Biochemical Systematics and Ecology 68 (2016) 23–26

Contents lists available at ScienceDirect

Biochemical Systematics and Ecology

journal homepage: www.elsevier.com/locate/biochemsyseco

Flavonoids from *Casearia sylvestris* Swartz variety *lingua* (Salicaceae)

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ARTICLE INFO

Article history:
Received 3 February 2016
Received in revised form 3 June 2016
Accepted 4 June 2016
Available online 5 July 2016

Keywords:
Casearia sylvestris variety *lingua*
Salicaceae
Glycosylated flavonoids

1. Subject and source

Casearia sylvestris Sw. (Salicaceae) is the most widely studied species of the genus *Casearia* Jacq, due to its use in folk medicine and its unique biological properties (Santos et al., 2010). *C. sylvestris* is highly adaptable, its range extending from Mexico to South America. Based on the external morphological differences of *C. sylvestris* specimens, Sleumer (1980) proposed two varieties of this species: *C. sylvestris* var. *sylvestris*, which inhabits humid and dense forests; and *C. sylvestris* var. *lingua*, which commonly occurs in open and xeric habitats (Cavallari et al., 2010). Most previous studies of the biological activities of *C. sylvestris* have employed *C. sylvestris* var. *sylvestris*. To date, *C. sylvestris* var. *lingua* has not been the primary focus of phytochemical investigations.

In the present work, leaves of *Casearia sylvestris* var. *lingua* (Salicaceae) were collected in August 2012 in the city of Araraquara (São Paulo State, Brazil; 21°49.300′S, 48°11.460′W). A voucher specimen (IAC 55839) was deposited at the Herbarium of the Agronomic Institute of Campinas. After collection, the leaves were immediately dried at 40 °C in an oven with air circulation and were then crushed with liquid nitrogen in an analytical mill. The resulting powder was stored at room temperature until used in the phytochemical procedures. For comparison, leaves of *Casearia sylvestris* var. *sylvestris* (IAC 55840) were also collected from the same field and were processed and deposited under the same conditions described above for *C. sylvestris* var. *lingua*.

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http://dx.doi.org/10.1016/j.bse.2016.06.002

2. Previous work

Previous chemical investigations of the genus *Casearia* have led to the identification of more than 285 compounds, including terpenoids, steroids, phenylpropanoids, and flavonoids. These include 152 clerodane diterpenes, which are considered taxonomic markers for this genus (Xia et al., 2015). Phytochemical investigations of *C. sylvestris* revealed the presence of approximately 41 clerodane diterpenes including casearins, (Itokawa et al., 1990; Morita et al., 1991; Carvalho et al., 1998; Santos et al., 2007, 2010; Wang et al., 2009a), casearvestrins (Oberlies et al., 2002), and casearisides (Wang et al., 2009b), among others. These compounds were found in the leaves, stem, stem bark, roots, and seeds of the plant (Carvalho et al., 2009). In addition to clerodane diterpenes, other compounds observed in this species include non-clerodane diterpenoids, sesquiterpenoids, phenylpropanoids, and phenolic compounds (Xia et al., 2015). Four ellagic acid derivatives were identified in *C. sylvestris* aqueous extracts (Da Silva et al., 2008a), and compounds found in alcoholic extracts include two gallic acid derivatives (Da Silva et al., 2008b), tyrosol (Wang et al., 2009c), and rutin (Silva et al., 2006).

3. Present study

Powdered leaves (27 g) of *C. sylvestris* var. *lingua* were successively extracted three times, at room temperature, using 175 mL of a 50:30:20 (% v/v) mixture of water/ethanol/isopropanol (Bueno et al., 2015). After evaporation of the alcohol, the extracts were lyophilized to obtain 5.6 g of crude dry extract (CDE). A portion (3 g) of this extract was fractionated using a medium-pressure chromatography system (Puriflash 4100, Interchim) equipped with two C-18 flash columns (Puriflash C18HQ, 15 μm, 35 g, Interchim). Elution was performed using a gradient of water (A) and methanol (B): 5% of B from 0 to 8 min, 5–25% of B to 35 min, 25–100% of B to 65 min, and 100% of B to 110 min. The flow rate was 8 mL/min and the UV detection wavelength was 254 nm. Fractions 2, 3, and 4 were combined and lyophilized to yield 2.3 g of dry material (denoted CDE-F1). The isolated CDE-F1 fraction was submitted to solid phase extraction (SPE) with polyamide, and the resulting methanolic fraction was dried to give 113 mg of dry matter. Finally, the methanolic fraction was dissolved in water/acetonitrile (80:20, v/v) at a concentration of 19 mg/mL, filtered through a 0.22 μm nylon filter (Millipore), and fractionated by preparative HPLC using a Shimadzu LC-8A chromatograph equipped with an SCL-10Avp controller and an SPD-M10Avp diode array detector. The compounds were separated on a Luna C-18 chromatographic column (5 μm, 150 × 21.5 mm, Phenomenex) using isocratic elution with an 80:20 (v/v) mixture of water/acetonitrile containing 0.1% (v/v) formic acid. The injection volume and flow rate were 0.4 mL and 10 mL/min, respectively. Under these conditions, 15 compounds were isolated and putative identification was achieved for ten of them (Table 1) using high resolution mass spectrometry (HRMS) and MS/MS in negative mode (micrOTOF-Q II, Bruker Daltonics). For five of the substances (1–5), structural elucidation was carried out by means of ^{1}H, ^{13}C, and 2D NMR analyses (Bruker Advance III HD 600, 14.7T), in order to confirm the putative identities and the glycosylation

