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Use of a LC-DAD-QTOF system for the characterization of the phenolic profile of the argentinean plant *Zuccagnia punctata* and of the related propolis: New biomarkers

Eliana Rita Solorzano [a], Claudio Bortolini [a], Sara Bogialli [a,*], Iole Maria Di Gangi [a], Gabriella Favaro [a], Luis Maldonado [b], Paolo Pastore [a]

[a] Department of Chemical Sciences, University of Padua, Via Marzolo, 1, 35131 Padova, Italy
[b] Estación Experimental Agropecuaria Famaillá, Instituto Nacional de Tecnología Agropecuaria, Ruta provincial 301, km 32, Famaillá, Tucumán, Argentina

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ABSTRACT

Argentinean northwestern propolis were included into the national Food Code, some of that were sometimes associated with *Zuccagnia punctata*, an endemic plant with interesting biological properties. An analytical protocol for characterizing the phenolic profile of *Z. punctata* by LC coupled to DAD-QTOF detection systems was proposed.

Mass spectrometric and chromatographic selectivity allowed to recognize various compounds including isomers. Eleven compounds never mentioned before for this shrub were highlighted. Among them, two uncommon dihydrochalcones, i.e. 4′-hydroxy-2′-methoxydihydrochalcone and 2′,4′-dihydroxydihy drochalcone, were described for the first time as major constituents of *Z. punctata*, suggesting a peculiar biosynthetic pathway.

Only the two propolis collected in the "Del Monte" phytogeographical region showed large amounts of molecules present in *Z. punctata*, which were proposed as markers of propolis type *Z. punctata.* Since *Z. punctata* exhibits a very interesting bioactivity, propolis derived from this plant can be used as nutraceutical food.

1. Introduction

From ancient times, medicinal plants are the main source for developing new medicinal substances. According to the World Health Organization, about 65–80% of the world population use mainly plants for their primary health care. However, for health safety issues, a relatively small number of plants were scientifically investigated for assessing their quality and efficacy (Calixto, 2005). Among the Latin American countries, Argentina produced a large number of studies on plants which produce secondary metabolites promoting their ability to grow at high altitudes and to resist to extreme environmental conditions (Alonso-Amelot, 2008) (Sülsen et al., 2007; Villagra et al., 2011). *Zuccagnia punctata* Cav. (Fabaceae) has been one of the most investigated mountain species in terms of bioactivity. *Z. punctata* is a shrub commonly known as "jarilla macho" or "puspus" and represents the only species of this genus that is endemic of central and western semi-arid regions of Argentina (Cabrera, 1971). Currently, it still continues being widely used in folk medicine mainly against bacterial and fungal infections, asthma, arthritis, rheumatism (Cabrera, 1971; Svetaz et al., 2007; Vattuone, Soberon, Sgariglia, Quiroga, & Sampietro, 2013; Villagra et al., 2011; Álvarez et al., 2012). The main bioconstituents described in *Z. punctata* belong to phenolic compounds, more exactly, flavonoids and caffeic acid derivatives (Agüero et al., 2010; Svetaz et al., 2004).

Flavonoids are secondary metabolites with a wide range of biological activities and serve as chemotaxonomic marker compounds. All flavonoids share a basic C6—C3—C6 phenylbenzopyran backbone. The three-carbon bridge between the phenyl rings (A and B rings) can be cyclized to form a third ring (C-ring). They can be classified into several groups, where chalcones are described as open-chain flavonoids (Cuyckens & Claeys, 2004; Hahlbrock & Grisebach, 1975). Caffeic acid derivatives are esters consisting of caffeic, ferulic, cinnamic or coumaric acids linked by the acidic hydroxyl group to aromatic or non-aromatic

* Corresponding author.
E-mail addresses: elianarita.solorzano@unipd.it (E.R. Solorzano), claudio.bortolini@unipd.it (C. Bortolini), sara.bogialli@unipd.it (S. Bogialli), iolemaria.digangi@unipd.it (I.M. Di Gangi), gabriella.favaro@unipd.it (G. Favaro), maldonado.luismaria@inta.gob.ar (L. Maldonado), paolo.pastore@unipd.it (P. Pastore).

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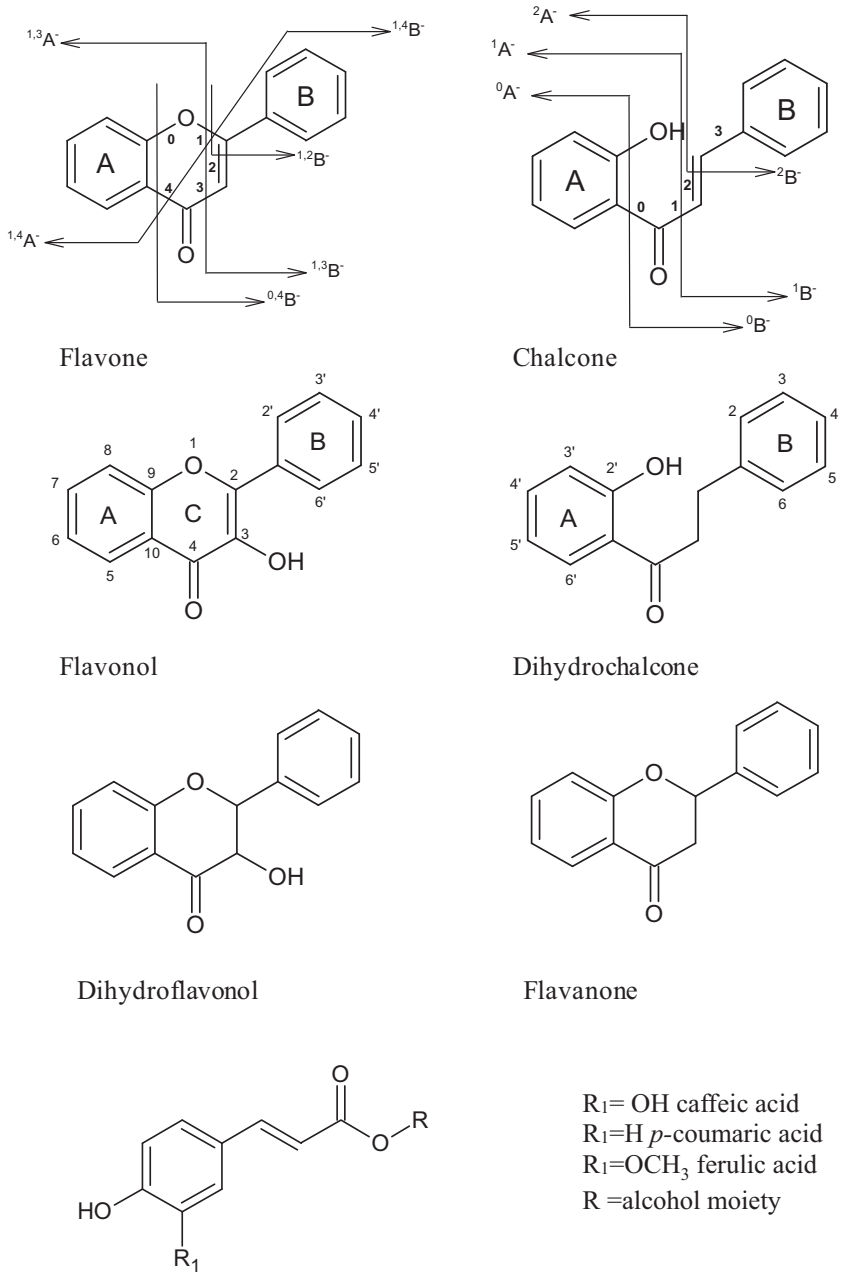

Fig. 1. Structures of flavonoids and caffeic acid derivatives. Typical cleavages for flavonoids and dihydro/chalcones are described on the numbered bonds (bold) according the nomenclature used by Fabre et al. (2001). Note that numbering of carbon positions (italic) is different among open chain flavonoids and chalcones.

moieties (Fig. 1). Some caffeic acid derivatives were reported having optimal antimicrobial and antiproliferative activities (Banskota et al., 2002; Natella, Nardini, Di Felice, & Scaccini, 1999).

