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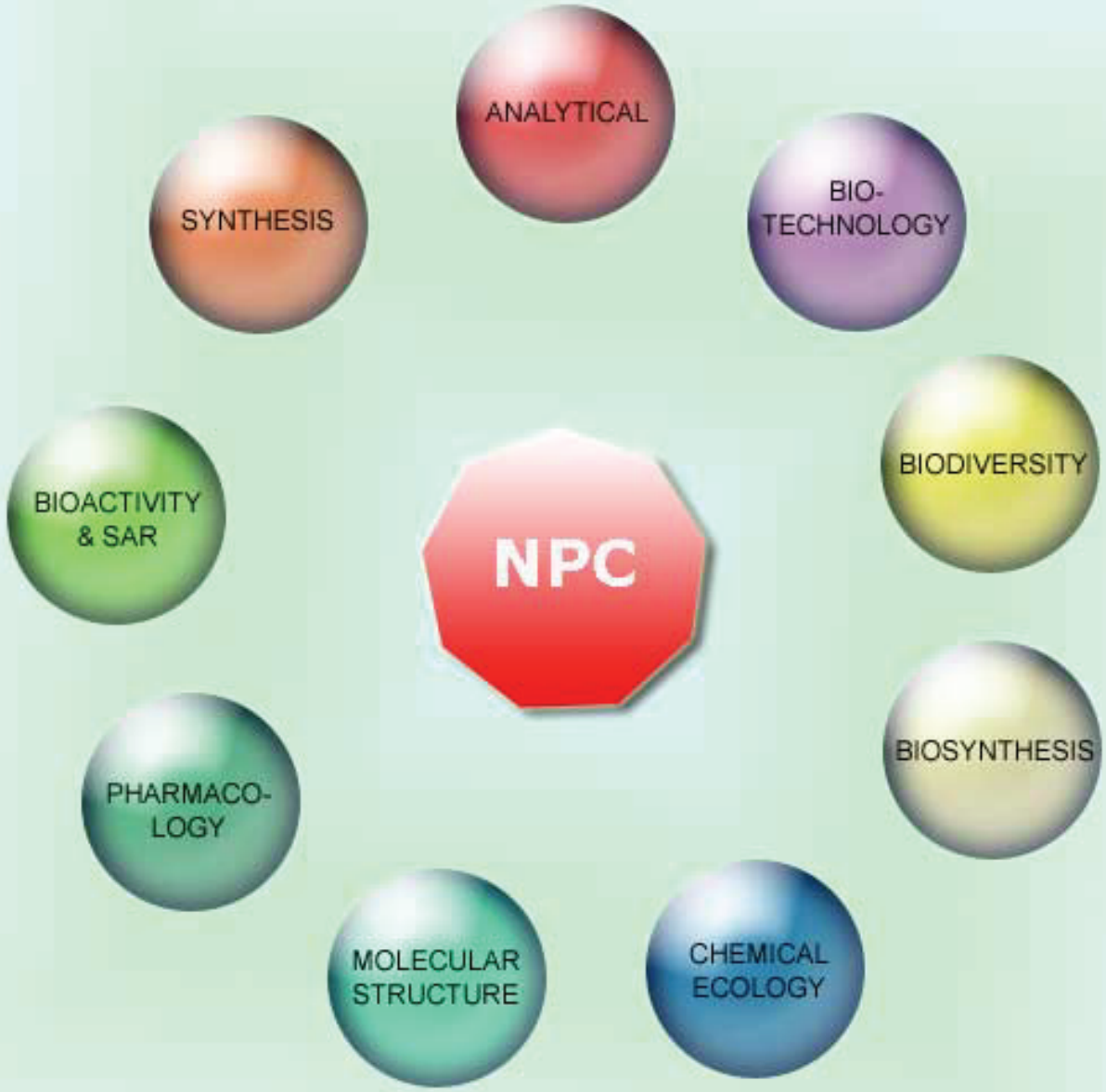

Volume 7. Issue 7. Pages 815-970. 2012
ISSN 1934-578X (printed); ISSN 1555-9475 (online)
www.naturalproduct.us

NPC Natural Product Communications

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NPC Natural Product Communications

2012
Vol. 7
No. 7
879 - 882

Chalcones in Bioactive Argentine Propolis Collected in Arid Environments

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Received: March 5th, 2012; Accepted: May 30th, 2012

