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Chemical Composition of Argentinean Propolis Collected in Extreme Regions and its Relation with Antimicrobial and Antioxidant Activities

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This paper reveals, for the first time, the functional properties of propolis from an extreme region of Argentine (*El Rincón*, Province of Catamarca, Argentina), as well as the isolation and identification of bioactive compounds. The antioxidant activity was determined by the ABTS method and β-carotene bleaching. The antibacterial activity was determined on methicillin resistant *Staphylococcus aureus* (MRSA) by the microdilution method and bioautographic assays. Twelve compounds were isolated and identified by NMR spectroscopy. The main bioactive compounds were 2',4'-dihydroxy-3'-methoxychalcone (**3**), 2',4'-dihydroxychalcone (**9**), 2',4',4-trihydroxy-6'- methoxychalcone (**8**), 5-hydroxy-4',7-dimethoxyflavone (**4**) , 4′,5-dihydroxy-3,7,8-trimethoxyflavone (**10**) and 7-hydroxy- 5,8-dimethoxyflavone (**11**). All compounds were active against clinical isolates (MIC_{50} 10 µg/ mL) and displayed antioxidant activity (SC_{50} values of 20 µg/mL). The MIC and SC_{50} values of the isolated compounds were lower than those obtained with crude propolis extracts, chloroform sub-extracts and isolated fractions.

Keywords: Argentinean propolis, antibacterial activity, antioxidant activity, bioactive compounds, chalcone.

Propolis (bee glue), one of the most valuable bee products, was incorporated as a dietary supplement into the Argentinean Food Code in May 2008 by means of Res. 357/08 of the Secretaría de Agricultura, Ganadería, Pesca y Alimentos and 94/08 of the Ministerio de Salud. Propolis has been used in different preparations to treat human diseases for centuries. It has proved to possess a wide range of antimicrobial, antiinflammatory, antitumor, and antioxidant activities due to its different constituents [1a-1g]. The chemical composition of propolis is highly variable and depends mainly on the local flora [1a,1b,1d].

Numerous studies led to the differentiation of two propolis groups, one from temperate regions and another from tropical areas, with completely different chemical characteristics. Propolis samples from a temperate zone are characterized by a similar composition, the main and biologically active constituents being phenolics: flavonoids, aromatic acids and their esters [2a]. On the other hand, the bee glue from tropical regions is chemically diverse and its constituents are different: phenolics, prenylated *p*-coumaric acid, flavonoids, benzophenones and terpenes [2a-2c]. Thus, characterization of propolis from different areas is necessary to assess its pharmacological properties, as well as the natural compounds involved in such activity. Argentina is a subtropical region that has a wide biodiversity and the chemical composition and biological properties of propolis is different according to the phytogeographical regions where it is found. In previous papers, propolis samples from three phytogeographical regions of Argentina with different altitudinal levels, Santiago del Estero (100 masl), Tucuman (1200 masl) and San Juan (600 masl), were characterized by their antioxidant capacity and antimicrobial activity [3a-3h]. Bee glue was collected in the beekeeping production area of the province of Catamarca (*El Rincón*, Monte phytogeographical region-Prepuna), in extreme warm and dry climatic conditions at 1300 masl. The regional flora is characterized by the combination of grass forms and shrubs in different proportions [4]. The principal plant species are *Larrea divaricata*, *Bulnesia retama*, *Fabiana densa*, *Stipa speciosa*, *Arístida speciosa*, *Panicum urvillanum*, *Cassia crassiramea*, *Gochnatia glutinosa*, *Aphylloclados spartioides*, *Bougainvillea spinosa*, *Zuccagnia puntacta* and *Bulnesia schickendanzzi*.

The aims of the present report were the isolation and structure elucidation of bioactive phenolic compounds of propolis from *El Rincón*, Catamarca, Argentine (Ri-Pr) and demonstrate their functional properties (antioxidant and antibacterial).