The remarkable biological effects described for *Z. punctata* such as antifungal cytoprotective, antibacterial, anti-genotoxic, were mainly ascribed to two open-chain flavonoids, i.e. 2′,4′-dihydroxy chalcone and 2′4′-dihydroxy-3′-methoxychalcone (Agüero et al., 2010; De la Rocha, María, Gianello, & Pelzer, 2003; Svetaz et al., 2004; Iris C. Zampini, Vattuone, & Isla, 2005; Iris Catiana Zampini, Villarini, Moretti, Dominici, & Isla, 2008). Indeed, research on chalcones gained a global interest for being simple structures with widespread spectrum of bioactivities, suggesting their use as templates for the rational design of new drugs (Orlikova, Tasdemir, Golais, Dicato, & Diederich, 2011; Vogel, Barbic, Jürgenliemk, & Heilmann, 2010).

In addition, several propolis collected in some arid regions of Argentina, e. g. in the Catamarca and Tucumán provinces, were somehow associated to *Z. punctata* (Agüero et al., 2010; Solórzano et al., 2012; Vera et al., 2011) however, it is not possible to categorize a propolis as "*Z. punctata*–type propolis" on the basis of a generic chemical similarity. Propolis characterization and quality control is more reliable when related to a recognized botanical source, as plants maintain sufficiently constant their phytochemical profiles (Bankova, 2005; Bankova, Popova, Bogdanov, & Sabatini, 2002; Sforcin & Bankova, 2011; Solórzano et al., 2012). Furthermore, since northwestern argentinean propolis was included into the Argentinian Food Code for its potential applicability as food preservative, nutraceutic as well as dietary supplement, its characterization is particularly important (Bankova, 2005; Vera et al., 2011).

The characterization of phenolic metabolites in plants is commonly based on liquid chromatography with diode array detection (LC-DAD) since they exhibit characteristic UV absorptions. Their identification, anyway, requires the use of mass spectrometry (MS) and NMR techniques after a suitable semi-preparative chromatographic process. In this context, LC coupled to the quadrupole-Time of Flight (QTOF)-MS is an excellent technique to elucidate the structure of natural products (de Rijke et al., 2006; Marston & Hostettmann, 2006).

In this work the description of *Zuccagnia*-type propolis will be proposed on the basis of the characterization of the phenolic profiles of alcoholic extracts of *Z. punctata*. This will be performed by means of a LC-DAD-QTOF-MS system directly on extracts of both vegetal materials and propolis, without any fractionation and concentration step. The use of high resolution (HR) MS empowered the selective and sensitive analysis of non-target compounds, revealing eleven new phenolic compounds both in plant and propolis. The major constituents were proposed as biomarkers specific for *Z. punctata*. This straightforward approach will allow the identification of many other relevant potentially bioactive compounds in plants-derived food products and will simplify the quality control of foodstuff containing these propolis.

2. Materials and methods

2.1. Reagents and materials

7-hydroxyflavanone (**1**∗), 2′,4′-dihydroxy-4-methoxychalcone (**4**), 2′-hydroxychalcone (**5**), 4′-hydroxychalcone (**6**), 3,5,7-trihydroxyflavone (galangin, **20**), 4′,5,7-trihydroxyflavone (apigenin, **21**), 3,5,7,3′,4′-pentahydroxyflavone (quercetin, **24**) and 5,7-dihydroxyflavone (chrysin, **25**), 5,7-dihydroxy-4′-methoxyiso flavone (biochanin, **27**), 3-(3,4-Dihydroxycinnamoyl) quinic acid (chlorogenic acid, **31**) were kindly obtained by prof. Raffaella Filippini and prof. Guglielmina Froldi (University of Padua). 2′,4′-dihy droxy-3′-methoxychalcone was isolated from *Z. punctata* extract according to Agüero et al. (2010). Standard stock solutions at concentration of 1 mg/mL were prepared dissolving dry extracts of each compound in a 50/50 (v/v) methanol/water or DMSO solution. Successive dilutions were made with 50/50 (v/v) methanol /water down to a final concentration of 100 ng/mL. All solvents and reagents were of analytical grade (Sigma Aldrich, Saint Louis, MO, USA).

2.2. Sample collection and preparation

Z. punctata Cav. and propolis samples were collected in different seasons during 2009–2011 in the northwestern provinces of Argentina, Tucumán (T) and Catamarca (C). Propolis samples named T1 and C2 were from Amaicha del Valle and Santa María, respectively, belonging to the "Del Monte" phytogeographycal region. T2 and C1 were collected in "Selva de las Yungas" and "Prepuna" phytogeographycal regions, respectively (Supporting Information Table S1). The vegetal species was identified by Lic. Alejandra Rodriguez and compared to authenticated specimens (voucher 605935 IML) from Facultad de Ciencias Naturales e Instituto Miguel Lillo, Universidad Nacional de Tucumán.

Aerial parts (leaves and stems) of *Z. punctata* and propolis collected were gathered to prepare single samples. Samples were prepared according to Solórzano et al. (2012). Dried leaves (2 g) of *Z. punctata* and raw propolis samples (2 g) were cut into small pieces and extracted with 20 mL of 80% ethanol using an ultrasonic device (30 min, 80w potency). The extracts were centrifuged for 20 min at 9000*g* in a refrigerated centrifuge (Sorvall RC50) and the supernatant was separated. Successive extractions of the residue were made to a final volume of 100 mL. Ethanolic extracts were dried by evaporation under vacuum at 50 °C. A 1 mg/mL solution was prepared by dissolving the dry extract in 80/20 (v/v) methanol/water. Dilutions were prepared with a 50/50 (v/v) methanol/water solution down to a final concentration of 20 μg/mL. The resulting solutions were named *Z.p.*E for the final methanolic extract of the plant and C1-E, C2-E, T1-E and T2-E for the final propolis extracts.

2.3. Instrumental analysis

LC-QTOF analysis were performed with a HPLC system (Agilent Series 1200; Agilent Technologies, Palo Alto, CA, USA), consisting of vacuum degasser, autosampler, a binary pump and a column oven coupled to both DAD and QTOF mass analyzer (Agilent Series 6520).

The chosen analytical column was a Kinetex pentafluorophenyl (PFP) (100 mm × 2.1 mm, 2.6 μm particle size, Phenomenex, Italy) thermostated at 30 °C. The sample injected volume was 5 μl. The mobile phase components A and B were water and methanol, respectively, both acidified with 1 mM formic acid. The eluent flow rate was 0.25 mL/min. The mobile phase gradient profile was as follow: 0–16 min, 20–100% B; 17–19 min, 100% B; 19–20 min, 20% B; 21–27 min, 20% B. During the optimization of the chromatographic separation, mainly a Kinetex C18, 150 mm × 2.0 mm I.D. was also evaluated (Phenomenex, Italy).

DAD signals were recorded in the 210–600 nm range, and three wavelengths, (254, 270 and 320 nm) were chosen for acquiring specific DAD channels. The QTOF system was equipped with an electrospray ionization interface (ESI), operating in dual ESI mode and negative ESI acquisition, with the following operating parameters: capillary voltage, 3500 V; nebulizer pressure, 35 psi; drying gas, 10 L/min; gas temperature, 350 °C; fragmentor voltage, 120 V; skimmer 65 V. The QTOF calibration was daily performed with the manufacturer's solution. For all chromatographic runs the *m*/*z* 112.9856, always present as impurity residue of formic acid dimer, was set as reference mass for accurate mass analysis. The instrument provided a typical resolving power (FWHM) of about 18,000 at *m*/*z* 311.0805. Mass spectra at MS level were recorded over the range *m*/*z* 80–1000 with a scan rate of 4 spectra/s, and for MS/MS over the range *m*/*z* 50–1000 with a scan rate of 8 spectra/s. Collision energies for precursor ions were set within the range 15–30 eV, after a preliminary optimization by injecting *Z. punctata* extracts. The list of preferred precursor ions used for AutoMS/MS analysis with raw formulas, molecular weights, retention times for target analysis (permitted tolerance ±0.2 min) and collision energies is reported in ESM, (Supporting Information Table S2). Mass spectra acquisition and data analysis was processed with Masshunter Workstation B 04.00 software (Agilent Technologies).

