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Histochemical localization and characterization of chalcones on the foliar surface of *Zuccagnia punctata* Cav. Insights into their physiological role

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ABSTRACT

Dihydroxychalcones as well as other metabolites synthesized via the phenylpropanoid pathway have a wide range of biological activities. Although this class of phenolic compounds is found in very large amounts in some tissues, their physiological significance remains unclear.

This approach focused on the chemical analysis of *Zuccagnia punctata* leaf rinse extract in which dihydroxychalcones (99.25 μg 2′,4′-dihydroxychalcone/cm^2 and 73.38 μg 2′,4′-dihydroxy-3′-methoxychalcone/cm^2) are the main constituents. Histochemical analysis (fluorescence microscope and emission scanning electron microscope coupled with an energy dispersive X-ray spectrometer) revealed a high flavonoid concentration on the foliar surface. The high accumulation of phenolic compounds, flavonoids or chalcones in the cuticle of *Z. punctata* leaves would act as defense mechanisms against UV radiation for the protection of photosynthetic tissues against oxidative stress.

Contents

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1. Introduction

Zuccagnia punctata Cav. (Fabaceae, Caesalpinieae), commonly known as jarilla pispito, puspus and jarilla macho, belongs to a monotypic genus comprised of this Argentine endemic species, characteristic of xerophytic plants from the Biogeographic Province of Monte. This plant species is widely distributed in arid and semiarid areas of western Argentina (Jujuy, Salta, Tucumán, Catamarca, La Rioja, San Juan, Mendoza and San Luis) (Ulibarri, 2005; Zuloaga and Morrone, 1999). *Z. punctata* inhabits the same environment as *Larrea divaricata* and *Larrea cuneifolia*, and forms a natural arid community named "jarillal".

Z. punctata aerial parts have been extensively used as a traditional medicine for the treatment of bacterial and fungal infections, asthma, arthritis and rheumatism (Ratera and Ratera, 1980; Toursarkissian, 1980).

The constituents of *Z. punctata* aerial parts include phytochemicals such as flavonoids (flavanones, flavones and mainly chalcones), oxygenated monoterpene-rich essential oils (Álvarez et al., 2012) and caffeoyl esters (Agüero et al., 2010; Pederiva et al., 1975; Pederiva and Giordano, 1984; Svetaz et al., 2004).

Z. punctata extracts and chalcones were reported to have antioxidant (Morán Vieyra et al., 2009), antibacterial (Zampini et al., 2005, 2012), antifungal (Agüero et al., 2010; Nuño et al., 2014; Quiroga et al., 2001; Svetaz et al., 2004, 2007), antiulcerous (De la Rocha et al., 2003) and antigenotoxic (Zampini et al., 2008) properties. Chieli et al., (2012), suggested an impact of *Z. punctata* extract and some of its components on the pharmacokinetics of drugs, which are P-gp substrates, as well as a potential role on multidrug resistance modulation.

To date, approximately 200 dihydroxychalcones are known to be formed in over 30 plant families (Hermoso et al., 2003; Hilt et al., 2003). Numerous studies have described the potential benefits of dihydroxychalcones in human health. However, their role in plant species still remains unresolved.

In an attempt to better understand chalcone physiological roles in *Z. punctata*, we first assessed their tissue location by using Neu's reagents. In addition, we investigated the foliar surface chemical composition by performing FESEM-EDS analyses and extracts of foliar washes, which were characterized by HPLC fingerprints.

2. Material and methods

2.1. Plant Material

Z. punctata aerial parts (leaves and stems) were collected from January to February 2013 at 2000 m above sea level (masl) in Amaicha del Valle, Tucumán, Argentina (Fig. 1A). Voucher specimens (LIL 605935) were deposited at the Herbarium of Fundación Miguel Lillo, Tucumán, Argentina. *Z. punctata* was authenticated by Dra. Ana Soledad Cuello, INQUINOA, CONICET.

Fresh plant material was used after harvesting to avoid degradation of chemical compounds.

