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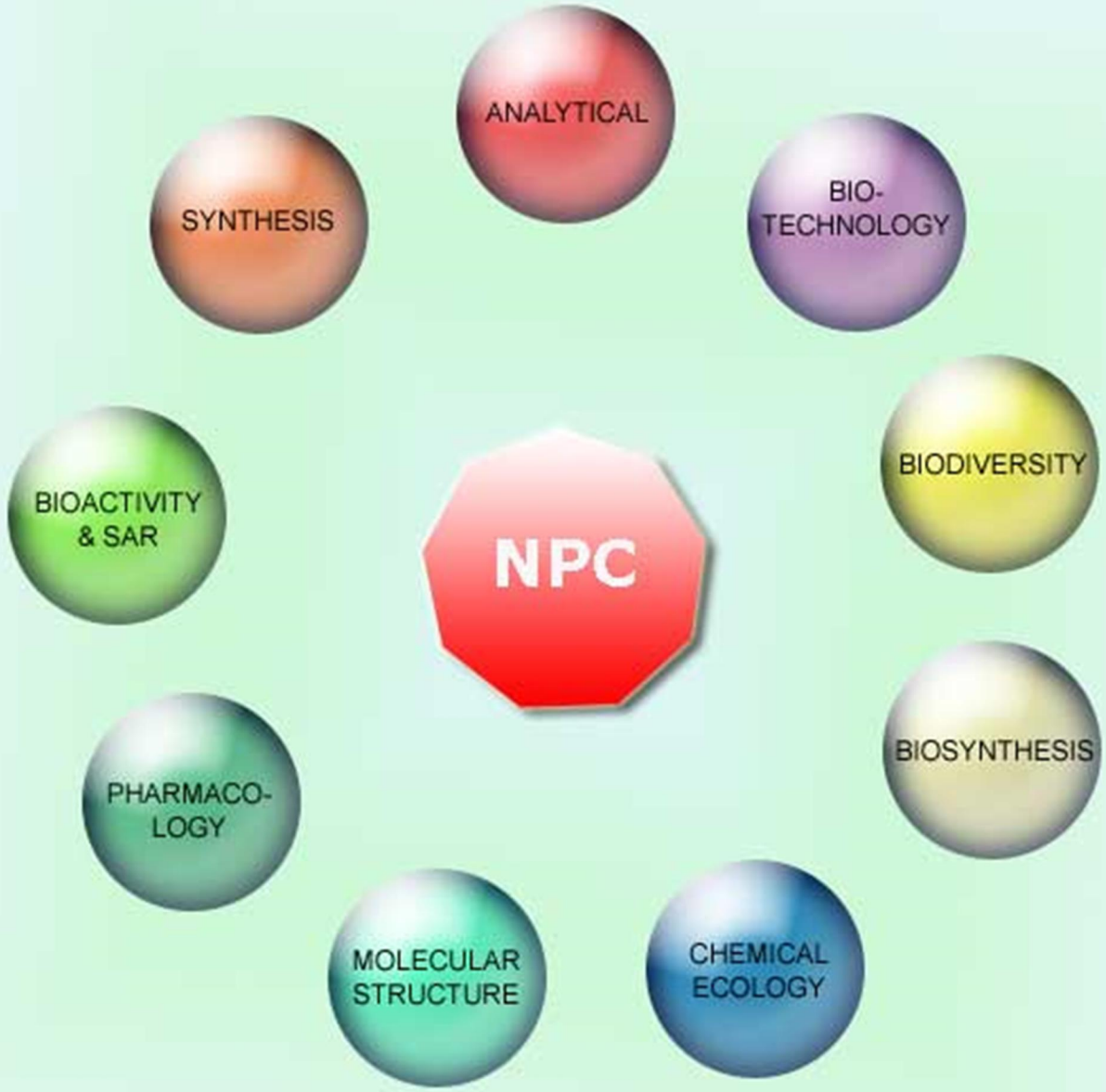

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Determination of Botanical Origin of Propolis from Monte Region of Argentina by Histological and Chemical Methods

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Propolis production by honey bees is the result of a selective harvest of exudates from plants in the neighborhood of the hive. This product is used in Argentina as a food supplement and alternative medicine. The aim of this study was to determine the botanical origin of propolis from the arid regions of Monte of Argentina using rapid histochemical techniques and by comparison of TLC and HPLC-DAD chromatographic profiles with extract profiles obtained from *Zuccagnia punctata*, *Larrea divaricata* and *Larrea cuneifolia*, plant species that grow in the study area as a natural community named "jarillal".