3. Results and discussion

3.1. Optimization of the experimental conditions

Compounds (**1**), (**1**∗), (**2**), (**2**∗) (flavonoids group), (**32**) and (**33**) (caffeic acid derivative group) were reported as the most abundant in *Z. punctata* extracts so that they were initially selected as representative compounds for method optimization and as preferred precursor ions in auto MS mode (Table S2 and Table 1).

The optimization mainly consisted in the chromatographic separation improvements of the (**1**), (**1**∗), (**2**), (**2**∗), (**32**) and (**33**). These phenolic compounds were previously characterized with C18 columns (Cuyckens & Claeys, 2002; Marston & Hostettmann, 2006), however PFP columns with 2.6 μm particle size allowed a better efficiency and resolution of both caffeic acid derivatives

Table 1
Results from analysis carried out on *Z. punctata* extract (*Z.p.*-E) and on propolis extracts collected in the provinces of Tucumán (T1-E and T2-E) and Catamarca (C1-E and C2-E). Confidence in MS identification (MSi) is here described as target (**t**), suspect (**s**) and non-target (**nt**) analysis (Schymanski et al., 2015) with accuracy experimented at MS level ≤ 3 ppm, while t_r stands for retention times. Previous reports referred to *Z. punctata* and/or propolis (prop) are also reported. The relative abundance of each compound in *Z. punctata* and propolis is related to the most abundant in the plant extract, i.e. 4′-hydroxy-2′-methoxydihydrochalcone **(38)**.

MSi	Compounds	Reports		Relative area, %				
				Z.p.-E	T1-E	T2-E	C1-E	C2-E
Chalcones and dihydrochalcones								
s	**(1)** 2′,4′-dihydroxychalcone	*Z.p.*, prop.	Agüero et al. (2010), Vera et al. (2011)	95.3	104.3	–	–	59.7
t	**(2)** 2′,4′-dihydroxy-3′-methoxychalcone	*Z.p.*, prop.	Agüero et al. (2010), Jimenez et al. (2014), Vera et al. (2011)	34.5	13.4	–	–	2.0
s	**(3)** 2′,4′,4-trihydroxy-6′-methoxychalcone	prop.	Vera et al. (2011)	10.0	2.1	–	–	9.1
Flavanones								
t	**(1*)** 7-hydroxyflavanone	*Z.p.*, prop.	Agüero et al. (2010), Jimenez et al. (2014), Vera et al. (2011)	57.9	24.9	–	–	24.8
s	**(2*)** 7-hydroxy-8-methoxyflavanone	*Z.p.*, prop.	Agüero et al. (2010), Jimenez et al. (2014), Vera et al. (2011)	22.3	34.1	–	–	8.9
s	**(3*)** 7,4′-dihydroxy-5-methoxyflavanone	prop.	Agüero et al. (2010)	2.0	15.7	–	–	1.3
s	**(10)** pinocembrin (5, 7-dihydroxyflavanone)	*Z.p.*, prop.	Agüero et al. (2010), Isla et al. (2005), Vera et al. (2011)	2.3	13.9	–	–	7.1
s	**(11)** pinostrobin (5-hydroxy-7-methoxyflavanone)	*Z.p.*, prop.	Agüero et al. (2010)	–	–	–	–	–
s	**(12)** 7-hydroxy-5,8-dimethoxyflavanone	prop.	Vera et al. (2011)	–	–	–	–	–
Flavones								
s	**(13)** Izalpinin (3,5-dihydroxy-7-methoxyflavone)	*Z.p.*, prop.	Agüero et al. (2010)	–	–	–	–	–
s	**(14)** 3,7-dihydroxy-8-methoxyflavone	*Z.p.*, prop.	Jimenez et al. (2014), Vera et al. (2011)	6.3	4.2	–	–	4.5
s	**(15)** rhamnocitrin (3,5,4′-trihydroxy-7-methoxyflavone)	*Z.p.*, prop.	Agüero et al., 2010	2.3	3.7	5.1		2.3
s	**(16)** 3-hydroxy-7,8-dimethoxyflavone	*Z.p.*, prop.	Agüero et al. (2010), Vera et al. (2011)	–	–	–	–	–
s	**(17)** 7-hydroxy-3′,4′-dimethoxyflavone	Z.p.	Jimenez et al. (2014)	–	–	–	–	–
s	**(18)** 7-hydroxy-5,8-dimethoxyflavone	prop.	Vera et al. (2011)	–	–	–	–	–
s	**(19)** 5-hydroxy-4′,7-dimethoxyflavone	prop.	Vera et al. (2011)	–	–	–	–	–
t	**(20)** galangin (3,5,7-trihydroxyflavone)	*Z.p.*, prop.	Agüero et al. (2010), Vera et al. (2011)	22.5	36.7	61.4	5.4	12.7
t	**(21)** apigenin (5,7,4′-trihydroxyflavone)	prop.	Isla et al. (2005)	–	–	–	–	–
s	**(22)** 3,5-dihydroxy-7,8-dimethoxyflavone	prop.	Vera et al. (2011)	–	42.1	64.2	13.9	5.6
s	**(23)** 4′,5-dihydroxy-3,7,8-trimethoxyflavone	prop.	Vera et al. (2011)	–	–	–	–	–
t	**(24)** quercetin (3,5,7,3′,4′-pentahydroxyflavone)	prop.	Isla et al. (2005)	–	–	–	–	–
t	**(25)** chrysin (5,7-dihydroxyflavone)	prop.	Isla et al. (2005)	3.7	77.7	34.8	19.5	7.0
s	**(26)** 3,7-dihydroxyflavone	*Z.p.*	Jimenez et al. (2014	5.3	5.6	–	–	5.3
Acids and esters								
s	**(28)** 1,1-dimethylallyl caffeic acid	prop.	Isla et al. (2005)	–	11.9	36.9	6.8	8.4
s	**(29)** Ferulic acid	prop.	Isla et al. (2005)	–	–	–	–	–
s	**(30)** caffeic acid	prop.	Isla et al. (2005)	–	–	–	–	–
s	**(32)** 1-methyl-3-(4′-hydroxyphenyl)-propyl caffeic acid ester	*Z.p.*	Svetaz et al. (2004)	21.9	35.0	0.4	–	24.2
s	**(33)** 1-methyl-3-(3′,4′-dihydroxyphenyl)-propyl caffeic acid ester	*Z.p.*	(Svetaz et al. (2004)	36.8	76.0	–	–	51.1
Proposed non-target compounds								
nt	**(34)** 7,8-dihydroxyflavanone	–	–	8.6	10.3	–	–	7.4
nt	**(35)** 3,7-dihydroxyflavanone	–	–	5.2	5.0	–	–	3.5
nt	**(36)** pinobanksin-5-methyl ether (3,7-dihydroxy-5-methoxyflavanone)	–	–	3.6	20.4	14.7	2.6	5.9
nt	**(37)** 3,7,8-trihydroxydihydroflavanone	–	–	6.3	27.7	96.3	7.8	3.8
nt	**(38)** 4′-hydroxy-2′-methoxydihydrochalcone	–	–	100.0	68.5	–	–	53.3
nt	**(39)** 2′,4′-dihydroxydihydrochalcone	–	–	23.6	23.3	–	–	14.5
nt	**(40)** 4′-terbutyloxyphenyl *p*-coumaric acid ester	–	–	0.9	0.6	–	–	0.5
nt	**(41)** 1-methyl-3-(4′-hydroxyphenyl)-propyl *p*-coumaric acid ester	–	–	22.8	25.3	–	–	18.2
nt	**(42)** 3,7-dimethyl-2,6-octadienyl caffeic acid ester (geranyl caffeate)	–	–	9.4	25.8	1.3	1.6	20.7
nt	**(43)** 1-methyl-3-(3′,4′-dihydroxyphenyl)-propyl ferulic acid ester	–	–	3.4	8.9	–	–	4.8
nt	**(44)** 2-methyl-3-(3′-hydroxy-4′-methoxyphenyl)-propyl caffeic acid ester	–	–	4.9	3.8	–	–	2.3

and flavonoids, including positional isomers of **(10)** at *m*/*z* 255.0663 (Fig. 2B). The presence of the pentafluorophenyl group in the stationary phase reduced secondary interactions with the silanolic residues of the support and enhanced specific interactions with the phenolic moieties, even limiting intramolecular bonds existing among phenolic groups. For instance, the elution order