The aim of this study was to assess the chemical and biological profile of propolis samples collected in arid environments of north-western Argentina. The samples were from two phytogeographical regions (Prepuna and Monte de Catamarca Province). Propolis ethanolic extracts (PEE) and chloroform (CHL), hexane (HEX) and aqueous (AQ) sub-extracts of samples from three regions (CAT-I; CAT-II and CAT-III) were obtained. All PEE exhibited antioxidant activity in the DPPH radical scavenging assay (SC_{50} values between 28 and 43 µg DW/mL). The CHL extract was the most active (SC_{50} values between 10 and 37 µg DW/mL). The antioxidant activity in the β-carotene bleaching assays was more effective for PEE and CHL (IC_{50} values between 2 and 9 µg DW/mL, respectively). A similar pattern was observed for antibacterial activity. The highest inhibitory effect on the growth of human Gram-positive bacteria was observed for CHL-III and CHL-I (Monte region) with minimal inhibitory concentration values (MIC_{100}) of 50 to 100 µg DW/mL. Nine compounds were identified by HPLC-PAD. Two of them (2', 4'- dihydroxychalcone and 2',4'- dihydroxy 3'-methoxychalcone) were found only in propolis samples from the Monte phytogeographical region. We consider that the Argentine arid region is appropriate to place hives in order to obtain propolis of excellent quality because the dominant life forms in that environment are shrubby species that produce resinous exudates with a high content of chalcones, flavones and flavonols.

Keywords: Chalcones, Bioactivity, Argentine propolis, Arid environments.

Propolis is a hive material produced by bees (*Apis mellifera*). They collect resin from buds and shoots of various trees and plants and then combine it with salivary secretions [1]. Bees use this material to seal cracks, and coat the walls of the hive to create a protective barrier against intruders and infective agents [2]. A large number of scientific studies have shown that propolis chemical composition is associated with the local surrounding flora. Hence, the chemical composition of propolis depends on the location of the hive [1,3a]. Bankova *et al*. [4] classified propolis into two groups: temperate and subtropical. Propolis from tropical regions, like Brazil (green propolis), Venezuela and Cuba, has prenylated *p*-coumaric acids, diterpenes and prenylated benzophenones as bioactive compounds [5] and its flavonoid content is very low. Propolis from temperate regions, like that containing poplars and birches, has other bioactive compounds: flavonoids, flavanones, flavones, phenolic acids, and their esters [5].

Argentina shows a great biodiversity and at least four climate types: temperate, arid, tropical and cold or their combinations. The arid climate includes Puna, Andes (Catamarca, La Rioja and San Juan provinces), the pre-Andean area, the extra-Andean Patagonia and the phytogeographic regions of Monte and Prepuna (Figure 1) [6a,b]. The most characteristic plant community of the Monte phytogeographical region is the "jarillal" with *Larrea divaricata* and *L. cuneifolia* as predominant species. Other important plant species in this region are the "algarrobales" of *Prosopis flexuosa*

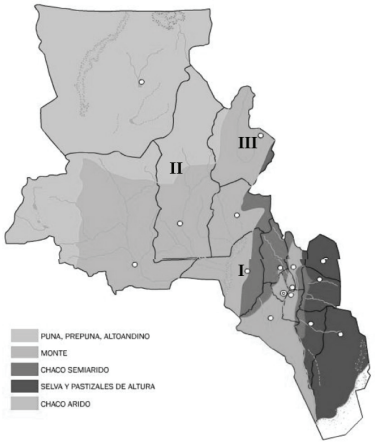

Figure 1: Phytogeographical regions of Catamarca and the area where the propolis samples were collected (Map extracted from Roig *et al*. [6a]).

and *P. chilensis*, and xerophytic shrubs. Jarilla (*L. divaricata*), retama (*Bulnesia retama*) and pus-pus (*Z. punctata*) are shrubs that grow in the Monte and Prepuna regions. Plants from arid regions synthesize many chemical compounds (phenolic compounds as flavonoids) that allow them to survive and endure adverse

Table 1: Phytogeographic origin, total phenolic and flavonoids content, and antioxidant activities of extracts and sub-extracts of Catamarca propolis samples.