2.2. Histochemical analysis

A qualitative method was used to determine flavonoid localization in plant tissues. Leaflets of *Z. punctata* were embedded in 3% agarose (type II) before cutting for histochemical examination. Cross-sections (25 μm) were obtained by using a microtome. Sections were observed as fresh samples under the light microscope or stained with conventional histological methods (Mercado et al., 2013). Neu's reagent (2-aminoethyl-diphenylborinate, Sigma) 1% in absolute methanol, a standard reagent for phenolic compounds (Neu, 1957), was also used. Cross-sections were immersed (10 min) in Neu's reagent and then mounted in glycerol–water (50:50 v/v) solution.

Sections stained with Neu's reagent were analyzed under a fluorescence microscope (Nikon Optiphot) with UV light (filter UV-1A: 365 nm excitation filter, 400 nm barrier filter). Under these conditions, flavonoids were detected by a yellowish fluorescence (Mondolot-Cosson et al., 1997). Photographs were taken with a digital Nikon Coolpix 4500 camera.

For scanning electron microscopy (SEM) samples were fixed in glutaraldehyde phosphate 5% buffered with 0.1 M sodium cocadylate at pH 7, and post-fixed in 1.5% osmium tetroxide buffered with 0.1 M sodium cocadylate at pH 7.2. Leaflets were dehydrated in a graded acetona series, dried by CO_2 critical point drying method and covered with a thin gold layer (200 Å) by using an ion-sputter.

Fresh leaflets and purified 2′,4′-dihydroxychalcone (DHC) and 2′,4′-dihydroxy-3′-methoxychalcone (DHMC) were examined with

Fig. 1. Photographic image of *Zuccagnia punctata*. A. General aspect. B. Flower and details of leaflet. Scale bar = 1 cm.

a field emission scanning electron microscope (FESEM-ZEISS SUPRA-55 VP) coupled to an energy dispersive X-ray spectrometer (EDS) for elemental characterization.

Electronic microscopy observations were performed at the Centro Integral de Microscopía Electrónica (CIME), CONICET, Tucumán, Argentina.

2.3. Phytochemical analysis

2.3.1. Phenolic compounds and wax extraction

Z. punctata leaves were extracted by immersions in chloroform for 20 s. The extract obtained was evaporated to dryness in a rotatory evaporator, then, residues were dissolved in methanol.

