J. Braz. Chem. Soc., Vol. 22, No. 6, 1096-1102, 2011.
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0103 - 5053 $6.00+0.00

Article

An HPLC-DAD Method to Quantification of Main Phenolic Compounds from Leaves of *Cecropia* Species

Geison M. Costa, Caroline F. Ortmann, Eloir P. Schenkel and Flávio H. Reginatto*

Departamento de Ciências Farmacêuticas, Centro de Ciências da Saúde, Universidade Federal de Santa Catarina, 88040-900 Florianópolis-SC, Brazil

Um método eficiente e reprodutível por CLAE-DAD foi desenvolvido e validado para quantificação simultânea dos compostos fenólicos majoritários (ácido clorogênico, isoorientina, orientina e isovitexina) presentes nas folhas de duas espécies de *Cecropia, C. glaziovii* e *C. pachystachya.* Das folhas de *C. glaziovii* e *C. pachystachya* foram isolados os flavonoides *C*- glicosídeos isoorientina e isovitexina e identificados em ambas as espécies o ácido clorogênico (ácido 3-*O*-cafeoilquínico) e o flavonoide *O*-glicosídeo isoquercitrina. O flavonoide *C*-glicosídeo orientina foi isolado apenas da espécie *C. pachystachya.* O ácido clorogênico mostrou-se como composto majoritário em ambas as espécies analisadas (11,1 mg g^{-1} de extrato de *C. glaziovii* e 27,2 mg g^{-1} de extrato de *C. pachystachya*), e em relação aos flavonoides quantificados, isovitexina se apresentou como o flavonoide *C*-glicosídeo majoritário para *C. glaziovii* (4,6 mg g^{-1} extrato) e isoorientina majoritário para *C. pachystachya* (17,3 mg g^{-1} de extrato).

An efficient and reproducible HPLC-DAD method was developed and validated for the simultaneous quantification of major compounds (chlorogenic acid, isoorientin, orientin and isovitexin) present in the leaves of two *Cecropia* species, *C. glaziovii* and *C. pachystachya.* From the leaves of *C. glaziovii* and *C. pachystachya* were isolated the *C*-glycosylflavones isoorientin and isovitexin and identified on both species chlorogenic acid (3-*O*-caffeoylquinic acid) and the *O*-glycosylflavonol isoquercitrin. The *C*-glycosylflavone orientin was isolated only from *C. pachystachya*. Chlorogenic acid was the major compound in both species (11.1 mg g^{-1} of extract of *C. glaziovii* and 27.2 mg g^{-1} of extract of *C. pachystachya*) and for the flavonoids quantified, isovitexin was the main *C*-glycosylflavonoid for *C. glaziovii* (4.6 mg g^{-1} of extract) and isoorientin the main one for *C. pachystachya* (17.3 mg g^{-1} of extract).

Keywords: *Cecropia glaziovii, Cecropia pachystachya,* HPLC-DAD, *C*-glycosylflavonoids, chlorogenic acid

Introduction

The genus *Cecropia* (Urticaceae) comprises around 60 trees species distributed throughout Latin America, some of them occurring in Brazil. *Cecropia glaziovii* Sneth. and *Cecropia pachystachya* Trécul. are the two most common species in the Southeast and South of Brazil.[1] These species are both popularly known as "embaúba", and although posses distinct morphologies, such as their height and color of the leaves, both are widely used in Brazilian folk medicine to treat cough, asthma, high blood pressure, inflammation, and as a diuretic.[2,3] The main pharmacological activities described in the literature for these species are hypotensive activity[4-6] and effects on the central nervous system, including anxiolytic and antidepressant-like activities.[7,8] In terms of chemical composition, flavonoids, procyanidins and catechins have already been reported in these plants.[9,10]

Although there are some phytopharmaceutical and homeopathic preparations that include species from this genus in some countries, such as Brazil and France,[11] as far as we are aware, there is no comparative evaluation of the chemical fingerprint of *C. glaziovii* and *C. pachystachya* leaves in the literature, especially from aqueous extract, which is used in folk medicine. Neither is there any individual quantitative analysis of their major phenolic compounds. Therefore, the development of chromatographic identification and assay methods based on the chemical constituents, particularly the phenolic compounds, may contribute to the standardization of the crude drug and extracts.

*e-mail: freginatto@hotmail.com

The aims of this work were to identify and isolate the main *C*-glycosylflavonoids in aqueous extracts from the leaves of *C. glaziovii* and *C. pachystachya*, and to quantify the main phenolic compounds from the leaves of *C. glaziovii* and *C. pachystachya* by high performance liquid chromatography (HPLC).

