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Development of a Bioproduct for Medicinal Use with Extracts of *Zuccagnia*-type Propolis

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Propolis with a botanic origin in *Zuccagnia punctata* Cav. (Fabaceae), a plant species widely distributed in the Argentinean Monte region, was used to produce a standardized propolis extract by biological activity and chemical composition. In this paper the effect on microorganisms isolated from otitis was determined. The extract was active against different *Staphylococcus* strains with MIC and MBC values (75 and 150 µg DE/mL, 200 and 600 µg DE/mL, respectively) and with MIC values of 800 µg DE/mL against *Proteus mirabilis*. A pharmaceutical product, ear drops containing this extract as bioactive was developed. Stability of ear drops was evaluated for 6 months, and their biological activity, physical and phytochemical properties were preserved. Ear drops showed inhibitory activity on pro-inflammatory enzymes such as lipoxygenase (IC_{50} values 90 and 100 µg DE/mL), free radical-scavenging effect (SC_{50} values 23 and 30 µg DE/mL), antihelmintic (LC_{50} values 70 and 71 µg DE/mL), anti-candida (400 µg DE/mL) and antibacterial activity against Gram-positive bacteria (200 µg DE/mL) at zero time and during six month-storage, respectively. The content of two chalcones, chemical markers of *Zuccagnia*-type propolis was quantified and its level was maintained while stored at room temperature. Its toxicity was also assayed. Our results are interesting since the extract and ear drops of *Zuccagnia*-type propolis developed could be promising for use in alternative medicine or phytomedicine as antibacterial, anti-candida, antihelmintic, anti-inflammatory and antioxidant.

Keywords: *Zuccagnia*-type propolis, 2′,4′-Dihydroxychalcone, 2′,4′-Dihydroxy-3′-methoxychalcone, *Zuccagnia punctata* Cav., Ear drops, Otitis externa, Antibacterial activity.

Propolis is a natural product produced by bees (*Apis mellifera*) from plant resins and exudates, and its color, consistency, chemical composition and biological activities are intimately related to the flora around the hive and to the season during which it is collected [1, 2a]. Propolis from Northwestern Argentina (Salta, Tucumán, Santiago del Estero and Catamarca) showed several biological properties that make it interesting for its medicinal use [2a-i].

An eco-region of Northwestern Argentina, the “Monte” is a warm and arid zone, where the predominant vegetation is the "jarillal", an association of *Zuccagnia punctata* Cav. (Fabaceae), *Larrea divaricata* Cav. (Zygophyllaceae), and *Larrea cuneifolia* Cav. (Zygophyllaceae). Propolis from the “Monte” was defined as “*Zuccagnia*- type propolis” according to its botanical origin [2e, 2i]. This propolis showed biological properties such as anti-inflammatory, scavenging free-radical, antimicrobial on human pathogens, antihelmintic and antimutagenic. In addition, this propolis was phytochemically characterized [2b-2e, 2g, 2h]. The major chemical components of this propolis are 2',4'-dihydroxy-3'-methoxychalcone (DHMC) and 2',4'-dihydroxychalcone (DHC) that were also found in *Zuccagnia punctata* resins, these bioactive chalcones being considered quality chemical markers of *Zuccagnia*-type propolis.

Otitis externa is an inflammatory pathology associated to oxidative stress and bacterial infection (*Staphylococcus aureus*, *Staphylococcus intermedius*, *Pseudomonas aeruginosa*, *Proteus mirabilis*), fungal infection (*Candida albicans*, *Malassezia pachydermatis*) or parasitic infection (*Octodectes cynotis*) in the external ear canal [3a-b]. These microorganisms showed high degree of resistance to commercial products in topical therapy [3-c]. For this reason, multipurpose products are frequently indicated particularly as a first-line treatment [3d].

The aim of this work was to determine the chemical composition of this propolis and its antimicrobial activity against microorganisms isolated from canine otitis and to formulate ear drops containing this propolis extract for veterinary use.

Table 1: Minimum inhibitory concentration (MIC), minimum bacterial concentration (MBC) of *Zuccagnia* -type propolis extract.