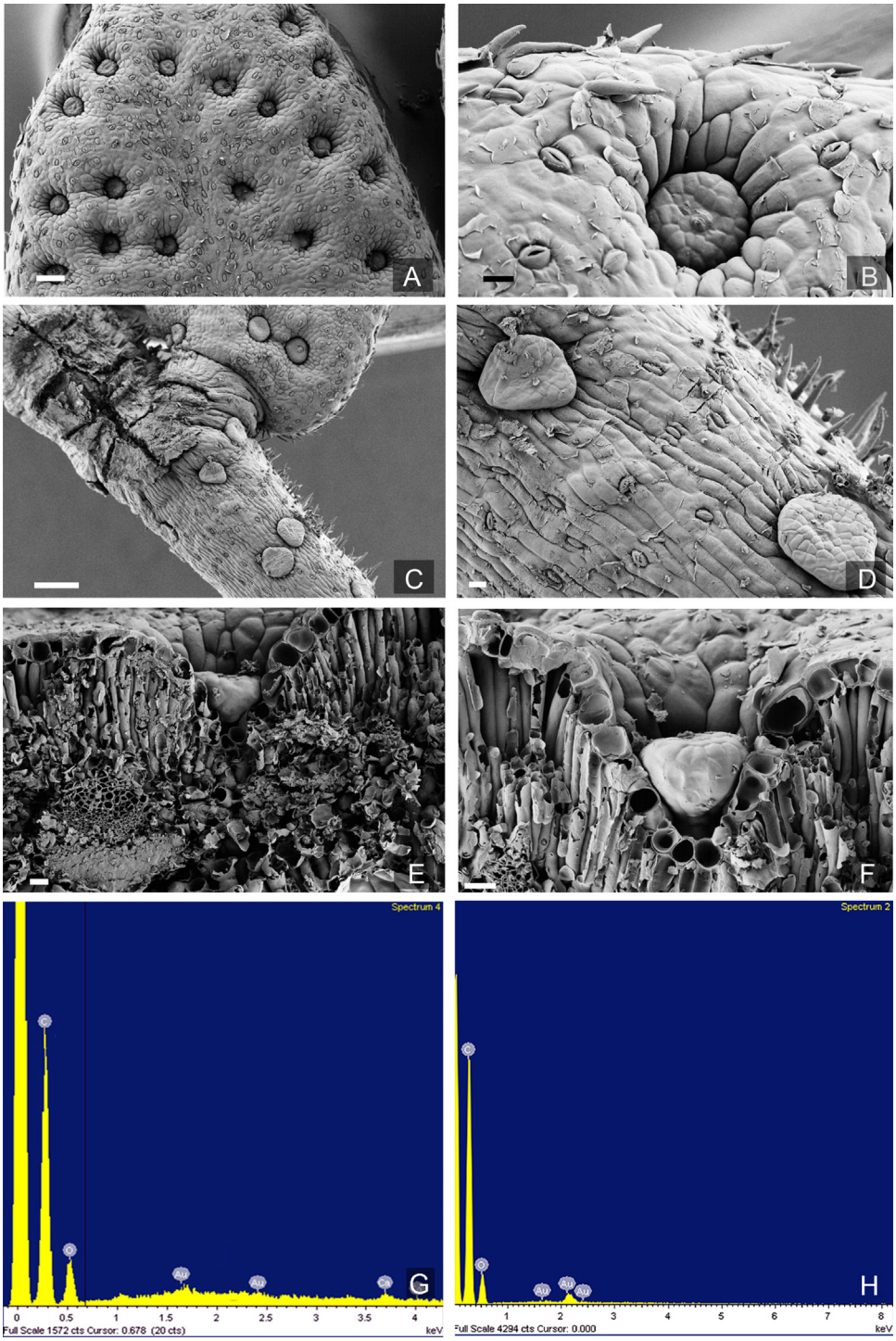

Fig. 2. SEM of *Zuccagnia punctata*. A and B Leaflet surface with cyclocytic stomata and sunken capitate glandular trichomes and non-glandular one-celled trichomes arranged in the margins. C and D Raquis suface showing stomata, non-glandular trichomes and capitate glandular trichomes. E. Leaflet section. F. Leaf section details of sunken capitate glandular trichome and thick cuticle. Scale bars *A* = 100 mm; *B*, *D*–*F* = 20 μm and *C* = 200 μm. G. EDS X-ray spectra of leaflet surface. H. EDS X-ray spectra of pure 2′,4′-dihydroxychalcone powder.

