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Nutritional and Functional Properties of Aqueous and Hydroalcoholic Extracts from Argentinean Propolis

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Bee propolis is a natural product extensively used as an ingredient in functional foods in amounts that may confer health benefits. The aim of this study was to investigate the nutritional and functional properties (antioxidant activity) of aqueous and ethanolic extracts of propolis samples from Santiago del Estero province, northern Argentina. All propolis extracts contained macronutrients (glucose, fructose, sucrose and proteins), micronutrients (Na, K, Ca, P and Mg) and trace elements (Fe). Spectrophotometric, TLC and HPLC-DAD analyses showed the presence of several phenolic, flavonoid and non-flavonoid compounds, but in all cases the flavonoids prevailed. The PCA of polyphenolic content provided a clear separation of propolis in Group 1 (SE1, 2, 3, 4 and 7) and Group 2 (SE5 and 6) associated with phenolic compound content and collection regions. Two compounds, pinocembrin and chrysin, which could be used as chemical markers of Santiago del Estero propolis, were identified in all samples. Propolis samples extracted with water presented better radical scavenging ability than ethanolic extracts, independent of the antioxidant method (scavenging activity of $ABTS^{\bullet+}$, $DPPH^{\bullet}$, $HO^{\bullet}$ and $O_2^{\bullet-}$ and β-carotene bleaching test). Such results correlated closely with the levels of total phenols and flavonoids in samples. The results justify the use of Argentine propolis as a functional dietary supplement.

Keywords: Argentine propolis, Macronutrients, Micronutrients, Functional compounds.

Bee propolis is a natural product extensively used as a phytochemical ingredient in functional foods in amounts that may confer health benefits. Since the chemical composition of propolis depends on the phytogeographical characteristics of the collection site, and as Argentina has a wide biodiversity, propolis obtained in different regions (northwest, northeast, Cuyo, center and Patagonia) at different times of the year is expected to have different chemical composition and, consequently, different biological activities. Several flavonoids and chalcones were identified in Argentine propolis, principally in northwestern Argentine propolis [1a-g]. These phytochemicals have antioxidant, anti-inflammatory, antibacterial and antifungal properties [1a-g, 2a-f]. However, up to now, the nutritional properties of propolis have not been analyzed, despite the fact that it is included in the Argentine Food Code.

Propolis ethanolic extract (PEE) is well known and has attracted much interest. Ethanol is the most commonly used solvent because the lipophilic compounds of propolis are easy to extract with it. However, this method has some drawbacks such as a strong residual flavor, adverse reactions and the intolerance to alcohol of some people [3a]. Propolis aqueous extract (PAE) has been featured in few reports, although PAE and its main constituents (including caffeoylquinic acids) have greater antioxidative effects and greater inhibitory activity against some enzymes than PEE and its constituents [3b]. The aim of this study was to investigate the nutritional and functional properties (antioxidant activity) of aqueous and ethanolic propolis extracts from Santiago del Estero province, Northern Argentina.

According to Sforcin & Bankova [4a], a critical step in propolis study is the selection and extraction of the propolis specimens that will be used. Propolis samples from different regions of Santiago del Estero, Argentina, were selected for this study. Water and 80° ethanol were used as extraction solvents. Water dissolved a small part of the constituents of propolis, about 3-10% of its weight, whereas 80° ethanol dissolved 30–50%, depending on the sample (Table 1). To our knowledge, propolis sugar content (sucrose, fructose and glucose) has been investigated in propolis from Kenia, Tanzania (≤ 1%) [4b], Greece, Cyprus (0.7 to 49.0 %) [4c] and Malta (0.8 to 37.2%) [4d], but not in Argentine propolis. All propolis from the SE exhibited the presence of sugars, with values from 5.2 to 10.8% for PEE and 1.5 to 2.1% for PAE (Table 1). Glucose, fructose and sucrose are believed to originate from either nectar or honey, introduced occasionally by bees, or hydrolyzed flavonoid glycosides and non-flavonoid glycosides [4b]. Mucilages were listed among potential sources of sugars in propolis [4e]. Until

Table 1: Content of macronutrients of SE-propolis by HPLC and dry weight of soluble principles extracted with ethanol and water.

