samples.

San Martin district had the highest contents of Ca, K, Na, and Mg. The concentration of Fe was higher in propolis from 25 de Mayo. The contents of Zn and Mn were quite similar in propolis samples from districts San Martin and 25 de Mayo. At the same time, the calcium levels in propolis from San Juan showed higher content (ranging from 1400 to 3900 mg/kg) except for the SP sample, which showed low values (42 mg/kg).

The macroelement levels of calcium, potassium, and magnesium were reported by González Rodríguez et al. (*8*) for different pharmaceutical preparations containing propolis. These preparations are mainly sold in health food stores. There are becoming more widely accepted by consumers as a supply of minerals and a complement to the general diet. The Argentinean Food Code suggests reference values for the daily ingestion of minerals. These are as follows: Ca, 1000 mg; Fe, 18 mg; Mg, 400 mg; Zn, 15 mg; Cu, 2 mg; Mn, 2 mg (*35*). Our results show that the mineral macroelements present in San Juan propolis could contribute to the requirements of daily consumption.

Chemometrics. Both LDA and KNN show that propolis samples from different districts can be distinguished (classified) with 100% certainty (data not shown). The application of forward stepwise LDA affords eight descriptors, galangin, pinocembrin, pinobanksin, chrysin, tectochrysin, DPPH, K, and Na, which allow differentiation between propolis samples from studied areas.

A graphical representation of studied propolis, in the plane defined by the first two canonical functions, is shown in **Figure 4A**, where it is statistically demonstrated that propolis samples from the same province can be clearly differentiated as a function of their district of sampling.

This work presents the first report on metal, total phenolic, and flavonoids contents, isolation of main flavonoids, and quantification by HPLC-PDA as well as free radical scavenging activity (DPPH) of propolis from the Provincia de San Juan (Argentina).

Scavenging activity of DPPH radical could be associated with the presence of phenolic compounds and main flavonoids. However, the Spearman test did not show significant correlations between DPPH activity and total phenolics or with total flavonoids. Furthermore, we did not find significant correlation between DPPH activity and any isolated compounds. Thus, characterization of other compounds present in lower concentrations is necessary to fully assess this last point. Chemometrics also shows that DPPH activity enables differentiation between propolis from San Martín (SM) and 25 de Mayo (M) districts, which are not differentiated by the analysis of individual flavonoids or by metal contents (**Figure 4B−D**). Thus, complete assessment of the geographical origin of propolis samples as well as their free radical scavenger activity is better accomplished by evaluating multiple parameters in association with chemometrics, rather than by the evaluation of groups of compounds (such as total phenolic or total flavonoids). This last point denotes the need for more complete profiling studies on different foodstuffs to assess their provenance and prevent frauds.

LITERATURE CITED

(1) Ghisalberti, E. L. Propolis: a review. *Bee World* **1979**, *60*, 59–84.

(2) De Castro, S. L. Propolis: biological and pharmacological activities. Therapeutic uses of this bee-product. *Annu. Rev. Biomed. Sci.* **2001**, *3*, 49–83.

(3) Bankova, V. Chemical diversity of propolis and the problem of standardization. *J. Ethnopharmacol.* **2005**, *100*, 114–117.

(4) Greenaway, W.; May, J.; Scaysbrook, T.; Whatley, F. R. Identification by gas chromatography-mass spectrometry of 150 compounds in propolis. *Z. Naturforsch.* **1990**, *46C*, 111–121.

(5) Sales, A.; Alvarez, A.; Rodriguez Areal, M.; Maldonado, L.; Marchisio, P.; Rodríguez, M.; Bedascarrasbure, E. Content of flavonoids in the Argentinean propolis. *J. Hazard Mater.* **2006**, *137A*, 1352–1356.

(6) Cushnie, T. T. P.; Lamb, A. J. Antimicrobial activity of flavonoids. *Int. J. Antimicrob. Agents* **2005**, *26*, 343–356.

(7) González, M.; Guzmán, B.; Rudyk, R.; Romano, E.; Molina, E. Determination of phenolic compounds in propolis. *Acta Farm. Bonaerense* **2003**, *22*, 343–348.

(8) Gonzalez Rodríguez, E.; Blázquez Abellán, G.; Orzáez Villanueva, M. T. Macroelements in dietetic products containing propolis. *Food Chem.* **1999**, *66*, 15–19.

(9) Hertog, M. G. L.; Hollman, P. C. H.; Putte van de, B. Content of potentially anticarcinogenic flavonoids of tea, infusions, wines and fruit juices. *J. Agric. Food Chem.* **1996**, *41*, 1242–1246.

(10) Nieva Moreno, M. I.; Isla, M. I.; Cudmani, N. G.; Vattuone, M. A.; Sampietro, A. R. Screening of antibacterial activity of Amaicha del Valle (Tucumán, Argentina) propolis. *J. Ethnopharmacol.* **1999**, *68*, 97–102.

(11) Nieva Moreno, M. I.; Isla, M. I.; Sampietro, A. R.; Vattuone, M. A. Comparison of the free radical-scavenging activity of propolis from several regions of Argentine. *J. Ethnopharmacol.* **2000**, *71*, 109–114.

(12) Ahn, M. R.; Kumazawa, S.; Usui, Y.; Nakamura, J.; Matsuka, M.; Zhu, F.; Nakayama, T. Antioxidant activity and constituents of propolis collected in various areas of China. *Food Chem.* **2007**, *101*, 1383–1392.

(13) Nieva Moreno, M. I.; Zampini, I. C.; Ordóñez, R. M.; Jaime, G. S.; Vattuone, M. A.; Isla, M. I. Evaluation of the cytotoxicity, genotoxicity, mutagenicity and antimutagenicity of propolis from Tucuman, Argentina. *J. Agric. Food Chem.* **2005**, *53*, 8957–8962.