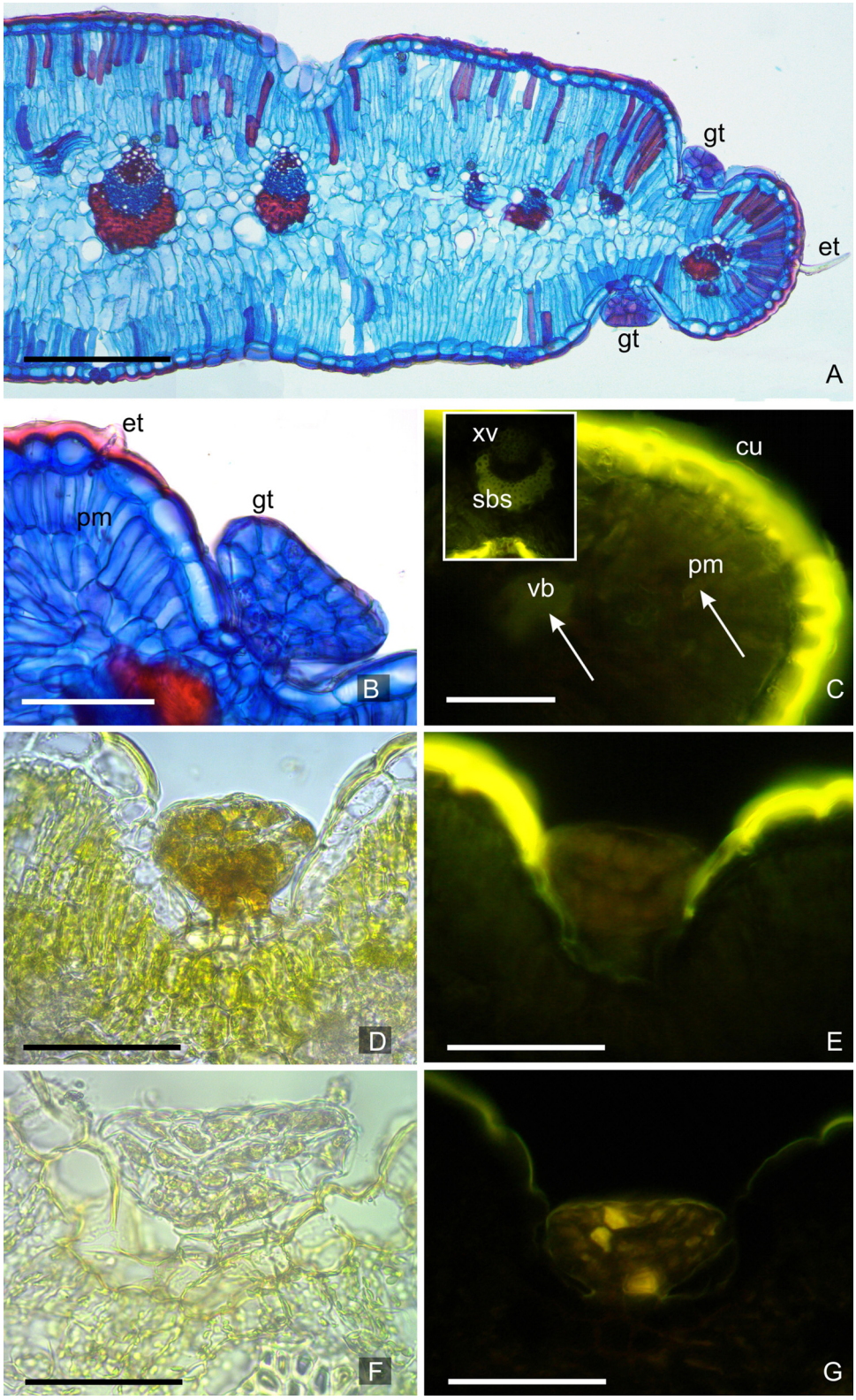

Fig. 3. Histochemical localization of flavonoids of *Z. punctata* leaflets. A. Astra blue- safranin, general aspect of glandular (gt) and eglandular (et) trichomes and palisade mesophyll (pm) by conventional anatomy techniques. B, C and D. Leaflet before foliar washes. B. Leaflet tissues treated with Neu's reagent and observed under UV light; note the intense fluorescence in the cuticle (cu) and weak fluorescence in palisade mesophyll (arrow, pm) and vascular bundles (arrow, vb). The inset shows details of vascular bundle; the bright yellowish fluorescence of the sclerenchyma of the bundle sheath cells (sbs) and xylem vessel (xv) walls is attributable to flavonoids bound to cell walls. C. Fresh material; a glandular trichome observed under light microscopy. D. Tissues stained with Neu's reagent observed under UV light; notice that glandular trichome fluorescence is secondary, masked by the brightness of the cuticle. E and F Leaflet after foliar washes. E. Fresh glandular trichome observed under light microscopy. F. Leaflet tissues treated with Neu's reagent and observed under UV light, notice the fluorescence in glandular trichome cells and the evident decrease in the cuticular fluorescence. Scale bars = 100 μm. Filter UV-1A: 365 nm excitation filter, 400 nm barrier filter.

The extract was subjected at −20 °C for 2 h to obtain the precipitation of waxes. Then, the extract was centrifuged. The supernatant was called ZpE. The pellet (waxes) was dissolved in a small volume of chloroform; the solvent was evaporated on a steam bath and the flasks maintained in a dessicator until constant mass. Extracted metabolite yield was determined.