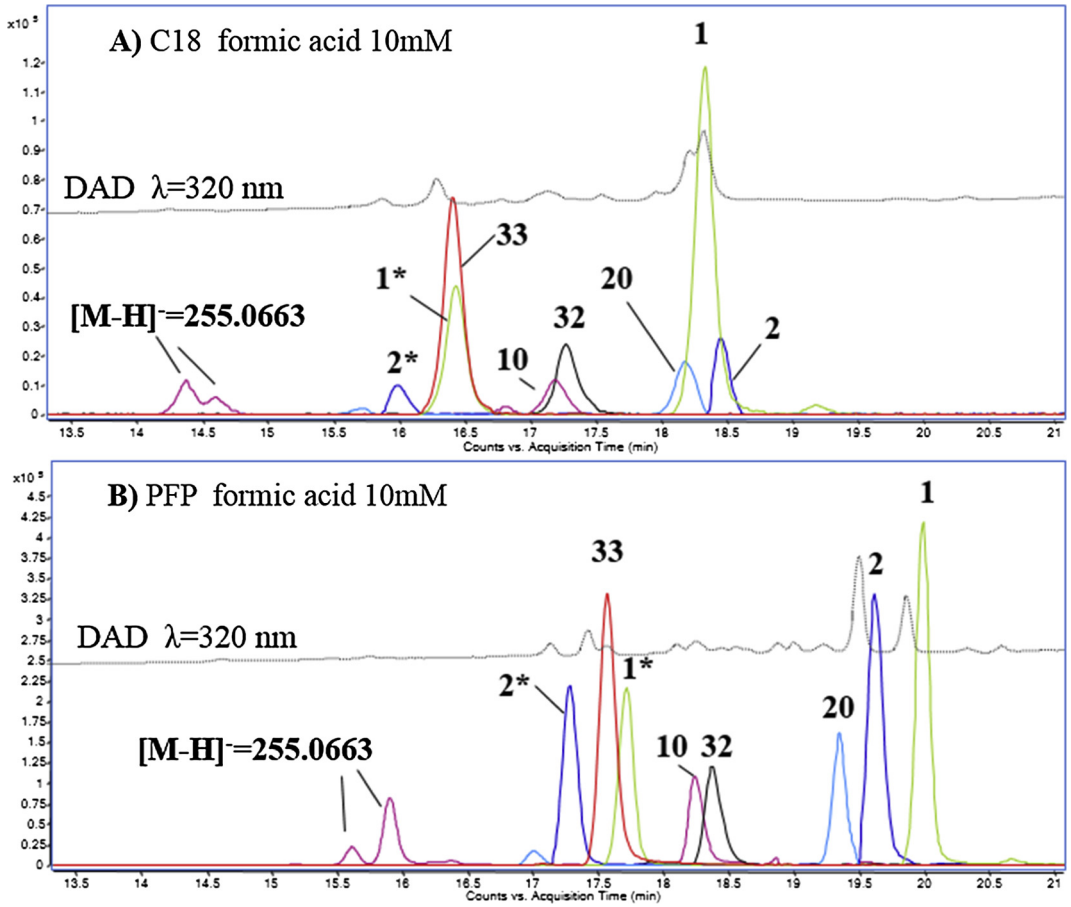

Fig. 2. Extracted Ion Chromatograms (accuracy 10 ppm) and DAD profile at 320 nm related to preliminary analysis of a *Z. punctata* extract. The analyses were performed with the C18 and the PFP column described in Section 2.3, using the same chromatographic gradient with different modifiers in the mobile phases. Numbering is reported in Table 1.

of chalcones (**1**) and (**2**) was inverted with the PFP column compared to that experimented with C18 (Fig. 2A). This fact could be ascribed to a stronger π interactions or H-bond for (**1**) with the stationary phase of the PFP column.

Despite the negative ionization, the acidified solvents generally incremented signal intensities of the selected compounds. This behavior is accounted to the strong tendency of phenolic compounds to form intramolecular and intermolecular bonds (Cuyckens & Claeys, 2004). Acidification also affected the chromatographic retention times. Flavonoids with -OH substituent in position 5 in the A ring may form intramolecular hydrogen bonding bridge with the carbonyl group in position 4 of the C ring producing retention times relative larger than corresponding positional isomers. Formic acid 1 mM was chosen as compromise between signal intensities and separation efficiency of isomers. The chromatographic behavior along to fragmentation patterns empowered the distinction of position-isomers at *m/z* 255.0663 from 5,7-dihydroxyflavanone (pinocembrin) (**10**) (Cuyckens & Claeys, 2002; Fabre, Rustan, De Hoffmann, & Quetin-Leclercq, 2001; Gardana, Scaglianti, Pietta, & Simonetti, 2007; Medana, Carbone, Aigotti, Appendino, & Baiocchi, 2008; Pellati, Orlandini, Pinetti, & Benvenuti, 2011) (Fig. 2B). Analogously, target isomers 2′-hydroxychalcone and 4′-hydroxychalcone exhibited very different retention times. The former was about two minutes more retained than the latter, confirming that the intramolecular bond with the vicinal carbonyl group is pivotal for the chromatographic behavior.

3.2. Identification scheme

The MS workflow used in this research consisted of three steps:

1. Determination of accurate MS signals in full scan analysis of suspect compounds selected from the relevant literature for *Z. punctata*.
2. MS identification of the largest peaks detected in DAD chromatographic profiles, with characteristic UV spectra.
3. Automatic MS fragmentation (AutoMS scan) of the previously selected MS precursor ions in order to characterize these compounds by fragmentation pattern.

The terms target **(t)** analysis here refers to the determination of compounds for which standards were available; suspect **(s)** screening to the identification of substances without standards, for which MS data and UV spectrum are inferable from the literature either on *Z. punctata* and northwestern propolis; non-target **(nt)** screening refers to compounds where no prior information is available. (Schymanski et al., 2015). In this last case, the tentative identification was made accounting for the MS fragmentation pattern, the UV spectra, the hydroxylation scheme as deriving from biological metabolism (Marston & Hostettmann, 2006; Hahlbrock & Grisebach, 1975), and the presence of intramolecular H-bond affecting the chromatographic behavior. Mass accuracy always ranged from 0.0 to 3.0 ppm in MS mode and from 0.0 to 8 ppm in tandem MS mode. Although high resolution MS spectra

improved the identification power, conventional low resolution values were often used through the text for sake of simplicity. All fragmentations of the identified compounds are reported in Table 2 with the relative nomenclature used in Fig. 1 (Fabre et al., 2001). The non-target compounds will be discussed in more detail.

3.3. Flavonoids

Flavonoids show a characteristic fragmentation behavior, such as loss of CO, CO_2, C_3O_2, by cleavage of two C—C bonds of the C-ring (Fig. 1). The biologically preferred hydroxylation is in positions 7 and 5 of the flavonoid backbone or in the 2′ and 4′ positions of chalcone backbone, respectively. On the other hand, the B-ring and the propyl chain (C3—C6) derive from the shikimic acid biosynthetic pathway whose typical hydroxylations are located at 4′, or 3′ and 4′, or 3′, 4′ and 5′ for mono, di- or tri- substituted, respectively (Fabre et al., 2001; Gardana et al., 2007; Hahlbrock & Grisebach, 1975; Medana et al., 2008).

Fragment ions on A-ring were typically observed at *m/z* 135 or 151 (*m/z* 135.0088 for $C_7H_4O_3$ and 151.0037 for $C_7H_4O_4$) with subsequent CO_2 loss, leading to the *m/z* 91 (*m/z* 91.0189) or 107 (*m/z* 107.0139), respectively (Fabre et al., 2001; Gardana et al., 2007; Medana et al., 2008). These ions are produced by fragmentation of $^{1,3}A^-$ or $^1A^-$ type for flavonoids or chalcones in negative ion mode according to the nomenclature used by Fabre et al. (2001) (Fig. 1, Table 2).