Microscopical analysis revealed the presence of several *Z. punctata* structures, such as multicellular trichomes, leaflets, stems and young leaves. Remarkable was the richness of the propolis in two bioactive chalcones, also present in *Z. punctata* resin; these compounds can be regarded as possible markers for propolis identification and justify its use as a dietary supplement, functional food and medicinal product. This study indicates that the source of resin used by honey bees to produce propolis in the Monte region of Argentina is only *Z. punctata*, a native shrub widespread in this phytogeographical region, while other more abundant species (*L. divaricata* and *L. cuneifolia*) in the region were not found, indicating that this propolis could be defined as a mono-resin, type-*Zuccagnia*.

Keywords: Argentine propolis, Botanical origin, *Zuccagnia punctate*, Chemo-histological techniques, HPLC-DAD, Chalcones.

Propolis is a resinous and aromatic material that bees produce from plant secretions (resin and volatiles constituents) and/or by using cut fragments of vegetative tissues mixed with metabolic products secreted by the insect (beeswax). Bees use propolis for diverse purposes, to close cracks and to coat the inside of the hive walls providing an aseptic internal environment. It is also used to coat the bodies of animals that have been introduced into the hive, avoiding decomposition [1].

Propolis is used as a food supplement and phytomedicine. In Argentina, propolis is included in the food code as an ingredient of sweets, honey, ethanolic extracts and as dietary supplements [2].

The chemical composition of propolis is extremely variable as the vegetation at the site of collection determines its chemical diversity [3a-c]. Probably few plant species contribute as major resin sources. Some investigations suggest common botanical sources and, consequently, similar chemical profiles for large geographical areas.

Bankova *et al*. [4a] classified propolis into two groups: temperate and subtropical. Propolis from temperate regions in Europe, North America and non-tropical regions of Asia, derived from poplars (*Populus* spp.) and birches (*Betula* spp.), have flavonoids, flavanones, flavones, phenolic acids, and their esters as main bioactive compounds, while propolis from tropical regions, like Brazil, Venezuela and Cuba, has prenylated *p*-coumaric acids, diterpenes and prenylated benzophenones as major bioactive compounds, with low flavonoids content [1, 4b-e].

Clusia minor was described as the botanical origin of Venezuela propolis [4f]. In Cuba, a brown-Cuban propolis from floral resin of *Clusia rosea*, and red-Cuban propolis from *Dalbergia* spp. were described. The major compounds are polyprenylated benzophenones and isoflavonoids, respectively [4g].

Brazilian propolis was classified into 12 types according to geographical origin, chemical composition and plant source [4b]. The most popular are green or Alecrim propolis, originating from *Baccharis dracunculifolia*, with artepillin C as the characteristic constituent, and red propolis, originating from resinous exudates of *Dalbergia ecastophyllum*. Propolis from Brazil also arises from *Hyptis divaricata* and *Populus nigra* [4b]. According to Koenig [4h] and Montenegro *et al*. [4i] the most frequent botanical origins of propolis from central Chile are *Salix humboldtiana* and *Eucalyptus globulus*.

Propolis from Northern Argentina (Santiago del Estero, Tucumán, Salta, Jujuy, Misiones and Catamarca provinces) was classified according to its chromatographic profiles into three types [5a]. Propolis collected from the Monte phytogeographical region in Argentina presented chalcones as major constituents [5b-e]. Recently it was demonstrated that these chalcones are located on the foliar surfaces and flowers of *Zuccagnia punctata* [5f-g]. Due to their chalcones content, propolis from the Monte region in Tucumán and Catamarca showed biological activity, such as antifungal, antibacterial, antioxidant, anti-inflammatory and antimutagenic [5a-e, 5h-m].

The aim of this research was to investigate by histological techniques, HPLC-DAD and TLC the botanical origins of propolis samples collected from the arid regions of Argentina belonging to the phyto-geographical province of Monte, province of Tucumán.