2.3.2. Total phenolic compounds and flavonoid quantification

ZpE was standardized by the determination of total phenolic compound content by using Folin–Ciocalteu reagent (Singleton et al., 1999) and flavonoids.

2.3.3. ZpE thin layer chromatography

An aliquot of ZpE (15 μg GAE) was spotted onto a TLC plate (8 cm × 6 cm) covered with silica gel (Merck F254). The TLC plate was developed in mobile phase (toluene:acetone:chloroform; 4.5:3.5:2.5). After drying, the plates were observed under UV light at 365 nm before and after development with Neu's reagent. Standards of hydroxylated chalcones (Indofine Chemical Company, Inc.) were used for comparative purposes.

2.3.4. HPLC fingerprints of ZpE

The HPLC system consisting of a Waters 1525 Binary HPLC Pump 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) was used to analyze the extracts. A XBridge™ C18 column (4.6 × 150 mm, 5 μm; Waters corporation, Milford, MA) with two gradient solvent system was used.

The system was composed of solvent A (9% acetic acid in water) and solvent B (methanol) (conditions: 25–45% B from 0 to 10 min and kept at 45% B from 10 to 20 min; 45–70% B from 20 to 40 min; 70–75% B from 40 to 50 min; 75–100% B from 50 to 55 min) were used for the component separation from extracts. Flow rate was set at 0.8 mL/min. A solution of 1 mg/mL was used. Data collection was carried out with Empower™ 2 software. The presence of phenolic compounds in extracts was confirmed by UV spectrometry (220–500 nm) in comparison with standard compounds and through co-injection of standard compounds with the extracts. The chalcon total amount per unit leaf area was calculated by using calibration curves with commercial chalcones and expressed as micrograms per total leaf area (mg/cm^2).

Calibration curves were obtained on the basis of five concentration levels of DHC and DHMC standards. A linear least square regression of the peak areas as a function of concentrations was performed to determine correlation coefficients (R^2).

2.3.5. GC–MS and NMR spectra

Waxes were analyzed by using a GC equipped with an Agilent 5973 N mass spectrometer (GC/MS) to produce electron ionization