Collection site and phytogeographic region	Samples	Total phenolic content [a]	Total flavonoid content [b]	DPPH assay[c]	β-Carotene bleaching assay[d]
CAT-I Monte Region (1300 m.a.s.l)	PEE-I	304.0±7.3	220.0 ± 9.1	43.0±3.0	2.0±0.3
	HEX-I	13.0±2.2	8.5 ± 0.4	NA	15.8±2.3
	CHL-I	175.0±8.7	97.6 ± 4.8	37.0±2	2.9±0.2
	AQ-I	7.2±4.4	0.5 ± 0.1	115.0±5.7	19.1±3.8
CAT-II Prepuna Region (1300 m.a.s.l.)	PEE-II	282.1 ±13.8	180.7 ± 9.1	38.0±2.2	8.2±3.1
	HEX-II	9.3±2.1	5.9 ± 0.6	84.2±6.3	6.8±1.9
	CHL-II	162.0±19.5	103.9 ± 5.2	17.3±2.8	2.5±0.7
	AQ-II	3.1±1.1	1.4± 0.2	NA	19.1±4.4
CAT-III Monte Region (900 m.a.s.l.)	PEE-III	321.0±13.2	268.0±12.4	28.0±2.2	8.4±1.4
	HEX-III	18.6±3.2	12.6.±0.6	158.0±12.0	9.1±1.6
	CHL-III	273.8±14.4	157.6±7.9	10.0±1.0	5.0±0.9
	AQ-III	2.0±0.4	0.3±0.1	220.0±17.0	17.0±3.1

NA: non active until 400 µg DW/mL; a) mg GAE/g propolis; b) mg QE/g ; c) SC_{50} (µg DW/mL); d) IC_{50} (µg DW/mL). Means ±SD

weather conditions [7]. Hence, the propolis collected from these regions could have the best biological activity and chemical composition. Previous papers have reported phenolic compound levels of 90 mg/g of raw propolis in samples from the northeastern and central regions (Chaco, Misiones and Santa Fe provinces). Santiago del Estero, a region with a semiarid climate, has values of 237 mg/g of raw propolis, as do some arid locations of Tucuman and Catamarca [8]. The authors reported a positive correlation between phenolic content (principally flavone and flavanone) and bioactivity in arid region propolis [3a, 8, 8c, 9, 9b, 6e]. A chalcone with antibacterial, antifungal and antioxidant activity [8c, 9a, 9d] has been reported in propolis extracts from some arid regions of Tucumán and Catamarca provinces.

The aim of this paper was to study the biological activity and preliminary chemical composition of propolis from two arid climate regions. Thus, we studied the activity of propolis samples from Catamarca against antibiotic-resistant human pathogenic bacteria and analyzed free radical scavenging activity using a standardized international methodology. The phenolic content and biological activities of propolis extracts obtained from two phytogeographic regions in Catamarca province, Argentina were examined.

Table 1 shows the collection sites, total polyphenol and flavonoid content of PEE, HEX, CHL and AQ sub-extracts. The content of polyphenols for all PEE (282.1 to 321.0 mg GAE/g propolis) was similar to that previously reported for north-western Argentine propolis (Table 2). These results are coincident with those of poplar or birch propolis from temperate regions of Europe and Asia, with a predominance of phenolic compounds [5]. The highest polyphenolic content was observed in the chloroform sub-extract, with values between 162.0 and 273.8 mg GAE/g propolis.

The chemical profile by TLC- NP/PEG and HPLC-PAD showed slight differences between PEE from Monte region (CAT-I and CAT-III) samples and those of the Prepuna region (CAT- II).

Nine compounds were identified in the PEE: pinobanksin, 7-hydroxy-flavanone, 3,7-dihydroxy 8-methoxyflavone, chrysin, 5-hydroxy 7-methoxyflavanone, 3,5-dihydroxy 7,8-dimethoxy-flavone, 2'4'-dihydroxychalcone, 2',4'-dihydroxy 3'-methoxy-chalcone, and 7-hydroxy 8-methoxyflavanone. One compound was undetermined.

Two chalcones (2', 4'- dihydroxychalcone and 2', 4'-dihydroxy 3'-methoxychalcone) were detected only in propolis samples from Monte region. These compounds were also recognized in propolis from Amaicha del Valle, Tucumán and in *Zuccagnia punctata*, a perennial shrub that grows between 700 and 2700 m.a.s.l. in Argentinean arid regions (Salta, San Luis, San Juan,

Table 2: Propolis phenolic compounds content and relation to climate and altitude of hive location.