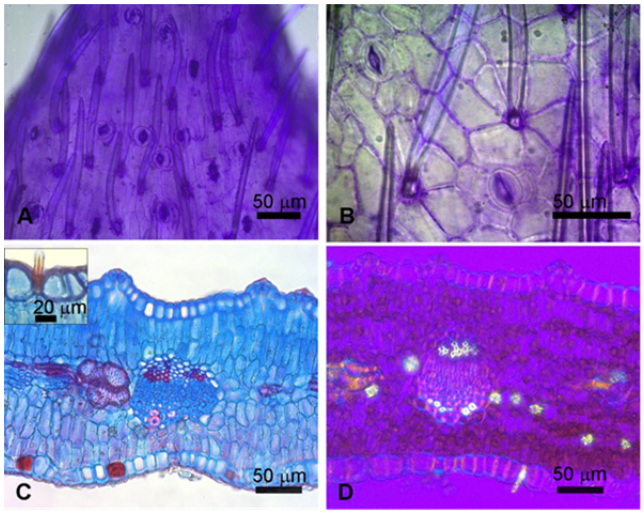

Figure 1: Plant material of *Larrea divaricata* A) Upper epidermal cells B) Anomocytic and cyclocytic stoma C) Cut of leaves and detail of the insertion of unicellular non-glandular trichome in the insert D) Free druses observed in the mesophyll under white and polarized light.

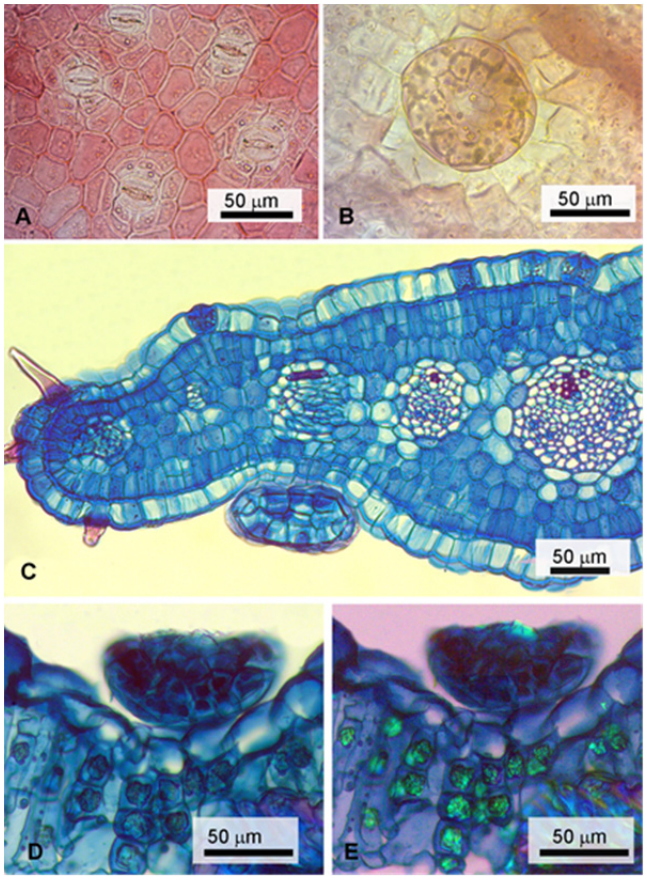

Figure 2: Plant material of *Zuccagnia punctata* A) Adaxial epidermis with stoma B) Multicellular capitate glandular trichomes C) Cut of leaves showing non-glandular marginal unicellular trichomes and capitate glandular trichomes in crypts D, E) Free druses observed in the palisade parenchyma under white and polarized light.

Botanical identification by microscopic analysis of fragments of leaves or other debris left by the bees during harvesting of plant exudates is one of the techniques used to determine the quality of propolis [6a]. The phytogeographical region of Monte in Northern Argentina is a temperate and arid zone, where the predominant vegetation is xerophytic and hallophytic shrub-steppe. The climax

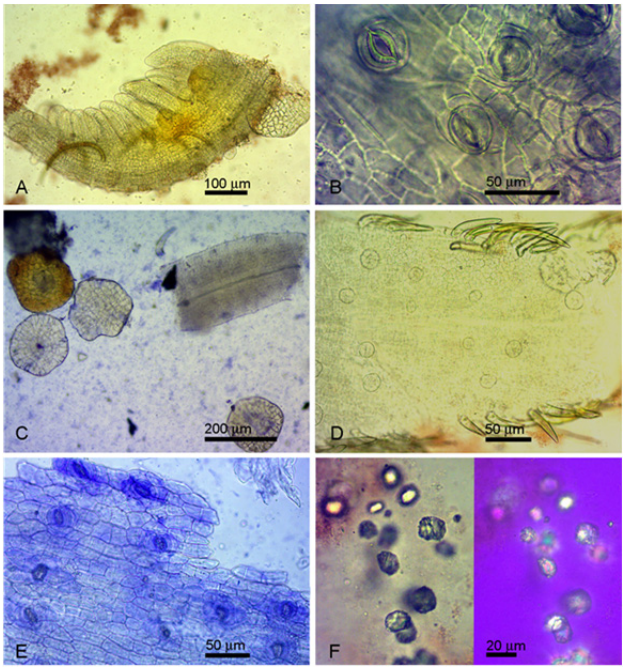

Figure 3: Botanical studies of propolis samples. Plant material identified as *Zuccagnia punctata* A) Leaf, raquis and leaflets B) Epidermal cells with straight anticlinal walls, thick and striated cuticle. Cyclocytic stoma C) Multicellular capitate, glandular trichomes D) Unicellular clothing trichomes, thick cuticle and blunt apex, located on the leaf margins E) Stems with rectangular epidermal cells. Anomocytic stoma F) Free druses observed under white and polarized light.