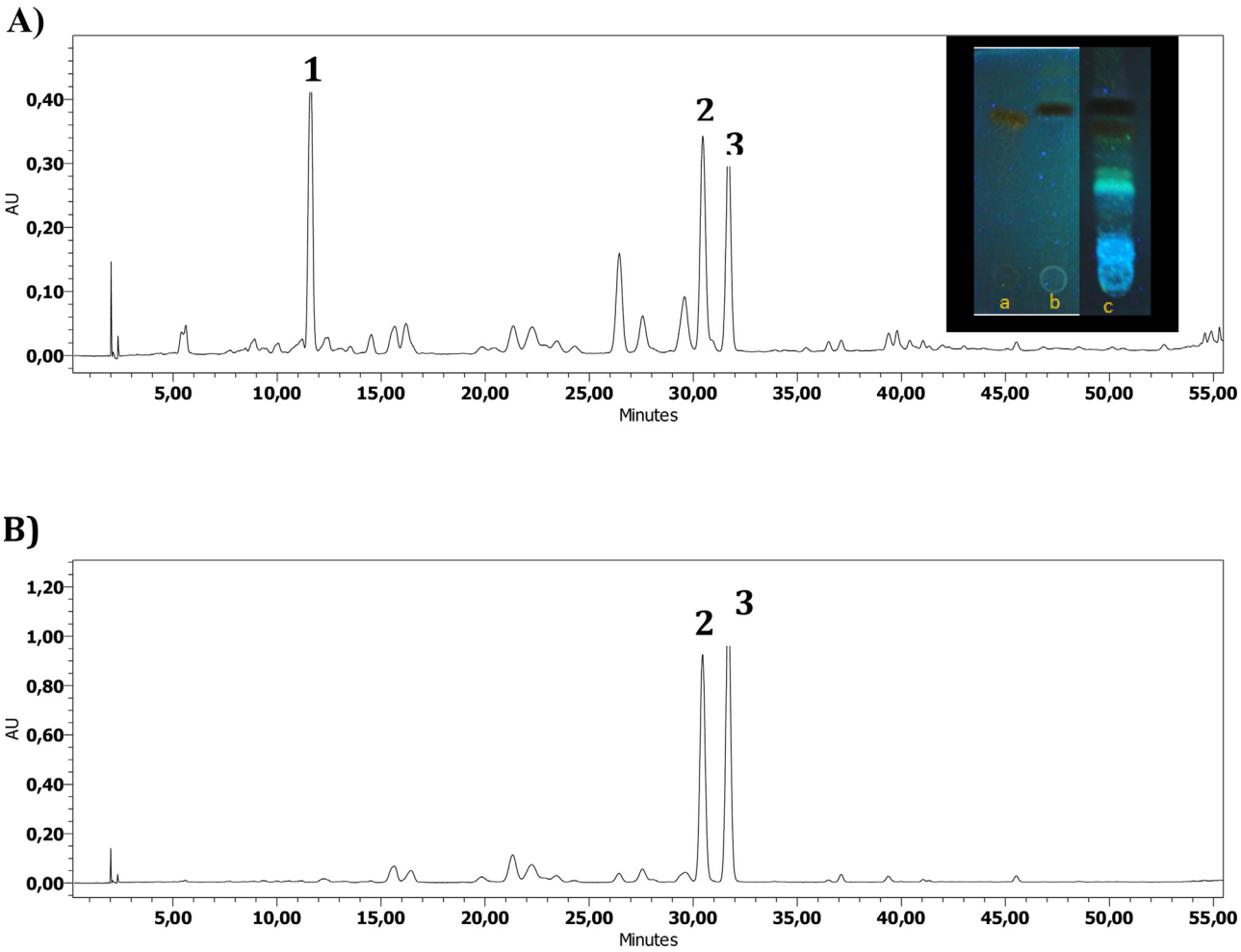

Fig. 4. High performance liquid chromatography profiles of foliar washing of *Zuccagnia punctata*. A) Fingerprint at 280 nm. B) Fingerprint at 330 nm. C1. Cinammic acid (RT 12.5 min); C2. 2′,4′-dihydroxychalcone (RT 30.47 min.); C3. 2′,4′-dihydroxy-3′-methoxychalcone (RT 31.69 min.). The inset of Fig. 1 A shows the TLC profiles corresponding to standards of a: 2′,4′-dihydroxychalcone, b: 2′,4′-dihydroxy-3′-methoxychalcone and c) foliar washing with chloroform. The solvent used as eluent was toluene: acetone: chloroform (4.5:3.5:2.5, v/v/v). The plate was visualized under UV-365 nm and developed with NP / PEG.

(EI) mass spectra of each peak. The individual components were separated by using a HP-5MS capillary column.

Compound identification was achieved by means of their GC retention indices, determined in relation to an homologous series of *n*-alkanes and fragmentation patterns in the mass spectra with those stored in our own library, in the GC–MS database and with literature data.

Wax total amount per unit leaf area was calculated as the total of all wax constituents including unknown peaks not identified by GC, and expressed as micrograms per total leaf area (mg/cm^2).

NMR spectra were recorded on a Bruker AC spectrometer operating at 200 MHz for ^{1}H. Mass spectra including high-resolution mass spectra were recorded on a JEOL JMS AX 500 spectrometer (HRCIMS).

2.4. Leaf area measurement

The total leaf areas were determined by using the software ImageJ 1.49 h Wayne Rasband, National Institutes of Health, USA. Java 1.7.0-45 (64 bits). Wax and phenolic compound contents are expressed by the ratio between wax or phenolic compound mass and leaf area (mg/cm^2), taking into account both adaxial and abaxial surfaces.

3. Results and discussion

Z. punctata has pseudo-paripinnate leaves (Fig. 1B) with subopposite leaflets, nanophyll with acuminate apex, rounded base and entire margin (Lersten and Curtis, 1996; Mercado et al., 2013).