Argentinean Provinces	Height (meters)	Climate	Total phenolic compounds (mg/g)	References
Buenos Aires	0-100	Temperate	17.5	9a
Entre Rios	0-100	Temperate	72.4-171.1	10a
Santa Fé	0-100	Temperate	8-32	10b
Corrientes	0-100	Subtropical	1.43-103	10c
Chaco	0-200	Subtropical	50-67	8a
Misiones	0-200	Subtropical	90	8a
Santiago del Estero	100-500	Subtropical	182-237	8a
Salta	200-500	Subtropical	100-197	8a
Salta	1000-3000	Arid	210-240	8a
Tucuman	1600-3000	Arid	190-358	8a
Tucuman	200-800	Tropical	32-199	8a
Catamarca	800-3000	Arid	200-330	8a; 8c
Mendoza	500-3000	Arid	181-348	10a; 10c
San Juan	500-3000	Arid	227-363	9c
Río Negro	0-3000	Arid	244.5-290	10a

Mendoza, Catamarca, Tucumán, La Rioja and Jujuy). Hence, *Jarilla macho* or *pus pus* was described as the botanical origin of Tucuman propolis [9d]. We propose that chalcones may be used as markers in order to identify *Zuccagnia type propolis*. These chalcones are recognized for their excellent antioxidant, antibacterial, and antifungal bioactivity [7a,c, 9d, 8c,10d,e].

CHL sub-extracts, as well as PEE, were more active as DPPH scavengers (SC_{50} values between 10 and 43 µg DW/mL) than HEX sub-extracts (SC_{50} values between 84.2 and 158.0 µg DW/mL). All propolis extracts and sub-extracts protected the lipids from oxidation with IC_{50} values between 2.0 and 19.1 µg DW/mL (Table 1). Propolis extracts from CAT-I and CAT-III showed a higher activity against Gram-positive bacteria than CAT-II extract. CAT-I was the most active against the tested Gram-negative bacteria. CHL-I and CHL-III sub-extracts were more active against *S. aureus* and *E. faecalis* with MIC values between 50 and 75 µg DW/mL and 100 µg DW/mL, respectively (Table 3).

Table 3: Antimicrobial activity (MIC_{100} µg DW/mL) of propolis ethanolic extracts and derived sub-extracts against human pathogenic methicillin resistant bacteria and control bacteria.

	CAT I			CAT II			CAT III			Phenotype of clinical isolate
Strains	**PEE**	**HEX**	**CHL**	**PEE**	**HEX**	**CHL**	**PEE**	**HEX**	**CHL**	
Staphylococcus aureus										
F16	100	200	50	100	200	100	100	100	50	*Met^s Oxa^s Gen^s Van^s*
F7	100	800	75	100	200	100	100	400	75	*Met^r Oxa^r Gen^r*
F30	100	800	75	100	400	100	100	400	75	*Met^s Oxa^r Gen^r Van^s*
ATCC 29213	100	800	75	100	800	100	100	400	75	*Control strain*
Enterococcus faecalis										
F203	100	800	100	100	800	100	100	800	100	*Gen^r Str^r Van^s Amp^s*
F208	100	800	100	100	800	100	100	800	100	*Str^r Van^s Amp^r Gen^r*
F226	100	800	100	100	800	100	100	800	100	*Van^s Amp^s Gen^s Str^s*
ATCC 29212	100	800	100	100	800	100	100	800	100	*Control strain*
Gram negative bacteria										
F301	1600	1600	1600	>1600	>1600	1600	1600	R	1600	*Lvx^r Cro^r Ctx^r Cxm^r*
ATCC 35218	1600	1600	800	R	R	1600	R	R	1600	*Control strain*
F302	800	1600	1600	>1600	>1600	1600	R	R	1600	*Lvx^r Tzp^r Cro^r Ctx^r*
F359	800	1600	400	800	1600	400	800	R	400	*Tzp^s Ctx^s Ipm^s*
F339	800	1600	800	ND	ND	ND	800	R	800	*Lvx^s Tzp^s Cro^s Ctx^s*
F364	1600	>1600	800	ND	ND	ND	R	R	R	Cro^r Ctx^r Cxm^r Fep^r
ATCC 700603	1600	1600	800	R	R	R	R	R	R	*Control strain*

R: resistant ND: not determined. S: susceptible

The hexane extract antimicrobial activity against Gram positive bacteria should be regarded as weak [10f]. The aqueous extracts in all cases were inactive. The MIC values obtained with Catamarca propolis were similar to those of propolis collected from arid regions like Amaicha del Valle (Tucumán) that have already been reported [3b]. Also, as shown in the HPLC profile, CAT-I and CAT-III propolis samples had the same bioactive compounds as Amaicha del Valle propolis (province of Tucumán): 2'4'-dihydroxychalcone and 2',4'-dihydroxy-3'-methoxychalcone. According to the result of the bioautographic assays both chalcones should be considered as responsible for MRSA growth inhibition by CAT-I and CAT-III.