Experimental

Chemical reagents

Methanol, ethanol, acetic acid, acetone and ethyl acetate (p.a.-grade) were provided by Vetec® (Brazil). Acetonitrile and acetic acid (HPLC-grade) were provided by Tedia® (Brazil). Water was purified with a Milli-Q system (Millipore®, Bedford, USA). All the solutions prepared for HPLC were filtered through a 0.45 μm membrane before use. Chlorogenic acid (3-*O*-caffeoylquinic acid, ≥ 98.0%) and isovitexin (4',5,7-tetrahydroxyflavone-6-glucoside, ≥ 98.0%) were purchased from Sigma-Aldrich® Co. (St. Louis, USA). Isoquercitrin (3',4',5,7-tetrahydroxyflavone-3-*O*-glucoside, ≥ 98.0%) was purchased from Carl Roth (Germany), and isoorientin (3',4',5,7-tetrahydroxyflavone-6-glucoside, ≥ 98.0%) and orientin (3',4',5,7-tetrahydroxyflavone-8-glucoside, ≥ 98.0%) were purchased from Extrasynthèse (France).

Plant material

Aerial parts of *C. glaziovii* Sneth. were collected in Florianópolis, in the State of Santa Catarina, Brazil, in September 2007, and a voucher specimen (FLOR 37143) was deposited in the Herbarium of the Universidade Federal de Santa Catarina, Florianópolis, Brazil. Aerial parts of *C. pachystachya* Trécul. were collected in Viamão, in the State of Rio Grande do Sul, Brazil, in March 2007 and a voucher specimen (ICN 150025) was deposited in the Herbarium of the Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil.

Extraction and isolation

The leaves of both *Cecropia* species were air dried at 35-40 °C for three days and the aqueous extracts were prepared separately, by infusion. Briefly, powdered leaf material (100 g) was extracted with boiled distilled water (1000 mL, 90 °C) for 30 min, and filtered. An aliquot of 100 mL of this extract was lyophilized and the residue dissolved in methanol:water (1:1 v/v) to obtain sample solutions for HPLC analysis. The remaining extract was evaporated under reduced pressure to a volume of 500 mL. The crude extracts were stirred with 50 g of Amberlite® XAD-16 for 1 h. Afterwards, the mixture was filtered and the resin stirred again (1 h) with 500 mL of methanol. This MeOH solution was then filtered and the solvent removed under reduced pressure. This procedure was repeated to yield 5 g of MeOH fraction from each *Cecropia* species.

MeOH fraction from *C. pachystachya* (5 g) was submitted to vacuum column chromatography over silica gel (Merck 60), using a gradient of ethyl acetate:methanol, starting with 100% ethyl acetate to 100% methanol. The fractions were pooled according to their TLC profile, yielding four sub-fractions (A, B, C, and D). Sub-fractions B (984 mg) and C (2,520 mg) were chromatographed, separately, on a silica gel column (40 cm × 2.5 cm i.d.; silica 70-230 mesh, 1:100 sample:stationary phase, m/m), and eluted with ethyl acetate:acetone:acetic acid:water (60:20:10:10 v/v, 6 mL each fraction), yielding 11 pooled fractions from each column. These fractions were purified on a Sephadex LH-20 column (30 cm × 2.5 cm i.d.; 50 g Sephadex), using a gradient with increasing polarity of ethanol:methanol [100% EtOH, EtOH:MeOH (1:1 v/v), 100% MeOH; 70 mL each gradient; 2 mL each fraction], resulting in compound **2** (36.4 mg), **3** (40.8 mg), **4** (3.3 mg).

MeOH fraction from *C. glaziovii* (5 g) was also submitted to vacuum column chromatography under the same conditions as those described above, yielding seven sub-fractions (A-G). Sub-fraction B (94 mg) yielded pure compound **4** (9.0 mg) after column chromatography on silica gel (25 cm × 1.5 cm i.d.; silica 230-400 mesh, 1:100 sample:stationary phase, m/m) using ethyl acetate:acetone:acetic acid:water (60:20:10:10 v/v, 2 mL each fraction) as mobile phase. **2** (9.0 mg) was isolated from sub-fraction D (104 mg) by column chromatography on silica gel (25 cm × 1.5 cm i.d.; silica 230-400 mesh, 1:100 sample:stationary phase, m/m) using ethyl acetate:formic acid:water (80:10:10 v/v, 2 mL each fraction) as mobile phase.

The identity of the compounds were established by TLC [Silica gel plates (10 cm for the development) using ethyl acetate:formic acid:water (80:10:10 v/v) as mobile phase and diphenylboryloxyethylamine 1% in methanol, followed by PEG 400 (5% m/v) (natural reagent) as color reagent], by HPLC-DAD co-chromatography (for chromatographic conditions, see HPLC analysis section), by UV spectra/shift reagents (PerkinElmer Lambda 25),[12,13] ^{1}H NMR (Varian AS, 400MHz), and by comparison with the literature data.