Table 1
Identification of the main compounds in *C. sylvestris* var. *lingua* by HRMS and MS/MS in negative mode.

Peak[a] (#)	Compound name	*RR*t (min)	UV max (nm)	Negative ionization $[M-H]^-$ (m/z)		Exact mass (experimental)	Exact mass (calculated)	Error (ppm)
				HRMS	MS/MS			
1	(+)-Catechin	2.6	279	289.0729	30 eV: 289 → 245; 221; 205; 203; 187; 179; 165	290.0802	290.0790	4.137
A	Isorhamnetin-3-*O*-trihexoside	9.4	254 (265 sh); 353	769.2203	55 eV: 769 → 314 (315)	770.2276	770.2270	0.779
B_1	Quercetin-3-*O*-dihexoside			579.1389	40 eV: 579 → 300 (301)	580.1462	580.1428	5.861
B_2	Kaempferol-3-*O*-dihexoside	9.6	256 (265 sh); 353	593.1537	50 eV: 593 → 284 (285)	594.1610	594.1584	4.376
B_3	Quercetin-3-*O*-hexoside			463.0907	30 eV: 463 → 300 (301)	464.0980	464.0955	5.387
2	Quercetin-3-*O*-rutinoside (rutin)	10.0	256 (265 sh); 353	609.1486	45 eV: 609 → 300 (301)	610.1559	610.1534	4.097
3	Isorhamnetin-3-*O*-neohesperidoside	10.6	254 (265 sh); 354	623.1646	45 eV: 623 → 314 (315)	624.1719	624.1690	4.646
4	Isorhamnetin-3-*O*-rutinoside (narcissin)	11.5	255 (267 sh); 354	623.1665	50 eV: 623 → 314 (315)	624.1738	624.1690	7.690
C	Quercetin-3-*O*-hexoside	12.0	256 (267 sh); 354	433.0811	35 eV: 433 → 300 (301)	434.0884	434.0849	8.063
D_1	Isorhamnetin-3-*O*-hexoside			477.1076	45 eV: 477 → 314 (315)	478.1149	478.1111	7.948
D_2	Isorhamnetin-3-*O*-[rhamnopyranosyl-3-hydroxy-3-methylglutaryl]-hexoside	12.4	254 (267 sh); 352	767.2065	30 eV: 767 → 623 ([(M-H)-144]$^-$); 314 (315)	768.2138	768.2113	1.953
D_3	Kaempferol-3-*O*-dihexoside			563.1448	35 eV: 563 → 284 (285)	564.1521	564.1479	7.445
5	Isorhamnetin-3-*O*-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranoside	13.2	254 (265 sh); 353	593.1553	40 eV: 593 → 314 (315)	594.1626	594.1585	6.901
E	Isorhamnetin-3-*O*-[3-hydroxy-3-methylglutaryl]-hexoside	13.7	254 (267 sh); 351	621.1502	55 eV: 621 → 477 ([(M-H)-144]$^-$); 314 (315)	622.1575	622.1534	6.590
F	Isorhamnetin-3-*O*-pentoside	14.1	255 (265); 351	447.0975	40 eV: 447 → 314 (315)	448.1048	448.1006	9.373

[a] Compounds B1, B2, B3, D1, D2, and D3 were identified in mixtures. For compounds D_2 and E, the neutral loss of 144 corresponds to a 3-hydroxy-3-methylglutaryl moiety. Compounds 1–5 were confirmed by ^{1}H, ^{13}C, and 2D NMR spectroscopy.