Samples	Ethanolic extracts					
	Total Sugars*	Sucrose**	Glucose**	Fructose**	Protein	Dry weight
SE 1	5.2 ± 0.3	1.0 ± 0.05	1.7 ± 0.08	2.4 ± 0.1	20.4 ± 1.1	55.0 ± 0.03
SE 2	6.4 ± 0.02	1.1 ± 0.06	2.1 ± 0.1	1.3 ± 0.07	22.3 ± 3.4	51.2 ± 0.02
SE 3	5.9 ± 0.4	1.0 ± 0.05	1.4 ± 0.07	1.0 ± 0.05	29.8 ± 2.7	51.2 ± 0.03
SE 4	6.5 ± 0.04	1.0 ± 0.05	1.3 ± 0.07	1.0 ± 0.05	15.9 ± 1.3	30.0 ± 0.02
SE 5	10.8 ± 0.1	1.0 ± 0.05	1.0 ± 0.05	0.9 ± 0.05	7.1 ± 1.7	46.2 ± 0.02
SE 6	7.7 ± 0.04	1.3 ± 0.07	2.2 ± 0.1	2.9 ± 0.1	13.5 ± 2.7	41.2 ± 0.02
SE 7	5.7 ± 0.6	1.0 ± 0.05	1.5 ± 0.08	1.0 ± 0.05	20.8 ± 0.6	45.0 ± 0.02
	Aqueous extracts					
SE 1	1.7 ± 0.1	0.1 ± 0.01	1.0 ± 0.05	1.1 ± 0.06	0.6 ± 0.06	5.7 ± 0.003
SE 2	1.5 ± 0.04	1.0 ± 0.05	1.0 ± 0.05	0.9 ± 0.05	0.9 ± 0.01	4.8 ± 0.01
SE 3	2.0 ± 0.3	0.1 ± 0.01	0.1 ± 0.01	1.0 ± 0.05	1.1 ± 0.03	4.7 ± 0.01
SE 4	2.1 ± 0.01	0.1 ± 0.01	1.0 ± 0.05	1.0 ± 0.05	1.4 ± 0.2	9.6 ± 0.01
SE 5	1.7 ± 0.1	2.5 ± 0.12	1.0 ± 0.05	1.0 ± 0.05	0.8 ± 0.02	3.3 ± 0.01
SE 6	2.1 ± 0.1	0.1 ± 0.01	0.1 ± 0.01	0.1 ± 0.01	0.7 ± 0.1	4.8 ± 0.01
SE 7	1.9 ± 0.1	ND	0.9 ± 0.05	0.9 ± 0.05	1.0 ± 0.1	5.8 ± 0.01

* all data were expressed as g/100 g of propolis. ND: not detected.

now, the hypothesis that some plant mucilages are additional sources of sugars seems better-founded because there is numerous proof that bees collect propolis from plant materials that contain flavonoid aglycones, but not glycosides [4b,4e].

Table 2: Phenolic compounds and flavonoid content in aqueous and ethanolic propolis extracts from different regions of Santiago del Estero.