Microorganisms	MIC/MBC (µg DE/mL)	Phenotype
Staphylococcus aureus 29213 (ATCC)	75.0±25.0[a]/300.0±100.0[a]	
Staphylococcus haemoliticus (A1)	150.0±50.0[a]/600.0±200.0[a]	$Ery^{S}Cli^{S}T^{S}Van^{S}Gen^{S}Cip^{R}Oxa^{S}Rfa^{S}TMS^{S}$
Staphylococcus aureus (A2)	150.0±50.0[a]/300.0±100.0[a]	$Ery^{S}Cli^{S}T^{S}Van^{S}Gen^{S}Cip^{S}Oxa^{S}Rfa^{S}TMS^{S}$
Staphylococcus aureus (A3)	100.0±0[a]/200.0 ±0[a]	$Ery^{S}Cli^{S}T^{S}Van^{S}Gen^{S}Cip^{S}Oxa^{S}Rfa^{S}TMS^{S}$
Staphylococcus epidermidis (A5)	150.0±50.0[a]/600.0±200.0[a]	$Ery^{S}Cli^{S}T^{R}Van^{S}Gen^{S}Cip^{S}Oxa^{S}Rfa^{S}TMS^{R}$
Staphylococcus aureus (A12)	150.0±50.0[a]/200.0±0[a]	$Ery^{S}Cli^{S}T^{S}Van^{S}Gen^{S}Cip^{R}Oxa^{S}Rfa^{S}TMS^{R}$
Staphylococcus aureus (A14)	150.0±50.0[a]/600.0±200.0[a]	$Ery^{S}Cli^{S}T^{S}Van^{S}Gen^{S}Cip^{S}Oxa^{S}Rfa^{S}TMS^{R}$
Staphylococcus aureus (A18)	150.0±50.0[a]/300.0±100.0[a]	$Ery^{S}Cli^{S}T^{S}Van^{S}Gen^{S}Cip^{S}Oxa^{S}Rfa^{S}TMS^{R}$
Staphylococcus aureus (A20)	150.0±50.0[a]/300.0±100.0[a]	$Ery^{S}Cli^{S}T^{S}Van^{S}Gen^{S}Cip^{S}Oxa^{S}Rfa^{S}TMS^{R}$
Staphylococcus aureus (A22)	75.0±25.0[a]/600.0±200.0[a]	$Ery^{R}Cli^{R}T^{R}Van^{S}Gen^{R}Cip^{S}Oxa^{R}Rfa^{S}TMS^{R}$
Proteus mirabilis (A6)	800.0±0[b]/R	$Am^{S}Cip^{S}Cef^{S}Cot^{S}Cli^{S}Ipm^{S}Mpm^{S}Nit^{R}Fox^{S}Ctx^{S}Amo+Clav^{R}$
Proteus mirabilis (A27)	800.0±0[b]/R	$Am^{S}Cip^{S}Cef^{S}Cot^{S}Cli^{S}Ipm^{S}Mpm^{S}Nit^{R}Fox^{S}Ctx^{S}Amo+Clav^{R}$

The values (mean ± SD, n = 3) in the same column followed by the same letter are not significantly different (Tukey's p> 0.05). Resistant (R), Susceptible (S), Erythromycin (Ery), Clindamycin (Cli), Oxacillin (Oxa), Gentamicin (Gen), Vancomycin (Van), Teicoplanin (T), Trimethoprim/ Sulfamethoxazole (TNS), Ciprofloxacin (Cip), Rifampicin (Rfa), Ampicillin (Am), Cephaloridine (Cef), Clotrimazole (Cot), Clindamycin (Cli), Imipenem (Ipm), Meropenem (Mpm), Nitrofurantoin (Nit), Cefoxitin (Fox), Cefotaxime (Ctx), Amoxicillin+Clavulanic acid (Amo+Clav).

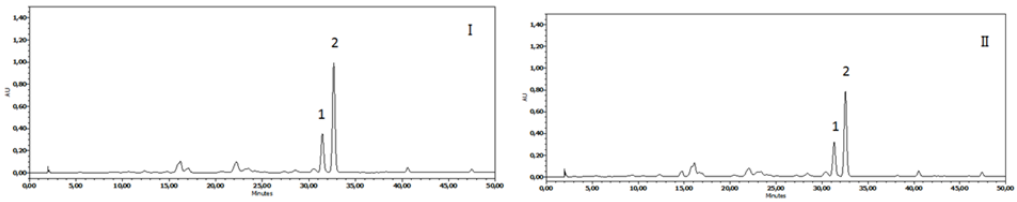

Figure 1: HPLC profiles of ear drops containing standardized *Zuccagnia*-type propolis extract I) Time= 0, II) Time = six month. Peak (1) 2′,4′-dihydroxy-chalcone. Peak (2) 2′,4′-dihydroxy-3′-methoxychalcone.