Our results and previous papers [8a; 8c] indicate that Argentine arid regions provide the optimum climate and native plant species with suitable resin content to obtain propolis with good antioxidant and antibacterial activity. For this reason, the climate would be an important factor when choosing the location of hives to enhance the biological activities of propolis.

Experimental

Propolis source: Propolis samples were collected from apiaries of "Red de Ensayos del INTA-PROAPI" (Programa Nacional Apícola) in Catamarca during 2000-2009: Monte (CAT-I and CAT-III) and Prepuna (CAT-II) (Table 1). The samples were stored at -20ºC until use. Voucher specimens are deposited at the Laboratorio de Investigación de Productos Naturales [LIPRON, INQUINOA (CONICET)], University of Tucumán, Argentina.

Propolis extracts preparation: Raw propolis samples (2 g) were cut into small pieces and extracted with 20 mL of 80% ethanol using an ultrasonic processor (30 min, 80w potency). The extractions were centrifuged for 20 mins at 9000 *g* in a refrigerated centrifuge (Sorvall RC50) and the supernatant was separated. Successive extractions of the residue were made to complete a final volume of 100 mL (labeled propolis ethanolic extract PEE). This extract was subjected to liquid-liquid extraction. Hexane (HEX), chloroform (CHL), and aqueous sub-extracts (AQ) were obtained. Each extract and sub-extract was dried by evaporation under vacuum at 50ºC. Extraction yields (% w/w) are given in Table 4. The dry extracts were dissolved in either methanol or dimethyl sulphoxide (DMSO) to prepare the stock solutions used for all determinations.

Table 4: Extraction yields (%, w/w) of PEE and successive extractions to obtain HEX, CHL and AQ sub-extracts.

	Yield (g DW/100 g propolis)		
	CAT-I	CAT-II	CAT-III
PEE	80	94	72
HEX	13	6	6
CHL	42	83	63
AQ	8	6	6

Total phenolic content: This was determined using the Folin–Ciocalteu (F-C) reagent [11]. Total phenolic content was expressed as mg of gallic acid equivalents (GAE)/g propolis.

Total flavonoid content determination: Total flavonoid contents in the extracts and sub-extracts were determined [12]. The results were expressed as g of quercetin equivalent (QE)/propolis.

Chemical profile by HPLC: PEE samples were dissolved in methanol (2 mg DW/mL) and filtered through a 0.45 µm nylon filter (Biopore, Germany) prior to injection of 20 µL. The HPLC analysis was performed using a Waters HPLC system with PDA detection equipped with a Bridge C-18 column (250 mm x 4.6 mm i.d., 5 µm). A binary gradient elution system composed of 2 solvents (solvent A: 5% acetic acid and solvent B: methanol) was used, starting with 30% B (0-15 min), 40% (15-30 min), 50% (30-45 min), 60 (45-65 min), 75% (65-80 min), 90% (80-90 min), 100% (90-100 min), with a flow rate of 0.8mL/min. Detection was carried out from 200 to 400 nm. The chromatograms were extracted at 268 nm and identification was made by comparison of retention times and UV spectral data with commercial standards and compounds obtained by Vera *et al.* [8c].

Antioxidant activity

DPPH free radical scavenging activity: The DPPH$^{\bullet}$ (1,1-diphenyl-2-picrylhydrazyl) scavenging assay was carried out according to Vivot *et al.* [13], with slight modifications. Reaction mixtures containing different concentrations of PEE and sub-extracts dissolved in DMSO (5 µL) and 95 µL of DPPH$^{\bullet}$ solution (0.125 mg/mL) in a 96-well microtiter plate, were incubated at 25°C for 30 min. Absorbance was measured at 550 nm in a microplate spectrophotometer (BioTek EL808). Scavenging activity of different propolis samples was determined by comparison with a DMSO control. SC_{50} values denote the sample concentration required to scavenge 50% DPPH$^{\bullet}$.