The EtOH extracts of collected raw propolis samples from different regions of Argentina were first assayed for antibacterial activities by agar diffusion methods and for antioxidant activity by DPPH methods, previously published [3c]. Ri-Pr was selected for the isolation of bioactive compounds because of its antibacterial and antioxidant activities.

Isolation and characterization of antibacterial and antioxidant compounds: Ri-Pr samples were powdered and extracted with 80% EtOH, *n*-hexane, $CHCl_3$ and distilled water at room temperature. The ethanolic extract and chloroformic sub-extract (CHL-SE) showed the highest phenolic compounds content, principally flavonoids (Table 1).

Table 1: Total phenolic compounds and flavonoids from ethanolic propolis extract (PEE), and chloroform (CHL), *n*-hexane (HEX) and aqueous (Aq) sub-extracts.

Samples	Total phenolic (µg GAE/mL)	Total Flavonoid (µg Q /mL)	Phenolic compounds (mg/g)	Flavonoids (mg/g)
PEE	182±11	133±8	364±10	266±8
HEX	63±11	48±8	126±10	96±8
CHL	174±21	110±9	348±10	220±9
Aq	47±7	1.1±0.2	94±8	2.2±0.2

[a]Mean ± SD.

The CHL-SE was more active than the other sub-extracts against ABTS radical cation ($ABTS^{+\bullet}$) and β carotene bleaching with SC_{50} values of 5 and 2.9 µg/mL, respectively (Table 2). The antimicrobial activity of the CHL-SE on methicillin resistant *S. aureus* (MRSA) was similar to that observed for PEE (MIC values of 65 µg/mL) (Table 2). For this reason, the CHL-SE was chromatographed on a silica gel column to give 16 fractions (Fr I to Fr XVI). All showed free radical scavenging activity on the $ABTS^{+\bullet}$ with SC_{50} values between 2 to 67 µg GAE/mL and inhibitory activity on β-carotene bleaching (1.9 to 74.4 µg/mL).

Fractions II to XV were active against MRSA (Table 2). All antimicrobial fractions were examined by HPLC. Simple profiles were observed in three bioactive fractions named IV, V and VI. These fractions were selected for isolation and structural identification of bioactive compounds.

Twelve phenolic compounds were isolated and identified. The ^{1}H NMR and UV spectra of compounds **1** (7-hydroxy-8-methoxyflavanone), **2** (7,4'-dihydroxy-5-methoxy-flavanone), **3** (2',4'-dihydroxy-3'-methoxychalcone), **4** (5-hydroxy-4',7-dimethoxyflavone), **5** (3,7-dihydroxy-8-methoxyflavone), **6** (3β,7-dihydroxy-5-methoxyflavanone, **7** (3,5-dihydroxy-7,8-dimethoxyflavone), **8** (2',4',4-trihydroxy-6'-methoxychalcone), **9** (2',4'-dihydroxy-chalcone), **10** *(4′,* 5-*d*ihydroxy-3*,*7*,*8-trimethoxyflavone), **11** (7-hydroxy-5,8 dimethoxyflavone), and **12** (7-dihydroxy-5,8-dimethoxyflavanone) are in agreement with reported data [3d,5a-5f,6a-6d].

Table 2: Biological activities of PEE, HEX, CHL and Aq extracts, and fractions obtained from CHL extracts.