Table 2: Physical, chemical and biological properties of ear drops at 1 day, 3 and 6 months at room temperature.

Physical properties	1 day	3 months	6 months
Viscosity (mPa s)	36.45±0.58[a]	37.03±0.29[a]	37.05±0.39[a]
Density (g/cm^3)	1.0405	1.0472	1.0488
pH	5.42	5.36	5.47
Color	I	I	I
Aroma	I	I	I
Appearance	I	I	I
Phytochemical characterization			
Phenolic compounds (mg GAE/mL)	10.9±0.4[a]	8.4±0.5[b]	7.7±0.4[b]
flavones and flavonols (mg QE/mL)	4.8±0.6[a]	3.5±0.1[b]	3.6±0.2[b]
flavonoids phenolic (mg GAE/mL)	4.7±0.1[a]	3.3±0.1[a]	3.3±0.1[b]
non-flavonoids phenolic (mg GAE/mL)	7.1±0.1[a]	4.3±0.1[a]	4.3±0.1[b]
DHC (mg/mL)	3.54±0.06[a]	3.44±0.04[a]	3.40±0.06[a]
DHMC (mg/mL)	4.50±0.08[a]	4.01±0.06[b]	3.83±0.06[b]
Biological properties			
S. aureus (A2)			
MIC (µgDE/mL)	200.0±50.0[a]	200.0±50.0[a]	200.0±50.0[a]
C. albicans			
MIC (µgDE/mL)	400.0±50.0[a]	400.0±50.0[a]	400.0±50.0[a]
Antihelmintic activity on *C. elegans* LD_{50} (µgDE/mL)	71.0±5.2[a]	70.0±2.3[a]	71.0±3.0[a]
Control Levamisol LD_{50} (µgDE/mL)	4.7±0.3		
ABTS$^{\bullet+}$ scavenging IC_{50} (µgDE/mL)	23.0±4.8[a]	33.0±2.0[a]	30.0±2.5[a]
Control Quercetin IC_{50} (µgDE/mL)	3.6±0.2		
LOX inhibition IC_{50} (µgDE/mL)	90.0±7.3[a]	100.0±3.2[a]	100.0±5.4[a]

The values (mean ± SD, n = 3) in the same line followed by the same letter are not significantly different (Tukey's p> 0.05). Physic appearance: (I) without changes, (II) slightly precipitated; (III) precipitated. Color and aroma: (I) without changes, (II) slightly modified; (III) modified.
Gallic acid equivalent (GAE), Quercetin equivalent (QE). Dry extract (DE).

The phytochemical composition of the propolis extract was determined. The propolis extract contained 4 g of dry extract (DE) per 100 mL (4%). A high level of total phenolic, flavonoid phenolic, non-flavonoid phenolic compounds, (592±15 mgGAE/g DE; 236±10 mgGAE/gDE; 357±13 mg GAE/g DE, respectively) and flavones and flavonols content (276±5 mgQE/gDE) were found.

In a previous work, we demonstrated several biological properties of *Zuccagnia*-type propolis such as anti-candida, antihelmintic, antioxidant and anti-inflammatory effect [2h]. In this study, the characterization of this propolis was followed. The antimicrobial activity of propolis extract was evaluated against bacteria obtained from dogs with otitis externa. The antibiotic resistant isolates included Gram positive strains (*S. haemolyticus*, *S. aureus*, *S. epidermidis*) and Gram negative strain (*P. mirabilis*) (Table 1). Propolis extract showed antibacterial activity against all Gram positive bacteria with MIC values between 75 and 150 µg DE/mL and MBC values between 200 and 600 µg DE/mL. The most resistant strain was *P. mirabilis* with MIC values of 800 µg DE/mL (Table 1). *Zuccagnia*-type propolis extract was more active against Gram positive bacteria than Brazilian Green propolis [3a].

Ear drops containing *Zuccagnia*-type propolis extract (2 g dry extract) was formulated by using 100 mL of different systems. The system 4 was selected as vehicle, because in this system the propolis extract showed the best solubility and stability. Physicochemical properties (physical appearance, color, aroma, viscosity, pH and density) of formulated ear drops were maintained constant for 6 months (Table 2).