community of Monte is the "jarillal", an association of *Z. punctata, L. divaricata, and L. cuneifolia* [6b].

Aerial parts of *Z. punctata*, *L. cuneifolia* and *L. divaricata* were analyzed by microscopic techniques (Figure 1 and 2). Microscopic analysis of propolis samples collected from Amaicha del Valle (semiarid valley, belonging to the phytogeographical province of Monte), showed several structures from *Z. punctata*, such as multicellular trichomes, leaflets, stems, young leaves (leaflets and rachis) and leaf primordia that clearly respond to the epidermal characteristics described for this species [6c-e]. Leaf primordium was observed with sub-opposite nanophyll leaflets (445.3 ± 258.1 mm long, 151.6 ± 77.1 mm lat), acuminate apex, rounded and symmetrical base and entire margin (Figure 3 A and D). Cyclocytic stoma (rarely paracytic or anomocytic) in both epidermal surfaces (31.49 ± 3.07 µm long and 29.80 ± 2.65 µm lat) (Figure 3 B) were observed. The epidermal cells showed straight anticlinal walls, and thick and striated cuticle (Figure 3 B). In foliar and rachis surfaces, sunken capitates pluricellular glandular trichomes (spherical or oval) (141.6 ± 96.1 µm of diameter) were observed (Figure 3 A, C and D). Non-glandular trichomes were arranged free on the adaxial base of the foliar surface and/or in the foliar and rachis margins (368.44 and 44.21 µm) (Figure 3 D). Free druzes (crystals of calcium oxalate) were evidenced with polarized light (Figure 3 F).

The stems showed rectangular epidermal cells with their major axis parallel to the organ surface and straight anticlinal walls. Anomocytic stoma (28.13 ± 2.13 long and 27.35 ± 3.21 µm lat) were observed (Figure 3 E). Large deviations in mean values in the longitude of leaflets and diameter of glandular trichomes could be attributed to different maturation stages. Structures of other plant species were not found.

HPLC-DAD, TLC and RPTLC profiles revealed that propolis ethanolic extracts (PEE) and *Z. punctata* ethanolic extract are

similar to each other, but different to extracts of *L. divaricata* and *L. cuneifolia*, two jarillas species that grow alongside *Z. punctata* in the Monte region (Supplementary material).

Predominant components of the propolis and *Z. punctata* extracts were two bioactive chalcones, 2', 4'-dihydroxychalcone (**1**) and 2', 4'-dihydroxy- 3'-methoxychalcone (**2**) (Table 1). A previous report described the chemical composition (cinnamic acid, caffeic acid prenyl ester, caffeoyldihydrocaffeate and caffeic acid 3,4-dihydroxyphenethyl ester, liquiritigenin, 2', 4'-dihydroxychalcone and 2', 4'-dihydroxy-3'-methoxychalcone) of these propolis samples [6c]. Nordihydroguaiaretic acid (**3**) was the major constituent identified in extracts of *L. divaricata* and *L. cuneifolia*. This compound was not identified in the propolis ethanolic extract (Table 1).

Our results suggest that the botanical origin of propolis obtained from the Monte region is *Z. punctata*. Thus, we propose to name this propolis as *Zuccagnia*-type.

Table 1: Identification of phenolic compounds in the propolis, *Zuccagnia punctata*, *Larrea divaricata* and *L. cuneifolia* ethanolic extracts by HPLC-MS/MS analysis.

Peak	Rt (min)	$[M-H]^-$	Fragmentation	Tentative identification
1	55.5	239	197, 135	2', 4'-dihydroxychalcone
2	55.8	269	254, 150, 106	2',4'-dihydroxy-3'-methoxychalcone
3	53.2	302	273, 122	nordihydroguayaretic acid

Experimental

Propolis samples: Propolis samples were collected in December 2012 and March 2013 from hives located at 2000 m.a.s.l., in the Monte Region, Tucumán (26°35'S, 65°55'W), Argentina (National Apiculture Program INTA-PROAPI, Argentina). Samples were weighed and frozen at -20ºC until processing.