Samples	ABTS assay SC_{50}[a] (µg/mL)	β-Carotene assay IC_{50}[a] (µg/mL)	Antibacterial activity MIC values[a] (µg/mL)
PEE	6.9 ± 0.6	2.0 ± 0.3	65 ± 20
HEX	44.7 ± 3.6	15.8 ± 2.3	700 ± 140
CHL	5.2 ±0.4	2.9 ± 0.2	65 ± 20
Aq	35.6 ± 2.8	19.1 ± 3.8	>1600
Fr I	ND	ND	ND
Fr II	6.6 ± 0.5	>80	ND
Fr III	8.8 ± 0.7	36.9 ± 5.5	ND
Fr IV	12.3 ± 0.9	46.5 ± 6.9	200 ± 50
Fr V	35.5 ± 2.8	64.5 ± 9.7	200 ± 50
Fr VI	67.1 ± 5.3	74.4 ± 11.2	50 ± 10
Fr VII	44.1 ± 3.5	24.6 ± 11.6	100 ± 20
Fr VIII	21.9 ± 1.7	21.8 ± 3.3	50 ± 10
Fr IX	11.6 ± 0.5	4.3 ± 0.6	100 ± 20
Fr X	9.5 ± 0.7	3.7 ± 0.5	100 ± 20
Fr XI	4.5 ± 0.2	3.4 ± 0.5	100 ± 20
Fr XII	1.6 ± 0.1	1.9 ± 0.3	50 ± 10
Fr XIII	4.1 ± 0.2	5.5 ± 0.8	50 ± 10
Fr XIV	1.7 ± 0.1	3.8 ± 0.6	50 ± 10
Fr XV	26.4 ± 2.0	23.5 ± 3.5	200 ± 50
Fr XVI	ND	43.6 ± 7.8	800 ± 150

[a]Mean ± SD.
ND: Not determined (at the concentrations tested did not show activity)

Compounds **3**, **4**, **8**, **9**, **10**, and **11** were more active as antibacterials by microdilution assays. The MIC_{50} values were around 10 µg/mL, and the MBC values 1000 µg/mL (Table 3). Compound 4 was the most abundant (7.3 mg), followed by compounds *3* (2.3 mg) and **11** (1.8 mg); these occur at concentrations that are about three fold higher than compounds **8**, **9** and **10** (0.7 mg).

All isolated compounds were active in the $ABTS^{+\bullet}$ assay (up to 90% of RSA at 20 µg/mL). Compounds **9** and **3** were previously isolated in an Argentinean propolis sample obtained in Tucuman province [3d,3h].

The literature bears witness to all the characteristics of compound **9**. It is antibacterial against antibiotic resistant Gram-positive bacteria [6c]; it is also active against phytopathogenic fungi [7a,7b] and shows free radical scavenging [7d] and antimutagenic properties [3d,7c]. Other studies indicate that this compound is neither genotoxic nor mutagenic [3d,7c].

Thus, our results support the hypothesis that the phenolic compounds present in the propolis studied could be the cause of its antibacterial and antioxidant activity. They also show that chalcones and flavones are very important in the biological activity of Argentinean propolis and

Table 3: Cellular viability (%) of different concentrations of bioactive isolated compounds.

		Cellular viability (%)			
	Flavonoids and Chalcones	10 (μg/mL)	50 (μg/mL)	100 (μg/mL)	1000 (μg/mL)
C 1	7-hydroxy-8-methoxyflavanone	66±6	59±5	61±7	35±4
C 2	7,4'-dihydroxy- 5-methoxyflavanone	68±6	58±4	47±4	19±1
C 3	2',4'-dihydroxy-3'-methoxychalcone	67±6	44±2	31±3	0
C 4	5-hydroxy- 4',7 -dimethoxyflavone	59±5	42±4	26±3	0
C 5	3,7-dihydroxy- 8-methoxyflavone	59±5	49±6	48±5	36±2
C 6	3β,7- dihydroxy-5-methoxyflavanone	60±5	53±6	42±3	13±1
C 7	3,5-dihydroxy-7,8-dimethoxyflavone	64±6	42±3	42±3	23±3
C 8	2',4',4-trihydroxy-6'-methoxychalcone	56±3	45±7	41±5	0
C 9	2',4'-dihydroxychalcone	51±4	29±3	29±3	0
C 10	***4',5-Dihydroxy-3,7,8-trimethoxyflavone***	57±4	48±4	40±2	0
C 11	7-hydroxy- 5,8-dimethoxyflavone	49±4	38±1	39±3	0
C 12	7-dihydroxy-5,8-dimethoxyflavanone)	65±6	69±6	67±5	33±3

reinforce previous evidence on the probable use of Argentinean propolis as a source of antibacterial and antioxidant compounds.