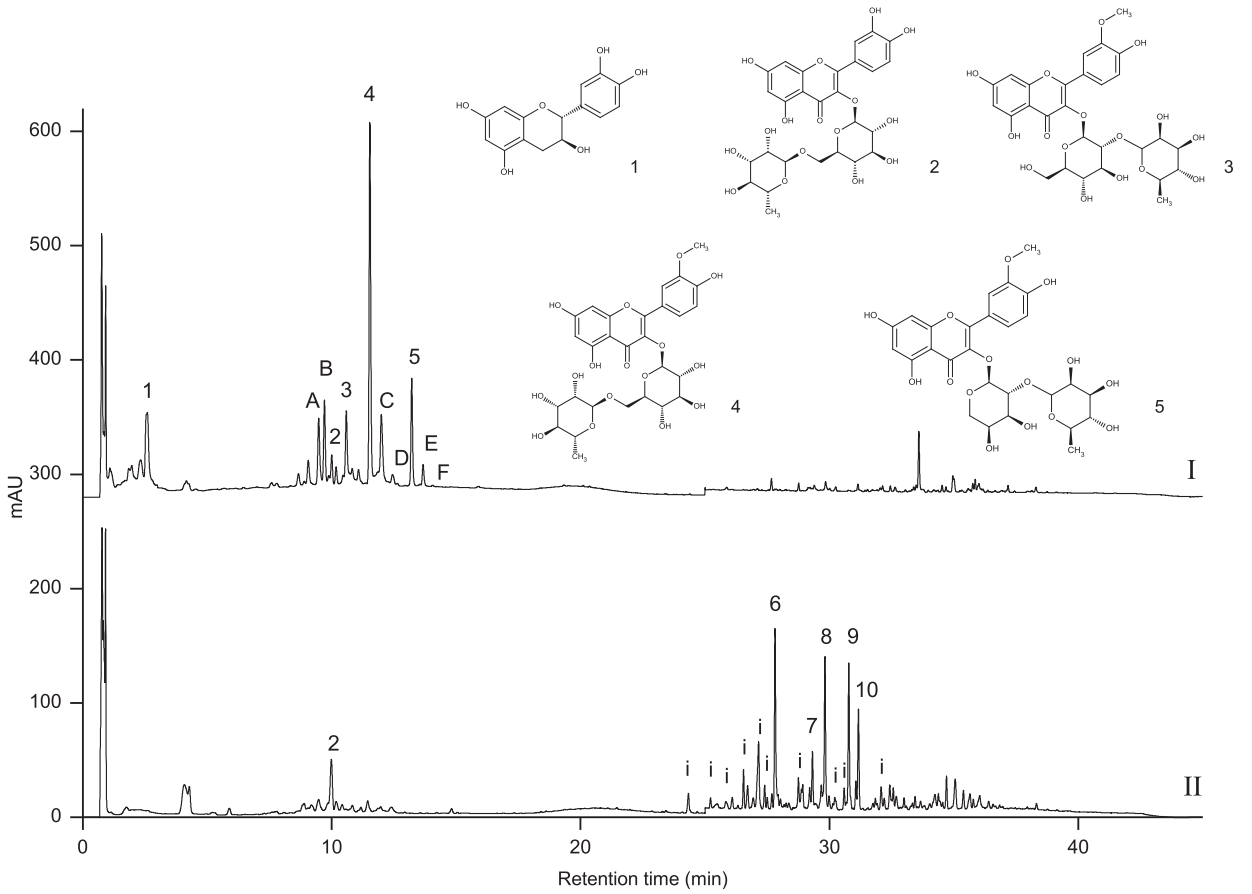

Fig. 1. Chromatogram of *C. sylvestris* var. *lingua* (I). Chromatogram of *C. sylvestris* var. *sylvestris* (II). Flavonoids from *C. sylvestris* var. *lingua*: (+)-catechin (**1**); quercetin-3-*O*-α-L-rhamnopyranosyl-(1 → 6)-β-D-glucopyranoside (rutin, **2**); isorhamnetin-3-*O*-α-L-rhamnopyranosyl-(1 → 2)-β-D-glucopyranoside (isorhamnetin-3-*O*-neohesperidoside, **3**); isorhamnetin-3-*O*-α-L-rhamnopyranosyl-(1 → 6)-β-D-glucopyranoside (narcissin, **4**); isorhamnetin-3-*O*-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranoside (**5**). Letters A to F correspond to the flavonoids identified by HRMS and MS/MS (putative identifications). Diterpenes from *C. sylvestris* var. *sylvestris*: casearin D (**6**); caseargrewiin F (**7**); casearin S (**8**); casearin I (**9**); casearin J (**10**). The symbol (i) corresponds to the clerodane diterpenes identified by means of their characteristic UV spectra (Bueno et al., 2015).