PEE	Phenolic compounds g GAE/100 g prop	Flavonoids g QE/100 g prop	Phenolic non flavonoids g GAE/100g prop
SE 1	34.8 ± 2.6	66.0 ± 1.5	17.5 ± 1.2
SE 2	37.9± 1.7	49.7 ± 1.6	15.3 ± 1.5
SE 3	31.8± 2.7	56.8 ± 2.5	23.0± 1.7
SE 4	40.9± 3.4	34.6 ± 0.9	13.1 ± 1.4
SE 5	27.3 ± 1.9	21.3 ± 0.1	5.7 ± 0.6
SE 6	27.8 ± 0.4	29.8 ± 0.6	9.0 ± 0.8
SE 7	42.4 ± 1.1	46.6 ± 0.8	14.2 ± 1.0
PAE	**Phenolic compounds g GAE/100g prop**	**Flavonoids g QE/100g prop**	**Phenolic non flavonoids g GAE/100g prop**
SE 1	0.9 ± 0.04	2.1 ± 0.04	0.8 ± 0.01
SE 2	1.5 ± 0.05	3.1 ± 0.4	0.8 ± 0.02
SE 3	1.3 ± 0.01	3.5 ± 0.2	1.1 ± 0.014
SE 4	2.2 ± 0.2	4.3 ± 0.3	0.9 ± 0.002
SE 5	0.9 ± 0.01	3.0 ± 0.3	0.6 ± 0.009
SE 6	1.0 ± 0.03	2.5 ± 0.1	0.6 ± 0.002
SE 7	1.4 ± 0.02	3.2 ± 0.4	1.0 ± 0.02

The protein content in PEE was between 7.1 and 29.1%, while in PAE the values were between 0.6 and 1.4% (Table 1). This is the first report about protein content in propolis samples. Micronutrients are involved in numerous biochemical processes and an adequate intake of some of them relates to the prevention of diseases [5a]. Mineral element characterization was carried out. Among the macroelements, K was the main mineral with 313.0-655.0 mg/ 100 g, followed by Mg (51.6-227.7 mg/100 g), P (29.7-77.4 mg/ 100 g) and Ca (12.0-60.4 mg/100 g). Na presented values between 24.9-136.6 mg/100 g. Regarding microelements, Fe presented high values (32.1-75.0 mg/100 g).

TLC analysis showed the presence of several phenolic, flavonoid (F) and non-flavonoid (NF) compounds, but in all cases the flavonoids were the major bands. Quantitative assays confirmed that flavonoids are the main phenolic compounds in all PEE (Table 2). PEE possessed significantly higher amounts of total phenols and flavonoids than aqueous extracts. The results indicated that the content of total phenols and flavonoids varied according to the collection zone. SE3 and SE1 propolis had the highest non flavonoid and flavonoid contents, respectively (Table 2). According to PCA, the PC1 divides SE propolis into group 1 (SE1, 2, 3, 4 and 7) and group 2 (SE5 and 6); both groups were significantly different mainly in their polyphenolic compound content (CCF). PC2 associates SE propolis 2, 4 and 7 with the phenolic compounds content, and SE1 and SE3 with F and NF. These two components explain 86.1% of the total variability of SE propolis (Figure 1).

The HPLC-DAD chromatographic pattern of all PEE was different in quantity and absorption intensity of peaks. SE3 extracts showed the major peak numbers with UV-spectra characteristic of flavonoids [5b], while the other extracts showed lower numbers of constituents. These results reveal a major structural diversity for SE3 propolis. The peaks at 19.6 and 21 min were identified as pinocembrin and chrysin, respectively, and were detected in all Santiago del Estero propolis extracts. To identify each peak, PEE was co-eluted with reference compounds. These same compounds were identified previously in ethanolic extract of Argentine propolis from other regions [1a,1d] and in propolis from other countries [4c].

A rapid TLC fingerprinting of Argentine propolis, together with reference substances, confirmed two main components (chrysin and pinocembrin) from their Rf values and UV fluorescence after spraying with NP. The UV profiles in HPLC analysis of aqueous extracts displayed similar results as those observed in quantitative

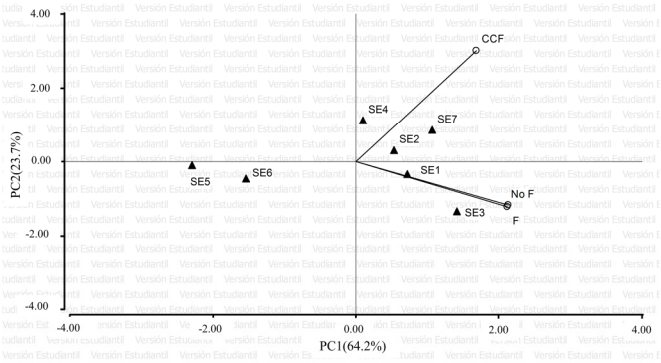

Figure 1: PCA of propolis from Santiago del Estero, Argentina.