3.4. Flavanones, flavones and dihydroflavonols

Compounds previously reported for *Z. punctata* can be observed in Table 1. Pinostrobin (**11**, *m/z* 269.0819), izalpinin (**13**, *m/z* 283.0612), 3-hydroxy-7,8-dimethoxyflavone (**16**, *m/z* 297.0768) and 7-hydroxy-3′,4′-dimethoxyflavone (**17**, *m/z* 297.0768) were reported by Agüero et al. (2010), Jimenez, Sampietro, Sgariglia, Soberón, and Vattuone (2014). The HR used in this work demonstrated that neither their nominal mass weight nor coherent UV or MS fragmentation were present.

3-Hydroxyflavanones undergo typical loss of H_2O with a very large relative intensity, due to the presence of the —OH group in carbon 3 position. Among the 3-hydroxyflavanones we tentatively identified for the first time in *Z. punctata,* three compounds: 3,7-dihydroxy-5-methoxyflavanone (pinobanksin-5-methyl ether) **(36)** at *m/z* 285.0768, 3,7,8-trihydroxyflavanone **(37)** at *m/z* 271.0594, and 3,7-dihydroxyflavanone **(35)** at *m/z* 255.0663. This latter ion presented three positional flavanone analogues. 3,7-dihydroxyflavanone **(35)** was discriminated from 7,8-

Table 2
Fragmentation pattern with the relative abundance in parenthesis for the proposed flavonoids and caffeic acid derivatives identified in *Z. punctata* extracts. For flavonoids, product ions assignments (bold) are named according (Fabre et al., 2001). All signals are referred to theoretical mass, with mass accuracy <8 ppm in tandem MS mode. MSi stands for mass spectrometric identification level and N° for compound numbering as reported in Table 1, while t_r stands for retention times.

MSi	N°	t_r (min)	Fragments, tentative assignments and abundances (%)
Chalcones and flavanones			
s	**1**	15.90	**[M−H]$^-$**: 239.0714 (100); **[M−H−C$_2$H$_2$O]$^-$**: 197.0608(20); **[^{1}A]$^-$**:135.0088 (37); **[^{1}A−CO$_2$]$^-$**:91.0189 (15)
t	**1***	13.50	**[M−H]$^-$**: 239.0714 (100); **[M−H−C$_2$H$_2$O]$^-$**:197.0608 (60);**[^{1,3}A]$^-$**: 135.0088 (37); **[^{1,3}A−CO$_2$]$^-$**:91.0189 (16)
t	**2**	15.54	**[M−H]$^-$**:269.0819 (18); **[M−H−CH$_3$].$^-$**:254.0585 (71); **[^{1}A]$^-$**: 149.9959 (100); **[^{1}A−CO]$^-$**: 122.0009 (9); **[^{1}A−CO$_2$]$^-$**: 106.006 (21); **[^{1}A−CO$_2$]$^-$**: 94.006 (27)
s	**2***	13.04	**[M−H]$^-$**:269.0819 (6); **[M−H−CH$_3$].$^-$**:254.0585 (84); **[^{1,3}A]$^-$**:149.9959 (100); **[^{1,3}A−CO]$^-$**: 122.0009 (8); **[^{1,3}A−CO$_2$]$^-$**: 106.0060 (22); **[^{1,3}A−CO−CO]$^-$**: 94.0060 (29)
s	**3**	14.54	**[M−H]$^-$**:285.0768 (19); **[M−H-CH$_3$].$^-$**:270.0534 (100); **[^{1}A−CH$_3$].$^-$**:149.9959 (43); **[^{1}A−CH$_3$−CO$_2$].$^-$**: 106.0060 (5); **[^{1}A-CH$_3$−CO−CO].$^-$**:94.0060 (5)
s	**3***	9.72	**[M−H]$^-$**:285.0768 (44); **[M−H-CH$_3$].$^-$**:270.0534 (52); **[^{1,3}A−CH$_3$].$^-$**: 149.9959 (100); **[^{1,3}A−CH$_3$−CO$_2$].$^-$**: 106.0060 (7); **[^{1,3}A−CH$_3$−CO−CO].$^-$**: 94.0060 (10)
s	**10**	14.08	**[M−H]$^-$**:255.0663 (100); **[M−H−CO]$^-$**:227.0714 (7); **[M−H−C$_2$H$_2$O]$^-$**: 213.0557 (6); **[M−H−CO$_2$]$^-$**:211.0764; **[M−H−CO$_2$−CO]$^-$**:183.0753 (10); **[^{1,3}A]$^-$**: 151.0037 (13); **[^{1,3}A−CO]$^-$**:123.0088 (10)
nt	**34**	11.29	**[M−H]$^-$**:255.0663 (100); **[M−H−CO]$^-$**:227.0714 (49); **[M−H−H$_2$O]$^-$**: 237.0557 (10); **[M−H−CO$_2$]$^-$**:211.0764; **[M−H−CO$_2$−CO]$^-$**:183.0753 (10); **[^{1,3}A]$^-$**: 151.0037 (23); **[^{1,3}A−CO$_2$]$^-$**:107.0139 (10)
nt	**35**	10.90	**[M−H]$^-$**:255.0663 (100); **[M−H−H$_2$O]$^-$**:237.0557 (56); **[M−H−H$_2$O−CO]$^-$**: 209.0608 (36); **[M−H−CO−CO]$^-$**:199.0720 (8); **[^{1,3}A]$^-$**: 135.0088 (14)
nt	**36**	11.34	**[M−H]$^-$**:285.0768 (100); **[M−H-CH$_3$].$^-$**:270.0489 (31); **[M−H−H$_2$O]$^-$**: 267.0663 (18);
nt	**37**	11.96	**[M−H]$^-$**:271.0612 (100); **[M−H−H$_2$O]$^-$**: 253.0489 (15); **[M−H−H$_2$O−CO]$^-$**: 225.0557 (4); **[^{1,3}A]$^-$**: 151.0037 (10); **[^{1,3}A−CO$_2$]$^-$**: 107.0094 (5); **[^{1,3}A−C$_3$O$_2$]$^-$**: 83.0139 (5)
Flavones			
s	**14**	14.46	**[M−H]$^-$**: 283.0613 (11); **[M−H-CH$_3$].$^-$**: 268.0367 (100); **[M−H−CO−CO]**: 212.0479 (6)
s	**15**	14.52	**[M−H]$^-$**: 299.0561 (32); **[M−H-CH$_3$].$^-$**: 284.0326 (85); **[M−H−CO]$^-$**: 271.0612 (100); **[M−H−CO$_2$]$^-$**: 255.0299 (59); **[M−H−CO−CO]$^-$**:243.0663 (29); **[M−H−CO−CO$_2$]$^-$**: 227.0305 (25); **[^{1,2}A]$^-$**: 178.0179 (22); **[^{1,3}A]$^-$**:165.0149 (33)
t	**20**	15.27	**[M−H]$^-$**: 269.0455 (100); **[M−H−CO−CO]$^-$**: 213.0557 (3); **[M−H−C$_2$H$_2$O]$^-$**: 227.0305 (2); **[M−H−CO−CO$_2$]$^-$**: 197.0608 (2)
t	**25**	15.46	**[M−H]$^-$** = 253.0506 (100); **[M−C$_3$O$_2$−C$_2$H$_2$O]$^-$** = 143.0502 (28); **[^{1,3}A−CO$_2$]$^-$** = 107.0139 (17); **[^{0,4}B−C$_4$H$_2$O$_2$]$^-$** = 65.0033 (18); **[^{0,4}B−C$_4$H$_4$O$_2$]$^-$** = 63.0240 (48)
s	**26**	12.58	**[M−H]$^-$**: 253.0506 (100); **[M−H−CO]$^-$**: 225.0557 (11); **[M−H−HCO$_2$]$^-$**: 208.0529 (24); **[M−H−CO−CO]$^-$**: 197.0608 (11); **[M−H−HCO$_2$−CO]$^-$**: 180.0581 (15)
Dihydrochalcones			
nt	**38**	14.91	**[M−H]$^-$**:255.1027(28); **[M−H−CH$_4$]$^-$**:239.0713(2); **[M−H−CH$_4$−PhCH$_2$]$^-$**:149.0244(9); **[^{1}A−CH$_3$].$^-$**:136.0165(100); **[M−H−CH$_4$−PhCH$_2$−CO]$^-$**:121.0290(2); **[^{1}A−CH$_3$−CO].$^-$**:108.0216(31); **[^{1}A−CH$_3$−CO−CO].$^-$**:80.0267(5)
nt	**39**	15.54	**[M−H]$^-$**:241.0870 (100); **[M−H−H$_2$O]$^-$**:223.0764 (6); **[M−H−CO$_2$]$^-$**:197.0971 (15); **[^{2}A]$^-$**:150.0322 (98); **[^{1}A]$^-$**:135.0087 (59); **[^{2}A−CO]$^-$**:122.0373 (69); **[^{1}A−CO]$^-$**:109.0295 (67); **[^{1}A−CO$_2$]$^-$**:91.0189 (36)
Caffeic acid derivatives			
s	**32**	14.29	**[M−H]$^-$**: 327.1238 (100); 179.0350 (16); 163.0401 (20); 135.0452(10); 134.03731 (7); 119.0502 (7)
s	**33**	13.41	**[M−H]$^-$**: 343.1187 (100); 181.0506 (3); 179.0350 (30); 163.0765 (3); 161.0244 (4); 135.0452 (22)
nt	**40**	14.63	**[M−H]$^-$**: 311.1289 (100); 253.0506 (10); 163.0401 (44); 145.0295 (36); 119.0502 (23); 118.0424 (7)
nt	**41**	15.06	**[M−H]$^-$**: 311.1289 (100); 163.0401 (63); 145.0295 (23); 119.0502 (25); 118.0424 (11)
nt	**42**	16.42	**[M−H]$^-$**: 315.1602 (8); 179.0350 (12); 178.0272 (10); 135.0452 (8); 134.0373 (100); 133.0289 (8)
nt	**43**	14.34	**[M−H]$^-$**: 357.1344 (23); 193.0506 (100); 179.0350 (7); 178.0272 (11); 163.0759 (21); 149.0608 (6); 134.0373 (54)
nt	**44**	14.52	**[M−H]$^-$**: 357.1344 (100); 342.1109 (7); 193.0506 (26); 179.0350 (67), 161.0244 (16); 135.0452 (43); 134.0373 (41)