(14) Isla, M. I.; Nieva Moreno, M. I.; Sampietro, A. R.; Vattuone, M. A. Antioxidant activity of Argentine propolis extracts. *J. Ethnopharmacol.* **2001**, *76*, 165–170.

(15) Isla, M. I.; Paredes-Guzman, J. F.; Nieva-Moreno, M. I.; Koo, H.; Park, Y. K. Some chemical composition and biological activity of northern Argentine propolis. *J. Agric. Food Chem.* **2005**, *53*, 1166–1172.

(16) Quiroga, E. N.; Sampietro, D. A.; Soberón, J. R.; Sgariglia, M. A.; Vattuone, M. A. Propolis from the northwest of Argentina as a source of antifungal principles. *J. Appl. Microbiol.* **2006**, *10*, 103–110.

(17) Tosi, E. A.; Re, E.; Ortega, M. E.; Cazzoli, A. F. Food preservative based on propolis: bacteriostatic activity of propolis polyphenols

and flavonoids upon *Escherichia coli*. *Food Chem.* **2007**, *104*, 1025–1029.
(18) Hernádez, O. M.; Fraga, J. M. G.; Jiménez, A. I.; Jiménez, F.; Arias, J. J. Characterization of honey from the Canary Islands: determination of the mineral content by atomic absorption spectrophotometry. *Food Chem.* **2005**, *93*, 449–458.
(19) Wunderlin, D. A.; Díaz, M. P.; Amé, M. V.; Pesce, S. F.; Hued, A. C.; Bistoni, M. A. Pattern recognition techniques for the evaluation of spatial and temporal variations in water quality. A case study: Suquía river basin (Córdoba - Argentina). *Water Res.* **2001**, *35*, 2881–2894.
(20) Heldrich, K. *Official Methods of Analysis of the Association of Official Analytical Chemists*; Association of Official Chemists: Arlington, VA, 1990.
(21) Chang, C. C.; Yang, M. H.; Wen, H. M.; Chern, J. C. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. *J. Food Drug Anal.* **2002**, *10*, 178–182.
(22) Tapia, A.; Rodriguez, J.; Theoduloz, C.; Lopez, S.; Feresin, G. E.; Schmeda-Hirschmann, G. Free radical scavengers and antioxidants from *Baccharis grisebachii*. *J. Ethnopharmacol.* **2004**, *95*, 155–161.
(23) Sánchez-Rabaneda, F.; Jáuregui, O.; Casals, I.; Andrés-Lacueva, C.; Izquierdo-Pulido, M.; Lamuela-Raventós, R. M. Liquid chromatographic/electrospray ionization tandem mass spectometric study of the phenolic composition of cocoa (*Theobroma cacao*). *J. Mass Spectrom.* **2003**, *38*, 35–42.
(24) Pascale, G.; Dufour, C.; Loonis, M.; Dangles, O. Quantitative kinetic analysis of hydrogen transfer reactions from dietary polyphenols to the DPPH radical. *J. Agric. Food Chem.* **2003**, *51*, 615–622.
(25) Foti, M. C.; Daquino, C.; Geraci, C. Electron-transfer reaction of cinnamic acids and their methyl esters with the DPPH radical in alcoholic solutions. *J. Org. Chem.* **2004**, *69*, 2309–2314.
(26) Middleton, E., Jr.; Kandaswami, C.; Theoharides, J. C. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease and cancer. *Pharmacol. Rev.* **2000**, *52*, 673–751.
(27) Leighton, F. Plant polyphenol antioxidants. *Biol. Res.* **2000**, *33*, 55–186.
(28) Feresin, G.; Tapia, A.; Gutierrez Ravelo, A.; Delporte, C.; Backhouse Erazo, N.; Schmeda-Hirschmann, G. Free radical scavengers, anti-inflammatory and analgesic activity of *Acaena magellanica*. *J. Pharm. Pharmacol.* **2002**, *54*, 835–844.
(29) Schmeda-Hirschmann, G.; Tapia, A.; Theoduloz, C.; Rodríguez, J. A.; Lopez, S.; Feresin, G. Free radical scavengers and antioxidants from *Tagetes mendocina*. *Z. Naturforsch.* **2004**, *59C*, 345–353.
(30) Agrawal, P. K. Carbon-13 NMR of flavonoids. *Studies in Organic Chemistry 39*; Elsevier: Amsterdam, The Netherlands, 1989; pp 564.
(31) Pederiva, R.; Kavka, J.; D'Arcangelo, S. Chalconas y flavanonas aisladas de *Larrea nitida* Cav. (*Zuccagnia punctata* Cav.) *An. Asoc. Quum. Argentina* **1975**, *53*, 85−90 (in Spanish).
(32) Kumazawa, S.; Hamasaka, T.; Nakayama, T. Antioxidant activity of propolis of various geographic origins. *Food Chem.* **2004**, *84*, 329–339.
(33) Volpi, N.; Bergonzini, G. Análisis of flavonoids from propolis by on-line HPLC-electrospray mass spectrometry. *J. Pharm. Biomed. Anal.* **2006**, *42*, 354–361.
(34) Gardana, C.; Scaglianti, M.; Pietta, P.; Simonetti, P. Analysis of the polyphenolic fraction of propolis from different sources by liquid chromatography−tandem mass spectrometry. *J. Pharm. Biomed. Anal.* **2007**, *45*, 390–399.
(35) http://www.alimentosargentinos.gov.ar/programa_calidad/Marco_Regulatorio/CAA/CAPITULOXVII.htm (Art. 1381).

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