pattern. The five compounds were identified as follows: (+)-catechin (**1**), quercetin-3-*O*-α-L-rhamnopyranosyl-(1 → 6)-β-D-glucopyranoside (rutin, **2**), isorhamnetin-3-*O*-α-L-rhamnopyranosyl-(1 → 2)-β-D-glucopyranoside (isorhamnetin-3-*O*-neohesperidoside, **3**), isorhamnetin-3-*O*-α-L-rhamnopyranosyl-(1 → 6)-β-D-glucopyranoside (narcissin, **4**), and isorhamnetin-3-*O*-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranoside (**5**).

Comparison of *C. sylvestris* var. *lingua* and *C. sylvestris* var. *sylvestris* was performed by chromatographic profiling using UHPLC-DAD (Ultimate 3000RS, Dionex), as described by Bueno et al. (2015). Briefly, 50 mg of powdered sample was extracted by sonication with 1 mL of a 50:30:20 (% v/v) mixture of water/ethanol/isopropanol. The mixture was centrifuged at 5000 *g* for 5 min, and 0.7 mL of the supernatant was filtered through a 0.22 μm nylon filter (Millipore) and immediately submitted to chromatographic analysis. Separation was performed using a Kinetex C-18 column (2.6 μm, 150 × 2.1 mm, Phenomenex). Gradient elution was performed with water (A) and acetonitrile (B), under the following conditions: 10–25% of B from 0 to 15 min, 25–90% of B to 35 min, 90% of B to 40 min, 90-10% of B from 40 to 42 min (return to the initial conditions), 10% B to 45 min (equilibration under the initial conditions). The flow rate, oven temperature, and injection volume were 400 μL/min, 35 °C, and 2 μL, respectively. The identification of the major chromatographic peaks corresponded to the clerodane diterpenes was carried out by means of their UV spectra and comparison with authentic standards.

4. Chemotaxonomic significance

In the present phytochemical study, fourteen glycosylated flavonoids and one catechin were isolated and identified from the leaves of *C. sylvestris* var. *lingua* (Table 1). The occurrence of flavonoids in *C. sylvestris* var. *lingua* was observed previously in genetic and intraspecific chemical variability studies undertaken by Silva et al. (2006) and Bueno et al. (2015), but only rutin (**2**) was identified to date.

Therefore, this is the first report to confirm the natural occurrence of (+)-catechin and 3-*O*-glycosylated flavonoids in *C. sylvestris* and provide new information on the chemistry of this species, especially the observed glycosylation patterns. With the exception of (+)-catechin, all the compounds were identified as 3-*O*-glycosylated isorhamnetin, quercetin, or kaempferol combined with one, two, or three sugar moieties, and two of the compounds (D2 and E, Table 1) contained a hydroxymethylglutaryl moiety. In earlier work, Mosaddik et al. (2006) also isolated catechin, kaempferol, and *O*-glycosylated flavonoids (kaempferol and quercetin) from the leaves of *Casearia grayi* Jessup.

The identification of these flavonoids in *C. sylvestris* var. *lingua* provided further differentiation from *C. sylvestris* var. *sylvestris*. Comparison of the chromatographic profiles of these two varieties (Fig. 1) evidenced significant differences in secondary metabolite composition. The findings corroborated studies of the intraspecific chemical variability of *C. sylvestris* that showed a prevalence of clerodane diterpenes in *C. sylvestris* var. *sylvestris*, while phenolic compounds predominated in *C. sylvestris* var. *lingua* (Bueno et al., 2015). In summary, the differential expression of these two classes of secondary metabolites in the two varieties illustrates their chemotaxonomic distinction. In addition to clerodane diterpenes, glycosylated flavonoids may be used as chemotaxonomic markers for distinguishing these *C. sylvestris* varieties.

Acknowledgements

The authors are grateful to Dr. Nivaldo Boralle and Dr. Lucineia Vizzotto Marconcini for the acquisition of NMR spectra and José Carlos Tomaz for the acquisition of mass spectra. The authors thank Dr. Juliana Rodrigues and João Luis Bronzel for laboratory assistance. This work was supported by grants from the São Paulo State Research Foundation (FAPESP, grants #2011/21440-3 and #2013/07600-3), the Brazilian Coordination for the Improvement of Personnel in Higher Education (CAPES), and the Brazilian Council for Scientific and Technological Development (CNPq).

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