Experimental

Propolis samples: Propolis samples were collected from beehives at *El Rincón*, Catamarca province (Northwestern Argentina), 1300 masl, in 2005-2009. The samples were stored at -20ºC until use. Voucher specimens are deposited at the Biological Studies Laboratory of INQUINOA (CONICET), University of Tucumán, Argentina.

Compounds isolation: A raw propolis sample (15 g) from Catamarca (Ri-Pr), Argentina was powdered and extracted three times with 80% EtOH (1:10; w:v) at room temperature for 24 h. The extract obtained (12 g) was extracted successively with *n*-hexane and $CHCl_3$ to obtain HEX-SE (1.5g), CHL-SE (5 g) and Aq-SE (0.5 g). The CHL-SE was chromatographed on a silica gel 60 (70-230 Mesh) column and eluted with $CHCl_3$ with increasing amounts of EtOAc (0-100%), and finally MeOH to give 16 fractions (Fr I–XVI).

Fraction IV (67.6 mg), eluted with $CHCl_3$-EtOAc (9:1, v:v), was processed again on a silica gel 60 column, eluted with $CHCl_3$ with increasing amounts of EtOAc (0-100%) and finally MeOH to give 6 fractions (Fr.1 to 6).

Frs 2-5 (54.7 mg) were processed by HPLC (column A). Elution was carried out using MeOH-H_2O-HAc (90:10:0.5) as mobile phase with a flow rate of 1.5 mL/min to obtain compounds **1** (1.5 mg), **2** (0.7 mg) and a mixture that was submitted to a new HPLC process [column B, MeOH-H_2O-HAc (90:25:0.5), 1.5 mL/min] to give compounds **3** (2.3 mg) and **4** (7.3 mg).

Fraction V (62.5 mg) was eluted with $CHCl_3$-EtOAc (8.5:1.5, v:v) and processed again on a silica gel column eluted with $CHCl_3$ with increasing amounts of EtOAc (0-100%) and finally MeOH to give 7 fractions (Fr.1 to 7). Fr. 3-7 (43 mg) were processed by HPLC [column A, MeOH-H_2O-HAc (90:25:0.5), 1.5 mL/min] to give compounds **5**.(0.5 mg), **6** (1.8 mg), **7** (0.7 mg), **8** (0.7 mg), **9** (0.7 mg) and **10** (0.6 mg).

Fraction VI (157.2 mg), eluted with a mixture of $CHCl_3$–EtOAc (8:2, v:v), was submitted to HPLC [column A, MeOH-H_2O-HAc (90:25:0.5), 2 mL/min] to give compounds **11** (11.3 mg) and **12** (0.7 mg).

NMR spectra were recorded on a Bruker AC spectrometer operating at 200 for ^1H, and 125 MHz for ^{13}C, with TMS as internal standard in $CDCl_3$. The mass spectra, including high-resolution mass spectra, were recorded on a JEOL JMS AX 500 spectrometer (HRCIMS). For HPLC separations, Gilson and Waters equipment was used. Detection was accomplished with UV and refractive index detectors. Columns: (A) Phenomenex Ultremex C8 (5 μm, 10 mm i.d. x 250 mm) and (B) Phenomenex Ultremex C18 (5 μm, 10 mm i.d. x 250 mm). Retention time was measured from the solvent peak. Analytical TLC (Kieselgel 60 F254 0.2 mm, Merck) was performed using two mobile phases: light petroleum–EtOAc (8:2) and $CHCl_3$–MeOH (9.8:0.2). The compounds were visualized by UV at 254 and 365 nm (UV Lamp Model UV 5L-58 Mineralight Lamp) and by spraying with saturated ceric sulfate solution followed by heating at 100°C. Fractions with similar TLC patterns were combined and evaluated for antibacterial and antioxidant activity.

Phenolic and flavonoid contents: Total phenolic compounds content was determined by the Singleton *et al.* method [8a]. Absorbance of the resulting blue color was measured at 765 nm. Results were expressed as gallic acid equivalents per mL (GAE/mL). Total flavone and flavonol contents were measured by a spectrophotometric assay based on aluminum chloride complex formation [8b]. Results were expressed as quercetin equivalents (QE).