dihydroxyflavanone **(34)** by its much larger peak intensity due to a water loss. The third analogue was assigned to pinocembrin **(10)** owing to the longer retention time and also by comparing the tandem MS spectra on the mz Cloud™ database (https://www.mzcloud.org/). The isoflavone biochanin (**27**), a target compound, was never identified in the samples.

3.5. Chalcones and flavanone isomers

In plants, chalcones are converted into the corresponding flavanones with a stereospecific reaction catalyzed by the enzyme chalcone isomerase. This close structural and biogenetic relationship explains why they often co-occur in natural products (Veitch & Grayer, 2006). Chalcone and flavanone isomers are distinguishable as the former have an UV absorbance in the 300–350 nm range and the latter at about 230 and 290 nm. The typical cleavages of chalcones are described in Fig. 1 and the fragmentation behavior is reported in Table 2.

They can be recognized by considering their relative retention times, since chalcones were more retained owing to their additional double bond. Anyway, in the case of isobaric compounds at *m/z* 239.0714, 7-hydroxyflavanone (**1**∗) was identified by a C_2H_2O loss (product ion at *m/z* 197.0608) larger than its open-chain analogue, 2′,4′-dihydroxychalcone (**1**). This fact is consistent with the presence of the C ring of the flavanones (Portet et al., 2008). The neutral loss of 104 corresponds to a vinylbenzene related to an unsubstituted B-ring. In the same way, $[M{*}{*}-H]^-$ at *m/z* 269.0819 presented two isobaric analogues comparable in terms of fragmentation pattern. The identification of these two compounds as 2′,4′-dihydroxy-3′-methoxychalcone **(2)** and 7-hydroxy-8-methoxyflavanone **(2∗)** was possible only on the basis of their UV spectra and by comparison with pure standards.

The double signal at *m/z* 285.0768 was assigned to 7,4′-dihydroxy-5-methoxyflavanone (**3**∗) and to 2′,4′,4-trihydroxy-6′-methoxychalcone (**3**). Compound (**3∗**) showed the product ion corresponding to $[^{1,3}A\text{-}CH_3]^{\cdot-}$ as base peak, whilst for (**3**) the loss of the CH_3 radical resulted the most probable MS cleavage. The assignment of hydroxyls position on the B-ring was chosen on the basis of their previous identification in propolis from Catamarca (Vera et al., 2011), although identification in *Z. punctata* was never verified before. Chalcones (**4**), (**5**) and (**6**), employed here as target compounds, were not found in any samples.

3.6. Dihydrochalcones

Chalcones and dihydrochalcones are sometimes described together with flavanones and dihydroflavonols, because they have similar UV absorption (Veitch & Grayer, 2006).

A very large peak at *m/z* 255.1027 with raw formula $C_{16}H_{16}O_3$ was found. It showed a peculiar UV spectra with λ_{max} at 228, 269 and 306 nm (Fig. 3A) comparable to those reported by Wollenweber, Dörr, Stelzer, and Arriaga-Giner (1992) for 4′-hydroxy-2′-methoxydihydrochalcone. No product ions typical of flavanones or chalcones were found (Fig. 3B), whereas fragment ions at *m/z* 163.0401, 149.0244, and 136.0166 (base peak) were ascribable to the $^2A^-$ fragmentation scheme described for dihydroxychalcones (Portet et al., 2008). Furthermore, the double loss of the CH_3 radical and CH_4 suggested the presence of acid hydrogens in the α carbon, thus indicating the possible presence of a methoxy group in position 2 of the A ring. Thus, the structure of 4′-hydroxy-2′-methoxy-dihydrochalcone (**38**) is proposed for the ion at *m/z* 255.1027.

Similarly, a compound with formula $C_{15}H_{14}O_3$ at *m/z* at 241.0870 showed the MS fingerprint of dihydrochalcones, with a water loss characteristic of two or more hydroxyl groups in the A-ring (Portet et al., 2008) (Fig. 3C). In this case we propose the structure of a 2′,4′-dihydroxydihydrochalcone (**39**). Product ions of the two dihydrochalcones are described in Table 2 with the relative assignments. To our best knowledge, this is the first time that these compounds are described for *Z. punctata*.

3.7. Caffeic acid derivatives

Like the B-ring of flavonoids, the hydroxylation pattern preferred for the caffeic acid derivatives derives from the shikimic acid metabolic pathway with OH groups located at 4′, or 3′ and 4′, or 3′,4′ and 5′ for mono, di- or try-substituted, respectively (Hahlbrock & Grisebach, 1975). The 1-methyl-3-(3′,4′-dihydroxyphenyl)-propyl caffeic acid ester at *m/z* 343.1187 (**33**) and 1-methyl-3-(4′-hydroxyphenyl)-propyl caffeic acid ester at *m/z* 327.1238 (**32**) were previously identified in *Z. punctata* by Svetaz et al. (2004) and they exhibited very good antifungal activity against *Phomopsis longicolla,* a soybean parasite. For compound (**33**) the fragment at *m/z* 163.0765 was ascribed to 1-methyl-3-(3′,4′-dihydroxyphenyl) moiety.

The compound with *m/z* 315.1602 (**42**) was tentatively assigned to a 3,7-dimethyl-2,6-octadienyl caffeic acid ester (geranyl caffeate). This hypothesis was supported by the relevant abundance of fragments at *m/z* 134 and 178, with ion at *m/z* 134 as the base peak, corresponding to the radical ions from fragmentation of the caffeic acid moiety. It is known that when a C atom linked to the carboxylic group is allylic, the product ions 134 and 178 are comparable or more abundant than the usual 135 and 179 ones, and 161, derived from a water loss of the deprotonated caffeic acid ion, is practically absent (Gardana & Simonetti, 2011; Jaiswal, Matei, Ullrich, & Kuhnert, 2011; Medana et al., 2008).