Both epidermal surfaces of the leaflets and the raquis present epidermal cells with straight anticlinal walls, thick cuticle, cyclocytic stoma, sunken capitate glandular trichomes located in crypts and non-glandular one-celled trichomes arranged in the margins (Figs. 2A–F and 3A and B) (Álvarez et al., 2012; Lersten and Curtis, 1996; Mercado et al., 2013). In section, leaflets are isolateral and amphistomatic (Figs. 2E and 3A). The middle vein presents a collateral vascular bundle with sclerenchymatous layers at the phloem pole (Fig. 3A). Idioblasts containing druses in the mesophyll are abundant (Fig. 3A).

Preliminary studies by vanillin sulfuric staining indicate that glandular trichomes could be associated with the synthesis of phenolic and terpenic compounds (Mercado et al., 2013). However, studies of the chemical composition of the extracts obtained from *Z. punctata* leaf surface and histochemical localization of chalcones have not so far been conducted.

3.1. Phytochemical analysis of foliar washing

Z. punctata foliar area was 9.01 ± 0.1 cm^2/leaf. The content of cuticular waxes was 88 μg/cm^2. In the foliar wax of *Z. punctata* leaves, the main alkanes constituents were *n*-heptacosane (C27), *n*-nonacosane (C29) and *n*-hentriacosane (C31). The content of total polyphenolic compounds on the foliar surface was 177 ± 13 μg GAE/cm^2. The foliar washing analysis (ZpE) by TLC showed a significant flavonoid pattern. TLC analysis revealed the presence of two major bands with Rf values coincident with commercial standards of chalcones (2′, 4′-dihydroxychalcone and 2′, 4′-dihydroxy-3′-methoxychalcone). The presence of cinammic acid (C1), 2′,4′-dihydroxychalcone (C2) and 2′,4′-dihydroxy-3′-methoxychalcone (C3) were confirmed (Fig. 4A and B) by HPLC–DAD, coinjection of reference compounds and H^1 RMN (data not shown).

The content of each chalcone was 99.25 μg DHC/cm^2 and 73.38 μg DHMC/cm^2. These compounds were previously described in ethanolic and DCM extracts of *Z. punctata* aerial parts (Agüero et al., 2010; Zampini et al., 2005).

3.2. Chalcone histochemical location

On the basis of previous results (Mercado et al., 2013), fresh leaflets of *Z. punctata* were subjected to histolocalization studies of polyphenolic compounds (flavonoids and chalcones). Neu's reagent, a standard reagent for phenols, forms complexes with phenolics which then emit a specific fluorescence. During immersion of cross-sections, this methanol-containing reagent dissolves chlorophyll and eliminates almost completely its red fluorescence, thus enabling better localization in situ of flavonoids by the specific yellowish fluorescence they emit under UV light.

In *Z. punctata* leaflets, an intense yellowish fluorescence was observed in the cuticle indicating a high flavonoid concentration and a low fluorescence in palisade parenchyma cells, glandular trichomes, xylem vessel walls and in the walls of sclerenchymatous bundle sheath cells (Fig. 3C and D). EDS X-ray sprectra of fresh leaflet surface showed the presence of 70% C and 20% O and a similar ratio was found for purified chalcone powder, suggesting the chemical nature of the products deposited on the leaflet surface (Fig. 2G–H).

In our assay conditions, after foliar washing with chloroform, the fluorescence under UV light and the thickness of the cuticle layer decreased on the epidermal layer, while the yellowish fluorescence remained in glandular trichomes (Fig. 3D–F). This observation indicates that a high level of chalcones accumulates on the foliar surface.

4. Conclusions

From chemical and histological results some interesting questions arose regarding the relationship between the localization versus the role of polyphenolic compounds. The purpose of the high accumulation of phenolic compounds, in particular chalcones, in the cuticle of *Z. punctata* would function as a defense mechanism against UV radiation (Agati et al., 2013; Beckman, 2000; Caldwell et al., 1983; Smith and Markham, 1998).

In brief, these compounds could be involved in various physiological processes such as defense against bioaggressors, redox homeostasis, dissipation of excess excitation energy and UV protection.

Studies on chemical composition of minor constituents of leaf rinse are being conducted.

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