β-Carotene bleaching assay: Antioxidant activity was determined according to the β-carotene bleaching method [14]. The initial absorbance at 470 nm was registered at zero time (t_0) and for 120 min. Antioxidant activity (AA%) was calculated as percent inhibition relative to control using the following equation:

$AA\% = [(R_{control} - R_{sample})/ R_{control}] \times 100$

where $R_{control}$ and R_{sample} are the bleaching rates of β–carotene in the reactant mix without antioxidant and in the presence of the extracts, respectively.

IC_{50} values denote the µg DW/mL required to inhibit 50% β–carotene bleaching.

Antimicrobial activity

Culture media and microbial identification: Clinical isolates of *Staphylococcus aureus* (*n* = 3; F 7, F16, F30), *Enterococcus faecalis* (*n* = 3; F203, F208, F226), *Escherichia coli* (*n* = 2, F301), *Klebsiella pneumoniae* (*n* = 1, F 364), *Proteus mirabilis* (*n* = 1, F359), *Enterobacter cloacae* (*n* = 1, F302), and *Morganella morganii* (*n* = 1, F339), were obtained from clinical samples supplied by Hospital Dr Nicolás Avellaneda, San Miguel de Tucumán, Tucumán, Argentina. The following reference strains were included in the study: *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 35218, and *Klebsiella pneumoniae* ATCC 700603. The strains were identified by the use of biochemical profiles according to the recommendations of the Manual of Clinical Microbiology [15]. Before testing, the suspensions were transferred to trypticase soy agar supplemented with 5% sheep blood (Difco) and aerobically grown overnight at 35°C. Individual colonies were isolated and suspended in 5 mL of 0.9% NaCl solution. The inocula were prepared by adjusting the turbidity of the suspension to match the 0.5 McFarland standards and diluted in CAMHB (cation-adjusted Müller–Hinton broth) in order to achieve the adequate inocula in each case. The cell number in CAMHB was estimated using a serial dilution technique, according to the recommendations of the CLSI [16]. MIC values were also determined for different commercial antibiotics. Resistance was defined for each case: levofloxacine (Lvx, MIC≥8µg/mL), piperacillin/tazobactam (Tzp, MIC≥128µg/mL), imipenem (Ipm, MIC > 16µg/mL), meropenem (Mem, MIC > 16µg/mL) , ceftriaxone (Cro, MIC > 128 µg/mL), cefotaxime (Ctx, MIC > 128µg/mL), cefuroxime (Cxm, MIC≥32 µg/mL), cefepime (Fep, MIC≥32 µg/mL), for Gram-negative bacteria and oxacillin (Oxa, MIC>16 µg/mL), streptomycin (Str, MIC≥300 µg/mL), ampicillin (Amp, MIC > 64 µg/mL), methicillin (Met, MIC > 16 µg/mL), gentamycin (Gen, MIC > 100 µg/mL) and vancomycin (Van MIC>6 µg/mL) for Gram-positive bacteria. The antimicrobial agents were supplied by Sigma Chemical Co (USA) and Laboratorio Britania S.A., Argentina. All experiments were carried out in triplicate.

Serial agar macrodilution method: The same volume (1 mL) of serial two-fold dilution of each extract and sub-extract was added to 9 mL of MHA (Müeller–Hinton agar) medium. After cooling and drying, the plates were inoculated in spots with 2 µL of each bacterial cell suspension (10^4 CFU) and incubated aerobically for

18–24 h at 35°C. A growth control of each tested strain was included. Controls of ethanol 80% were carried out. MIC_{100} was defined as the lowest concentration of extract at which no colony was observed after incubation.

Bioautographic assays: *In situ* comparisons were made of antimicrobial activity of PEE from CAT-I (1), CAT-II (2) and CAT-III (3), 2',4'-dihydroxychalcone (4) and 2',4'-dihydroxy-3'-methoxychalcone (5) on silica gel F_{254} plates [3b].

Statistical analysis: Results are mean values obtained from at least 3 independent experiments. The values were calculated using GraphPad Prism 5.0 software.

Acknowledgments - This research was partially supported by grants from Consejo de Investigación de la Universidad Nacional de Tucumán (CIUNT, Tucumán, Argentina), INTA-Famaillá and Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET; Buenos Aires, Argentina).

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