Two relevant isobaric signals at *m/z* 357.1344 were efficiently separated. Compound (**43**) was ascribed to a ferulic acid ester, considering diagnostic the presence of the product ions at *m/z* 193.0506 (base peak), 134.0372 and 149.0608, even considering the tandem MS spectra of the ferulic acid obtained from the mz Cloud™ database. The ion at *m/z* 163.0765 was attributed to 1-methyl-3-(3′,4′-dihydroxyphenyl) ester, as for (**33**). Thus, (**43**) was attributed to 1-methyl-3-(3′,4′-dihydroxyphenyl)-propyl ferulic acid ester. Its isobaric compound (**44**) showed a fragmentation pattern typical of caffeic acid, with remaining insaturations indicating a phenolic-derived ester. We hypothesized that the presence of the fragment at *m/z* 193.0506 can be attributed to the methyl ester of the caffeic acid, whilst the other methylation was assigned to a methoxy group in the phenolic alcohol, as suggested by the loss of a methyl radical (ion at *m/z* 342.1109). For this compound, we propose the structure of the 2-methyl-3-(3′-methoxy-4′-hydroxyphenyl)-propyl caffeic acid ester.

Ion at *m/z* 311.1289 showed two resolved signals, the main peak and a shoulder. The major signal at t_r 15.06 min (**41**) exhibited product ions typical of a coumaric acid (163 and 119) and the ion with *m/z* 145.0295, related to a water loss. Even in this case, we recognized a phenolic moiety in the esteric function, so that the structure of the 1-methyl-3-(4′-hydroxyphenyl)-propyl *p*-coumaric acid ester was proposed. The second compound (**40**) at t_r 14.63, presenting a further characteristic loss of terbutyloxyl function (−58), was identified as 4′-terbutyloxy phenyl *p*-coumaric acid ester. The chromatographic elution order confirms these hypotheses, with the propyl derivate more retained than the terbutyloxyphenyl one. Chlorogenic acid (**31**), a target compound, was not identified in the samples here studied. All identifications for caffeic acid derivatives are reported in Table 2.

3.8. Characterization of Zuccagnia punctata and related propolis

Z. punctata is a shrub growing in xerophilic environment, and synthesizes high levels of flavonoids and caffeic acid derivatives

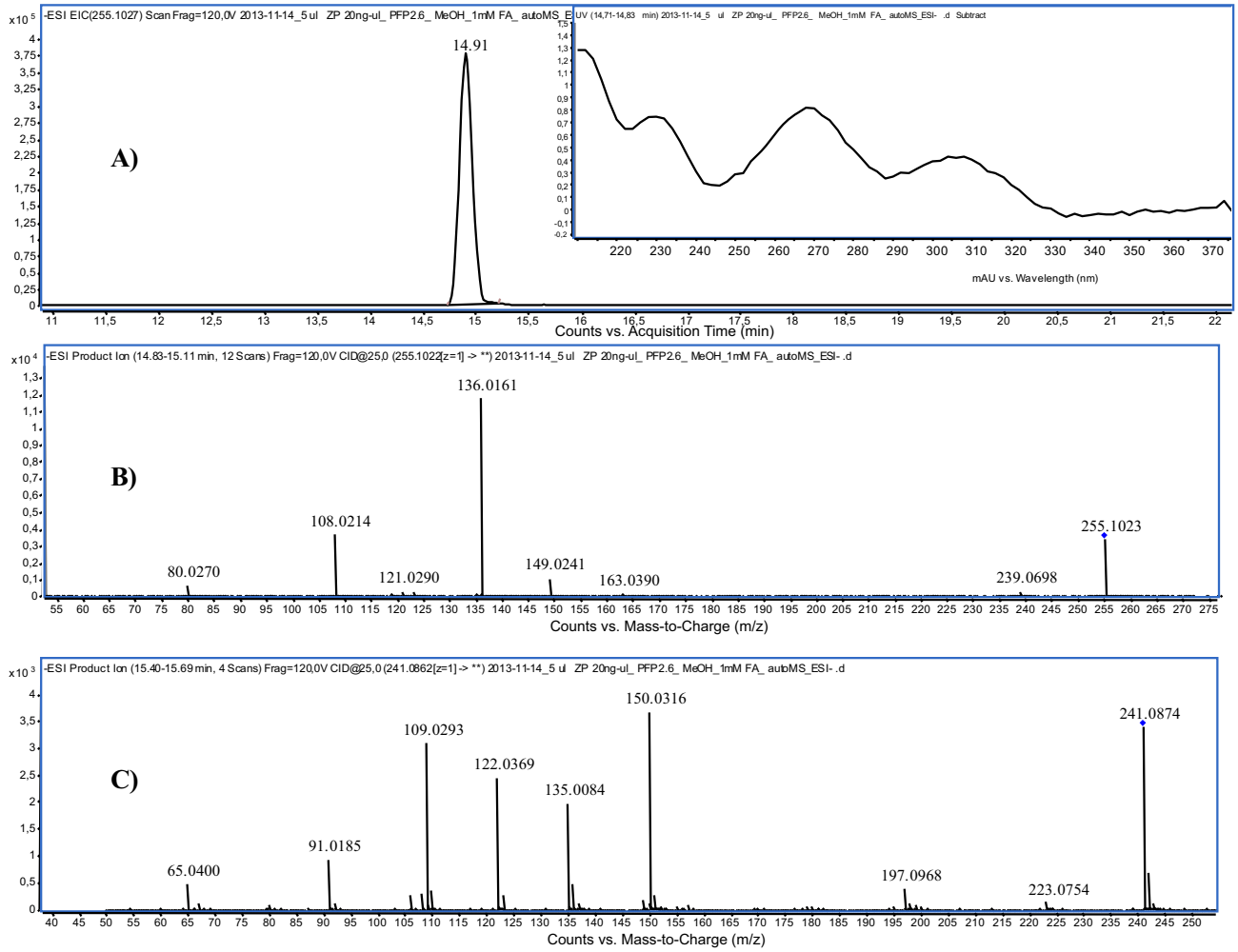

Fig. 3. Extracted Ion Chromatogram (accuracy 10 ppm) and UV spectra (A) related to signal at *m/z* 255.1027 with its tandem MS spectrum (B). Tandem MS spectrum of signal at *m/z* 241.0875 is reported in (C).

for protection against overexposure to UV light (Villagra et al., 2011). The major phenolic components reported for *Z. punctata* were so far restricted to the chalcones (**1**) and (**2**) and flavanone (**1**∗), so that the observed biological effects have been attributed to them (Agüero et al., 2010; Svetaz et al., 2004, 2007; Vattuone et al., 2013). Although also two caffeic acid derivatives (**32** and **33**) have been identified in *Z. punctata,* few details about their biological activities have been described (Svetaz et al., 2004). The present paper clearly pointed out the relevant presence of many other potentially bioactive compounds in *Z. punctata* extracts. In fact, eleven constituents were identified for the first time in this plant, and particularly 4′-hydroxy-2′-methoxydihydrochalcone (**38**) resulted to be the major constituent. They all are summarized in Table 1. The amount of each component is expressed as relative percentage area with respect to the most abundant one (**38**). According to these results, (**38**), recognized here for the first time in *Z. punctata*, constitutes the major metabolite together with **(1)**. The (**39**) is third compound that makes chalcones the most representative for *Z. punctata* (Fig. 4). Among the caffeic acid derivatives, which are the second group for abundance, the non-target compound here identified as (**41**) was comparable in terms of abundance to the already known (**32**). The amount of geranyl caffeate (**42**) was also significant, whilst the presence of a ferulic derivative (**43**) resulted notable. A typical marker in propolis type-poplar is caffeic acid phenethyl ester (CAPE), totally absent in *Z.p.*-E. Flavanones were the third group of compounds providing substantial abundance to the phenolic content, being the already described (**1**∗) and (**2**∗) the major components. The identification of the two dihydrochalcones and the flavanones related to the isomeric chalcones suggests that different biosynthetic pathways and environmental regulators affect dihydrocalcones or flavanones production starting from chalcones.