Plant samples: Aerial parts (bud and unexpanded leaf) of *Zuccagnia punctata* Cav., *Larrea cuneifolia* Cav. and *L. divaricata* Cav. were harvested from Amaicha del Valle, Tucumán, Argentina at 2000 m.a.s.l. The plants were identified by Dra Soledad Cuello, Laboratory of Investigation in Natural Products (LIPRON-INQUINOA-CONICET) and voucher specimens (*Z. punctata*: LIL 612170; *L. cuneifolia*: LIL 614829; *L. divaricata*: LIL 614299) were deposited at the Herbarium of Fundación Miguel Lillo (Tucumán, Argentina).

Preparation of propolis extracts: Samples of crude propolis (20 g) were extracted with 250 mL of ethanol: water 80:20 (v/v) at room temperature for 7 days. The extracts were named as propolis ethanolic extract 1 and 2 (PEE-1; PEE-2). The extracts were taken to dryness under reduced pressure, lyophilized and stored at -20ºC in the dark until analysis.

Preparation of plant extracts: Buds and unexpanded leaves (20 g) of *Z. punctata*, *L. cuneifolia* and *L. divaricata* were extracted with 250 mL of ethanol: water 80:20 (v/v) at room temperature for 7 days. The extracts were taken to dryness under reduced pressure, lyophilized, stored at -20ºC in the dark until analysis and named as *Z. punctata* extract (ZpE), *L. cuneifolia* extract (LcE), and *L. divaricata* extract (LdE).

Botanical analysis of propolis by microscopic techniques: Three samples of each crude propolis (0.5 g) were processed according to Barth [7a]. The samples were maintained in ethanol (15 mL) for 1 month, with occasional stirring.

The residual material (leaves, stems fragments) was treated with 10% KOH for 5 min [7b], followed by immersion in 50% sodium hypochlorite solution for 4 min. After these procedures, the material was washed with distilled water, stained with either cresyl violet or safranin and mounted in 50% glycerol [7c].

Observation of stained and un-stained material was made under a Zeiss Axiolab optical microscope equipped with a Zeiss Axiocam ERc 5s digital camera. Scales and measures were calculated using the software AxioVision Rel. 4.8.

Botanical identification was made by comparing observed structures with plant material (*Z. punctata*, *L. cuneifolia, L. divaricata*) collected at the site where the beehives were located and with relevant literature [5f, 7d-f].

Thin layer chromatography (TLC) and reversed phase thin layer chromatography (RPTLC): Plates of silica gel RP-18 F254 for RPTLC and silica gel F254 for TLC were used. The different extracts (40 µg of soluble principles) of propolis and plant extracts were applied to the lower edge of the plate. The plates were developed in ascending direction with toluene: $CHCl_3$: acetone (4.5:2.5:3.5, v/v/v). The separated components were revealed under UV light at 365 nm (UV Lamp Model GL-58 Mineralight Lamp) and 1% methanolic solution of diphenylboric acid aminoethyl ester [7g].

Identification of polyphenolic compounds: The HPLC system used consisted of a Waters 1525 binary HPLC pumps system with a 1500 Series column heater, a manual injection valve with a 20 µL loop (Rheodyne Inc., Cotati, CA) and a Waters 2998 photodiode array detector (PDA). An AXBridgeTM C18 column (4.6 x 150 mm, 5 µm; Waters corporation, Milford, MA) was used. The HPLC analyses were performed using a linear gradient solvent system consisting of 0.1% acetic acid in water (A) and 0.1% acetic acid in MeOH (B) as follows: 90% A to 43% A over 45 min, followed by 43% A to 0% A from 45 to 60 min, and remaining in 0% A during 5 min at 35°C. The flow rate was 0.5 mL/min and the volume injected was 20 µL of a solution of 1 mg/mL. Data collection was carried out with Empower$^{TM\,2}$ software. The extracts were monitored at 254 nm. The presence of different phenolic compounds in propolis and plant extracts was confirmed by UV spectrometry (220–500 nm) in comparison with standard compounds and co-injection of commercial compounds. Data were also recorded on a HPLC-ESI-MS/MS system, which consisted of the HPLC HP1100 (Agilent Technologies Inc., Santa Clara, CA-USA) connected through a split to the mass spectrometer Esquire 4000 Ion Trap LC/MS(n) system (Bruker Daltonik GmbH, Bremen, Germany).

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