In addition, HPLC fingerprints of ear drops showed the two major compounds considered biomarkers of *Zuccagnia*-type propolis [2h-2i], 2′,4′-dihydroxychalcone (DHC) and 2′,4′-dihydroxy-3′-methoxychalcone (DHMC). The ear drops showed a decrease of phenolic compounds and flavonoids content of about 30% and 25% respectively, after six months at room temperature, with respect to the values obtained to extract and ear drops at zero time (Table 2). However, concentration of DHC remained constant (3.54±0.06 to 3.40±0.06 mg/g DE) and DHMC (4.50±0.08 to 3.83±0.06 mg/g DE) decreased only 15% after six months. Our results revealed the stability of this bioactive product.

In vitro biological activity of ear drops was evaluated during six months and it was able to inhibit the growth of *S. aureus*, *C. albicans* (MIC values of 200 µg DE/mL and 400 µg DE/mL, respectively), showed antihelmintic effect on *C. elegans* (LC_{50} values between 70 and 71 µg DE/mL), antioxidant activity (SC_{50} values between 23 and 30 µg DE/mL) and ability to inhibit the activity of pro-inflammatory enzyme LOX (IC_{50} values 90 and 100 µg DE/mL) (Table 2). Pharmacological activities of ear drops were maintained constant for at least 6 months. Probably, the high antioxidant activity demonstrated could be responsible for the conservation of formulation during the storage. In this study a multi-purpose formulation was developed and characterized. Taking into account the biological properties demonstrated, it is necessary to know about the toxicity of active principles. For this reason, the acute toxicity of ear drops was evaluated. The formulation was not mutagenic on *S. typhimurium* TA98 and TA 100 until concentrations of 500 µg DE/mL and 125 µg DE/mL respectively, with a ratio of mutagenicity lower than 1.5 at these concentrations.

The ear drops containing *Zuccagnia*-type propolis extract keep their chemical, physical and biological properties for 6 months and do not show *in vitro* toxicity indicating the potential application of this product as phytomedicine in veterinary treatment as a multi-purpose product (anti-inflammatory, antioxidant, antibacterial, antifungal and antihelmintic), for the treatment of otitis. Our results would indicate that this product could be also applied in human medicine as antiinflammatory. A more thorough analysis including *in vivo* assays would be mandatory in order to establish its clinical application.

Experimental

Propolis samples: Propolis samples were provided by INTA-PROAPI from hives located at the Agrotechnical School in the Monte Region, Tucumán, Argentina (26°35'S, 65°55'W). The samples were collected in December 2012 and are representative of the collection time of raw material for phytotherapeutic purposes. Samples were weighed and frozen at -20 ºC until processing.

***Extraction of Zuccagnia-type propolis by maceration*:** 20 g of propolis were mixed with 250 mL of 80% ethanol (v/v) at room temperature for 7 days. After extraction, the mixture was filtered through Whatman N° 4 paper and an ethanolic extract was obtained. The extract was concentrated under reduced pressure at 40 °C, lyophilized to obtain a dry extract (DE) and frozen at -20 °C until further processing.

Phytochemistry characterization: Total phenolic compound (TP) concentration in propolis samples was determined spectrophotometrically according to the Folin-Ciocalteu colorimetric method [4a]. The content of non-flavonoid phenolics (NFP) and flavonoid phenolics (FP) was determined spectrophotometrically according to [4b]. The results were expressed as mg of gallic acid equivalent per g of dry (mg GAE/g). Flavone and flavonol concentrations were determined by aluminum chloride complex formation [4c]. Results were expressed as mg of quercetin equivalents per g of dry extract (mgQE/g).

HPLC-DAD fingerprints and quantification of chalcones: Propolis extract and ear drops were analyzed by HPLC coupled to a diode array detector. The HPLC system consisting of a Waters 1525 Binary HPLC Pumps system, a Waters 2998 photodiode array detector (PDA) and a XBridge™ C18 column (4.6 x 150 mm, 5 µm; Waters corporation, Milford, MA). The HPLC analyzes were performed by using a linear gradient consisting of methanol (solvent B) and acetic acid 9% (solvent A) (conditions: 25% B–75%A from 0 to 10 min and kept at 45% B-55% A from 10 to 20 min, 70% B–30% A from 20 to 40 min, 75% B–25% A from 40 to 50 min, 100% B from 50 to 55 min) to separate the components of ear drops. Flow rate was set at 0.8 mL/min. Data collection was carried out by using the Empower ™ software. The presence of the *Zuccagnia*-type propolis biomarkers DHC and DHMC were confirmed by UV spectrometry (220–500 nm) and coinjections with standard commercial chalcones (Indofine SRL). Chalcone concentrations were quantified by using calibration curves with commercial reagents.