Total antioxidant capacity assay: The antioxidant capacity assay was carried out by the improved $ABTS^{+\bullet}$ [9a]. $ABTS^{+\bullet}$ was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature (23ºC) for 12–16 h before use. $ABTS^{+\bullet}$ solution (1 mL; absorbance of 0.7 $\pm$ 0.02 at 734 nm) was added to each fraction (5 to 80 μg) dissolved in ethanol 80%. The reactive mixture was allowed to stand at room temperature and the absorbance reading was taken at 30°C exactly 6 min after initial mixing. The results were expressed as scavenging concentration of 50% (SC_{50}) and defined as the concentration at which 50% of $ABTS^{+\bullet}$ was scavenged. All experiments were carried out in triplicate.

Autographic assay: The components of the different fractions were separated by TLC using chloroform: ethyl acetate (8.8:0.2; 8.2:1.8; 7.6:2.4 and 5:5, v:v) as development solvents. Once the TLC plates were dried, the separated components were vizualized with $ABTS^{+\bullet}$ [9b].

β-Carotene-linoleic acid assay: The antioxidant activity of each fraction was determined [9c]. One mL of 0.2 mg/mL β-carotene dissolved in chloroform was added to round-bottom flasks (50 mL) containing 0.02 mL linoleic acid and 0.2 mL Tween 20. Each mixture was then dosed with either 0.2 mL of different sample dilutions or positive (BHT) or solvent control. After evaporating to dryness under vacuum at room temperature, distilled water (25 mL) was added. The mixture was shaken for 2 min and then subjected to thermal oxidation at 50ºC for 60 min. The absorbance of the solution was monitored at 470 nm in a spectrophotometer (Beckman DU-650) by taking measurements at 20 min intervals for 60 min. All samples were assayed in triplicate. β-Carotene bleaching rate was calculated using the following equation: AA= 100 (DRc-DRs)/ DRc where AA is the antioxidant activity, DRC is the control degradation rate (ln(a/b)/60), DRS is the degradation rate in the presence of the sample (ln(a/b)/60), a is the initial absorbance at time 0, and b is the absorbance at 60 min. IC_{50} was defined as the concentration at which 50% of β carotene was protected. All experiments were carried out in triplicate.

Bioautographyc assays: PEE, fractions (60 µg dry weight) and isolated compounds (25 µg dry weight) were separated by TLC under the same conditions that was used in autographic assays Then, the plates were dried overnight in a sterile room and the bioautographic assays were realized using MRSA (F7), according to Nieva Moreno [3a].

Broth microdilution susceptibility assay: PEE (25 to 800 µg/ mL) and isolated compound (10 to 1000 µg/ mL) dilutions were prepared in 96-well microplates. After evaporating all fractions to dryness, the residues were dissolved in dimethyl sulphoxide (DMSO). The wells were filled with Müller-Hinton broth. The final inoculum (MRSA) was $1x10^5$ CFU. The inoculated plates were incubated aerobically at 35ºC for 24 h [10]. After the broth microdilution susceptibility assay, 20 µL of methylthiazolyltetrazolium chloride solution (MTT) (12 mg/mL in PBS) was added to the wells and incubated for 1 h. Cellular viability was determined by absorbance at 550 nm.

Minimal inhibitory concentration (MIC) was defined as the lowest concentration of either propolis extract or isolated compound able to restrict growth to an absorbance <0.1 at 550 nm. To confirm MIC and to establish MBC, 10 µL of culture medium from each microplate well was inoculated in agar plates and incubated aerobically for 24 h at 36ºC. Then, the number of surviving organisms was determined.

MBC was defined as the lowest extract concentration at which 99.9% of the bacteria had been killed. All experiments were carried out in triplicate.

The data obtained in the cell proliferation and cytotoxicity assays were analyzed using the GraphPad Prism program (GraphPad Software, San Diego, CA).

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