Table 3: SC_{50} values (µgGAE/mL) of aqueous extracts (PAE) and ethanolic extracts (PEE) of propolis from different regions of Santiago del Estero.

Sample	$ABTS^{\bullet+}$ scavenging		$DPPH^{\bullet}$ scavenging		β-carotene bleaching	
	PEE	PAE	PEE	PAE	PEE	PAE
SE1	6.0±0.4	1.1±0.06	36.0±2.7	10.0±1.1	10.8±0.8	2.2±0.2
SE2	5.7±1.0	0.8±0.06	20.0±1.5	6.8±0.5	4.5±0.3	1.8±0.1
SE3	2.5±0.1	0.8±0.1	9.0±0.7	9.0±0.8	2.8±0.2	1.7±0.1
SE4	9.5±0.4	0.8±0.08	20.0±1.5	8.4±0.5	7.6±0.6	2.4±0.2
SE5	8.3±0.6	0.8±0.08	29.0±2.2	12.5±0.6	7.2±0.5	1.8±0.1
SE6	7.1±0.4	0.8±0.03	34.0±2.5	12.8±2.1	3.6±0.3	2.2±0.2
SE7	5.5±0.3	0.6±0.2	17.0±1.3	6.8±0.6	6.5±0.5	2.1±0.2

analysis of total phenolic and non-flavonoid phenolics. PAE showed lower peak numbers than PEE and the highest intensities tended to be crowded in places with low retention times.

PAE presented the highest scavenging activity with the lowest SC_{50} values (Table 3). All aqueous extracts were effective $ABTS^{\bullet+}$ scavengers in a concentration-dependent manner in SE samples from 0.5 to 2.5 µg GAE/mL (r^2: 0.99) with similar SC_{50} values and around 1 µg GAE/mL. On the other hand, SC_{50} values for ethanolic extracts were between 2.5 and 10 µg GAE/mL. SE3 ethanolic extracts were the most active $ABTS^{\bullet+}$scavenger. The results demonstrated that all extracts had marked electron donor properties for neutralizing free radicals by forming stable products.

SC_{50} values for aqueous and ethanolic extracts, BHT, and Trolox on the DPPH radical were 4 to 15 µg GAE/mL (r^2: 0.985), 9 to 36 µg GAE/mL (r^2: 0.813), 11.0 µg/mL (r^2: 0.994) and 10 mM (r^2: 0.970), respectively. SE3 ethanolic extract was the most active free radical scavenger. All extracts were able to protect linoleic acid from oxidation with IC_{50} values of 1.7-2.4 µg GAE/mL for PAE and 3.0-11.0 µg GAE/mL for PEE. The hydroxyl radical is the most reactive as it can attack and damage almost every molecule found in living cells. $OH^{\bullet}$ reactions include the ability to interact with DNA purine and pyrimidine. It can also abstract hydrogen atoms from biological molecules, including thiols, leading to the formation of sulfur radicals able to combine with oxygen to generate oxysulfur radicals, a number of which damage biological molecules. Only aqueous extracts were assayed as the reactants used to determine the scavenging capacity of $HO^{\bullet}$ and $O_2^{\bullet-}$ can react with most of the organic solvents and substances employed for buffer preparations. SC_{50} values of $HO^{\bullet}$ and $O_2^{\bullet-}$ for aqueous extracts were 10 to 100 µg GAE/mL and 53 to 275 µg GAE/mL, respectively (data not shown).