On the basis of the above considerations, (**1**), (**2**) and their analogues (**1**∗) and (**2**∗), (**38**), (**39**), together with caffeic acid ester (**32**), (**33**) and *p*-coumaric acid ester (**41**) are here proposed as the main phenolic components of *Z. punctata* (Fig. 4).

A preliminary survey was attempted on four samples collected from apiaries located in semi-arid mountain where *Z. punctata* grows (Table S1) in order to ascertain the primary botanical source of northwestern propolis. Results showed that all 25 compounds here identified for *Z. punctata* were also found with very similar proportions in extracts of T1 and C2 propolis. Indeed, the seven proposed markers resulted highly expressed in these propolis, resulting geranyl caffeate (**42**) even magnified.

Albeit pinostrobin (**11**), izalpinin (**13**), (**16**) and (**17**) were previously reported (Agüero et al., 2010; Jimenez et al., 2014; Vera et al., 2011), they were not found in the plant extracts with the applied method. When analysis of flavonoids is made with LC-DAD technique or with low resolution MS analyzers, misidentifications are often possible. For instance, previous papers reported pinocembrin (**10**) ($[M-H]^-$ = 255.0663) as a marker of northwestern argentinean propolis related to *Z. punctata* (Agüero et al., 2010; Isla,

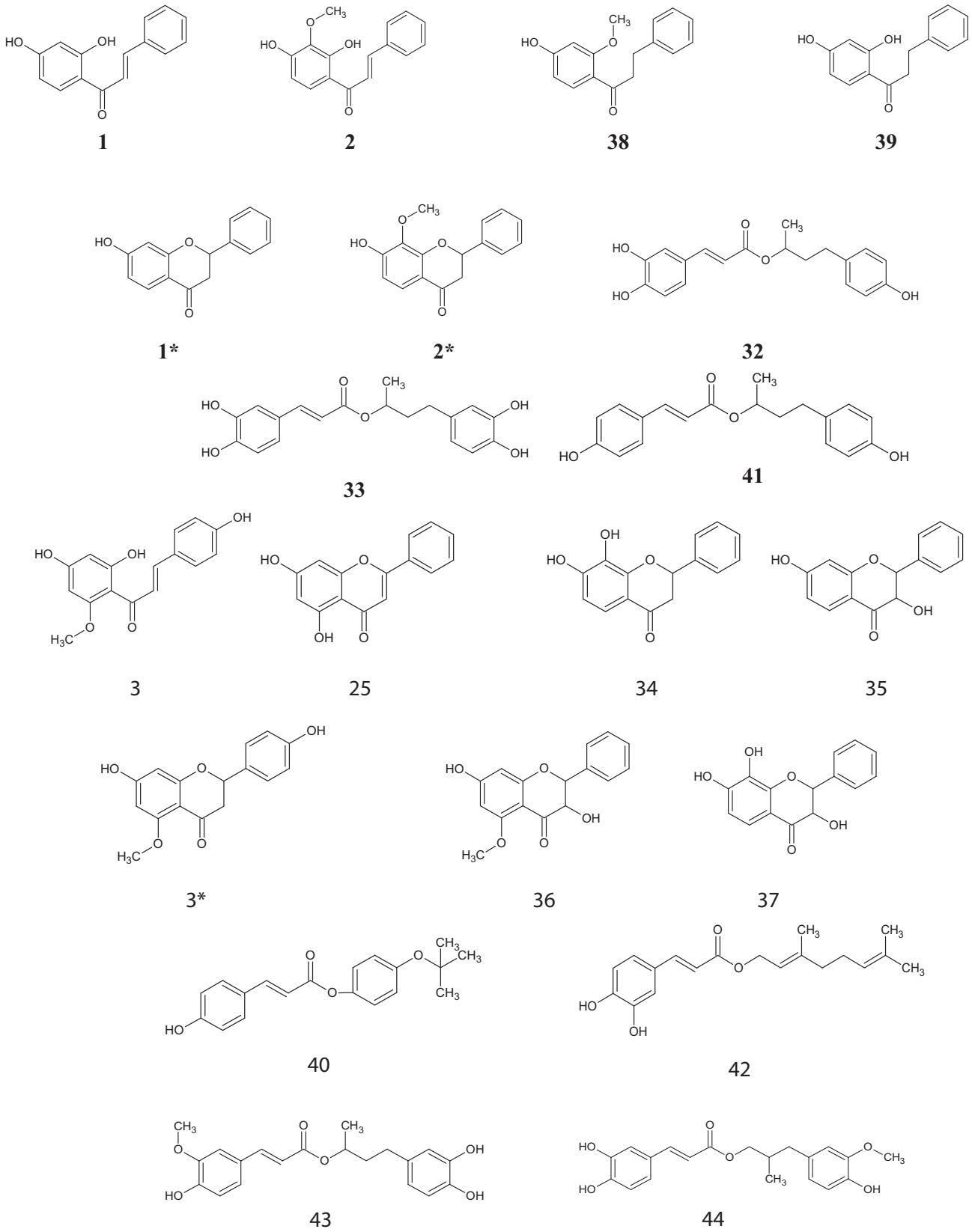

Fig. 4. Structural formulas of the phenolic compounds firstly identified in *Z. punctata* and proposed markers (in bold) for propolis type *Z. punctata*. Compounds (**1**), (**1***), (**2**), (**2***), (**32**) and (**33**) were already reported in literature (see Table 1, which also shows numbering).

Paredes-Guzman, Nieva-Moreno, Koo, & Park, 2005). Pinocembrin can be easily mistaken with other isobaric compounds in low resolution MS such as the cited 4′-hydroxy-2′-methoxydihydrochalcone (**38**) ($[M-H]^-$ = 255.1025). The high resolution MS analysis evidenced a limited contribution of pinocembrin in T1 and C2 propolis, whereas the ion at *m*/*z* 255.1025 was very intense.

Conversely, propolis named T2 and C1, although collected very close to the T1 and C2 did not exhibit significant similarities with the *Z. punctata* chemical profile. The phytogeographycal region was actually different and consequently even the biocomponents, so that a different primary botanical source has to be expected for the T2 and C1 samples. It is noteworthy that a significant chemical correspondence between *Z. punctata* and T1 and C2 does not exclude the participation of other plants species as a secondary source. Thus, a propolis can be categorized as type-*Zuccagnia* when (**1**), (**2**) and its related (**1**∗), (**2**∗), (**38**) and (**39**), together with (**32**), (**33**), and (**41**) are present as major components regardless of proportions.

4. Conclusions

The LC-HR tandem MS approach was used to assist the selective and reliable characterization of the botanical phenolic profile. The analysis performed on *Z. punctata*, a typical plant of the northwestern regions of Argentina with very promising bioactivity, revealed for the first time the characteristic signals assigned to two peculiar compounds belonging to the class of dihydrochalcones together with other chalcones, flavanones and caffeic acid derivatives. Eleven constituents were identified for the first time in this plant, and particularly the 4′-hydroxy-2′-methoxydihydrochalcone resulted present as major constituent. The HR approach was particularly useful to point out some misidentifications reported in the literature, obtained with low resolution MS techniques. The new identified compounds indicated an unusual phenolic profile that can be specifically ascribed to *Z. punctata*, and related to the particular phytogeographical area of this plant. This fingerprint was also recognized in two propolis collected where this plant grows, whereas other two propolis, collected in close sites with a lower diffusion of that plant, were significantly different. The presence of the bioactive compounds in propolis will allow their pharmaceutical use preserving the green plant. This is useful as *Z. punctata* plays an important role as hydric regulator of the ecosystem.

Since the northwestern propolis are included in the Argentinean Food Code, the well-known sweetness and anti-parasitical properties of several dihydrochalcones would be interesting for further nutraceutic applications.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jff.2017.04.003.

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