***Formulation and stability studies of ear drops*:** Ear drops were prepared by using five vehicle systems (systems 1 and 2, ethanol: propylene glycol, 1:1 and 1:5 (v/v) respectively; systems 3, 4 and 5, ethanol: glycerol: propylene glycol 1:1:1; 2:2:4 and 1:2:4 (v/v/v), respectively). Dry extract of propolis (2% w/v) was included as an active principle. A negative control of ear drops was prepared without extract. The stability was evaluated at the initial time, three and six months

Physical stability: A digital pH meter (ADWA AD1030) was used to determine the pH of ear drops. Density and viscosity of ear drops were determined by using a digital densitometer (Anton Paar model DMA 45) and a viscometer type Ubblohde (Schott Gerate, model AVS 400) and expressed as g/cm^3 and mPa s, respectively. All measurements were made at room temperature.

Phytochemistry stability of ear drops: Phenolic compound and flavonoid content was determined in ear drops according to [3a-c]. The content of chemical markers (DHC and DHMC) was determined by HPLC-DAD by using calibration curves with commercial reagents.

Antimicrobial activity evaluation

Culture media and microorganisms: The microorganisms used in this study were isolated from dogs with canine otitis from a veterinary clinic of San Miguel de Tucumán, Tucumán, Argentina. The strains tested were: *Staphylococcus haemoliticus* (n=1) (Lipron-A1), *Staphylococcus aureus* (n=7) (Lipron-A2, A3, A12, A14, A18, A20 and A22), *Staphylococcus epidermidis* (n =1) (Lipron-A5), *Proteus mirabilis* (n=2) (Lipron-A6 and A27), *Staphylococcus aureus* ATTC 29213 and *Candida albicans (Lipron-B)*.

***Broth microdilution method on S. aureus*:** The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of extracts and ear drops (25 to 1000 µg DE/mL) were determined according to [5a]. The final inoculum was 5×10^5 CFU/mL. The inoculated microplates were aerobically incubated at 35 ºC for 20 h. MIC was determined as the first well where no pellet appeared. The resistance profile to commercial antibiotics was also determined.

***Broth microdilution method on C. albicans*:** The antifungal effect of extracts and ear drops was evaluated against *Candida albicans* strain. The fungi inocula were prepared by adjusting the turbidity of culture suspension to 1-5 x 10^3 CFU/mL as described by [5b]. The ear drops were diluted to reach concentrations between 50 and 1000 µg DE/mL in the wells. The inoculated microplates were aerobically incubated at 28ºC for 48 h. Fungal growth was indicated by the presence of turbidity and a pellet on the well bottom. MIC was determined as the first well where no pellet appeared [5b].

***Free radical-scavenging activity*:** The antioxidant capacity assay was carried out by the ABTS cation radical ($ABTS^{\bullet+}$) method as described by [5c] with different concentrations of ear drops (10-50 µg DE/mL). SC_{50} values (sample concentration required to scavenge 50% of ABTS free radicals) were calculated.

Anti-inflammatory activity: Inhibition of lipoxygenase (LOX) activity was determined according to [5d], based on the enzymatic oxidation of linoleic acid to the corresponding hydroperoxide. The extract concentration necessary to inhibit 50% of enzymes was determined (IC_{50}).

***Antihelmintic activity*:** Nematode strains (*Caenorhabditis elegans*) N2 (Bristol) was obtained from the Genetics Center (University of Minnesota, Minneapolis, MN) and maintained at 20 °C on Nematode Growth Medium (NGM) supplemented with *Escherichia coli* strain OP50 (uracil requiring bacterial strain). The antihelmintic assay was determined according to [5e]. Results were expressed as percent of control.

Toxicity evaluation

Mutagenicity assay - Ames Test: The genotoxic effect was examined on *Salmonella typhimurium* strains TA98 and TA100 by Maron & Ames [6]. Different concentrations of propolis extract (125; 250; 500 µg DE/plate) were used. DMSO was used as negative control and the mutagens 4-nitro-o-phenylenediamine (4-NPD) was used as positive control (10 µg/plate). All tests were performed twice in duplicate. Results were expressed as number of revertant/plate and the mutagenicity ratio was also calculated (MR), which is the ratio of the number of plate induced revertants (IR) and number of spontaneous revertants on a control plate (SR),
RM = IR/SR. The ratio of mutagenicity: No mutagenic (less than 1.5), slightly mutagenic (1.5-2), mutagenic (more than 2).

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