In conclusion, SE propolis from Argentina includes nutrients such as sugars, proteins, and minerals that are essential in ATP- energy producing metabolism. Non-nutrients such as phenolic compounds, principally flavonoids, were found to play important roles in oxidative stress as antioxidants that scavenge reactive species related to various lifestyle-related diseases, e.g., obesity, type 2 diabetes or cardiovascular diseases.

Experimental

Propolis samples: Several samples from different areas of Santiago del Estero, Northwestern Argentina, were analyzed: Departmento Capital, El Palomar (SE1), Departmento Figueroa (SE2 and SE7), Departmento Banda (SE3 and SE5), Departmento Copo (SE4), and Departmento Robles (SE6). The samples were stored at -20ºC until use.

Sample processing: Two g of ground propolis was extracted with 25 mL distilled water for 5 min in an ultrasonic bath at room temperature. The suspension was boiled at 80°C for 2 h and centrifuged at 5000 *g*. The residue was re-extracted under the same conditions and the supernatants were combined to reach a final volume of 100 mL of propolis aqueous extract (PAE).

Two g of ground propolis was extracted with 15 mL 80° ethanol and heated at 70°C for 35 min. The preparation was centrifuged at 8000 *g* for 10 min. The insoluble material was re-extracted with ethanol and the supernatants combined to produce propolis ethanolic extract (PEE) [6a].

Thin-layer chromatography (TLC): PEE and PAE (3 µg and 5 µg, respectively) were spotted onto TLC plates (Kieselgel 60 F254 0.2 mm, Merck), which were developed using chloroform: ethyl acetate (80:20). The separated components were visualized under ultraviolet light (254 and 365 nm, UV Lamp Model UVGL-58 Mineralight Lamp, USA), followed by spraying with NP reagent [6b].

Chemical profile by HPLC analysis: Chromatography was performed using a HPLC system consisting of a Waters 1525 Binary HPLC Pumps system with a 1500 Series Column Heater, (Rheodyne Inc., Cotati, CA). A Waters 2998 photodiode array detector (PAD) was used to analyze the extracts. A XBridgeTM C18 column (4.6 x 150 mm, 5 µm; Waters corporation, Milford, MA) at 40ºC and a gradient solvent system consisting of solvent A (methanol) and solvent B (9% acetic acid) (conditions: 25–45% A from 0 to 10 min and kept at 45% A from 10 to 20 min; 45–70% A from 20 to 40 min; 70–75% A from 40 to 50 min; 75–100% A from 50 to 55 min and kept at 100% A from 55 to 60 min) were used for separation. The flow rate was set at 0.8 mL/min. The sample injection volume was 20 µL. PAD acquisitions were performed in the range 190–500 nm, and chromatograms were integrated at 280 nm.

Sugar determination by spectrophotometric method and HPLC: The phenol–sulfuric acid method [6c] was used for determination of total neutral sugars in PAE and PEE. Results were expressed as g of glucose/100 g dry weight (DW). The samples were also analyzed by HPLC (Waters) with an automatic injector {ALLIANCE Waters 2695, detector IR Waters 410 and column sugar pack (Waters)}. The elution solvents were water containing EDTA-Na. The flow rate was 0.5 mL/min. Temperature: 85°C. The retention times of identified compounds were checked by co-injection with commercial standards.

Protein determination: Soluble protein concentration was determined by the method of Bradford [6d]. Results were expressed as mg of bovine serum albumin (BSA) /100 g DW.

Minerals: Na, K, Ca, Fe and Mg were determined by atomic absorption spectroscopy using a Perkin Elmer 3110 spectrophotometer (acetylene air flame and hollow-cathode lamps). P was determined using an atomic absorption spectrometer 600 (Perkin Elmer). Results are expressed as mg/100 g propolis.

Determination of total phenolic and non-flavonoid phenolic content: Total phenolic content of the samples was determined using the Folin–Ciocalteu method [7a]. Results are expressed in g gallic acid equivalents (GAE) per 100 g DW. Non-flavonoid phenols were measured by determination of total phenol content remaining after precipitation of the flavonoids with acidic formaldehyde [7b]. Results are expressed in g GAE/100 g DW.

Total flavonoids: Total flavonoid content was determined with aluminum chloride [7c] and expressed as g quercetin equivalents (QE) per 100 g DW.

ABTS free radical scavenging activity: The antioxidant capacity assay was carried out by an improved $ABTS^{\bullet+}$ method [8a]. $ABTS^{\bullet+}$ solution (1 mL) was added to PAE (0.625 to 5 µg GAE/mL) and PEE (2.5 to 10 µg GAE/mL). Absorbance was read at 734 nm at 1 min and 6 min. SC_{50} was determined (concentration of total phenolic compound able to scavenge 50% of $ABTS^{\bullet+}$). Results were expressed as µg GAE/mL. BHT and Trolox were used as reference antioxidants.

DPPH free radical scavenging activity: DPPH radical scavenging activity was measured [8b]. DPPH solution (1.5 mL of 300 µM in 96º ethanol) was added to 0.5 mL of PAE (containing 5 to 20 µg of GAE/mL) and PEE (containing 2.5 to 10 µg of GAE/mL) and shaken for 20 min at room temperature. Absorbance was measured at 515 nm. The percentage inhibition $[(A_0–A_1/A_0) \times 100]$ was plotted against phenolic compounds content and SC_{50} values were determined (concentration of total phenolic compounds able to scavenge 50% of DPPH free radical). BHT and Trolox were used as reference radical scavengers.

β-Carotene bleaching assay: Antioxidant activity was determined according to the β-carotene bleaching method [8b]. The initial absorbance at 470 nm was registered at zero time (t_0) and at 120 min. Antioxidant activity (AA%) was calculated as percent inhibition relative to control. IC_{50} values denote the sample concentration required to inhibit 50% β-carotene bleaching.

Hydroxyl radical scavenging: The deoxyribose assay as described by Chobot [8c], with a slight modification, was applied to measure $HO^{\bullet}$ scavenger capacity. The aqueous extract was dissolved in a KH_2PO_4/KOH buffer solution (50 mM, pH 7.4) to yield final concentrations from 10 to 200 µg GAE/mL; 50 µL of a 10.4 mM 2-deoxy-D-ribose solution, 100 µL of $FeCl_3$ (50 µM) and 100 µL of 52 µM EDTA were added. To start the Fenton reaction, 50 µL of 10 mM H_2O_2 and 50 µL of 1.0 mM ascorbic acid were added. The mixture was incubated at 37°C for 60 min. Then, 500 µL of 1% 2-thiobarbituric acid dissolved in 3% trichloroacetic acid (w/v) was added to each vial and maintained at 100°C for 20 min. To extract the reaction product of malondialdehide (MDA) and thiobarbituric acid, 700 µL of *n*-butanol was added, and the mixture was vigorously vortexed. The *n*-butanol layers of the tubes, each 600 µL, were separated and the absorbance was determined at 532 nm. Assays were performed in triplicate. Reaction mixtures without the test compound served as positive control (100% MDA). The negative control contained the full reaction mixture except 2-deoxy-D-ribose. Controls without either EDTA or ascorbic acid were performed. Inhibition (I) of deoxyribose degradation in percent was calculated.

Superoxide anion radical ($O_2^{\bullet-}$) scavenging capacity: The $O_2^{\bullet-}$ were generated in 120 µL of sodium phosphate buffer (19 mM, pH 7.4), which contained 40 µL NADH (2 mM), 20 µL of NBT (1 mM), 40 µL of PMS (60 µM), PEE and PAE at different concentrations or distilled water and ethanol for controls. The color reaction was detected at 550 nm using a Microplate reader [8d]. SC_{50} values denote the µg GAE/mL required to scavenge 50% of superoxide free radicals.

Statistical analysis: Sampling and analyses were performed in triplicate, and the data are presented as mean ± standard deviation. The PCA and correlation between two variants by the Pearson test was realized using Infostat software, with the level of significance set at $p < 0.05$.

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