Isoorientin (**2**): 45.4 mg; TLC Rf: 0.57; HPLC Rt: 24.5 min; UV λ_{max} nm^{-1} (MeOH) 255, 270, 348; ^{1}H NMR (400 MHz; DMSO-d_6) δ 4.56 (1H, d, *J* 9.7 Hz, H-1''); 6.44 (1H, s, H-8) 6.64 (1H, s, H-3); 6.86 (1H, d,

J 8.2 Hz, H-5'); 7.37 (1H, d, *J* 2.1 Hz, H-2'); 7.40 (1H, dd, *J* 2.1 Hz, 8.2 Hz, H-6'); 13.55 (1H, s, OH-5).

Orientin (**3**): 40.8 mg; TLC Rf: 0.65; HPLC Rt: 25.7 min; UV λ_{max} nm^{-1} (MeOH) 255, 266, 348; ^{1}H NMR (400 MHz; DMSO-d_6) δ 4.68 (1H, d, *J* 9.7 Hz, H-1''); 6.22 (1H, s, H-6); 6.61 (1H, s, H-3); 6.83 (1H, d, *J* 8.0 Hz, H-5'); 7.55 (1H, d, *J* 2.1 Hz, H-2'); 7.57 (1H, dd, *J* 2.1 Hz, 8.0 Hz, H-6'); 13.17 (1H, s, OH-5).

Isovitexin (**4**): 12.3 mg; TLC Rf: 0.61; HPLC Rt: 28.8 min; UV λ_{max} nm^{-1} (MeOH) 270, 337. ^{1}H NMR (400 MHz; DMSO-d_6) δ 4.58 (1H, d, *J* 9.7 Hz, H-1''); 6.52 (1H, s, H-8); 6.71 (1H, s, H-3); 6.93 (2H, d, *J* 8.9 Hz, H-3', H-5'); 7.93 (2H, d, *J* 8.9 Hz, H-2',H-6'); 13.55 (1H, s, OH-5).

Quantitative high-performance liquid chromatography analysis

The quantitative analysis of phenolic compounds was carried out in a PerkinElmer Series 200 high-performance liquid chromatography (HPLC) system, equipped with diode array detection (DAD), quaternary pump, online degasser and autosampler. The data were processed using the TotalChrom® Workstation software. The injection volume was 10 µL. The baseline resolution was obtained at room temperature (24 ± 2 °C) using a PerkinElmer Brownlee Choice C_{18} column (150 × 4.6 mm i.d.; 5 µm) and a gradient combining solvent A (acetonitrile) and solvent B (acetic acid 1%, adjusted to pH 3.0) as follows: 0-30 min, linear change from A-B (5:95 v/v) to A-B (20:80 v/v); 30-40 min, isocratic A-B (20:80 v/v). The mobile phase was prepared daily and degassed by sonication before use. The flow rate was kept constant at 1.0 mL min^{-1} and the chromatograms were recorded at 340 nm while the UV spectra were monitored over a range of 450 to 200 nm. The peaks were characterized by comparing the retention time and UV spectra with the reference standards, and by the co-injection of the sample and authentic samples. The standard solutions were prepared in different concentrations, as follows: chlorogenic acid (3-*O*-caffeoylquinic acid), 2.5, 5.0, 10.0, 15.0, 25.0, 30.0 µg mL^{-1}; isoorientin, 0.8, 1.0, 2.0, 5.0, 7.0, 15.0, 20.0 µg mL^{-1}; orientin, 0.7, 1.0, 3.0, 7.0, 10.0, 20.0 µg mL^{-1} and isovitexin, 0.5, 1.0, 2.0, 5.0, 7.0, 10.0, 15.0 µg mL^{-1}. The concentration of the extracts analyzed were 1,000 µg mL^{-1} and fractions were 500 µg mL^{-1}, excepted for accuracy assay, which employed crude extracts at 1,500 µg mL^{-1} and 700 µg mL^{-1} for *C. glaziovii* and *C. pachystachya,* respectively. Quantification of the individual compounds was performed using a six-point regression curve ($r^2 > 0.995$). All standard solutions were analyzed in triplicate and the peak average areas measured.

Validation of analytical procedures

The validation of analytical procedures was performed according to Cass and Degani and the ICH guidelines.[14,15] The validated parameters were specificity, linearity, accuracy, precision (repeatability and intermediate precision), limit of quantification (LOQ) and limit of detection optical (LOD).

Results and Discussion

Isolation and identification

C. glaziovii has about 16 to 20 meters tall in their adult stage and leaves with green color on both sides. On the other hand, *C. pachystachya* reaches a maximum of 12 meters and its leaves have dense arachnoid indumentum on the lower surface, yielding a white-gray color. Although these species posses distinct morphologies, both are popular known in Brazil as "embaúba", fact that could lead to mistakes at collections. It is well accepted that medicinal plants can be distinguished from each other by comparing their chemical fingerprints. Analysis of both crude aqueous extract and its phenolic compounds enriched fraction by TLC and HPLC-DAD showed different profiles between *C. glaziovii* and *C. pachystachya*, based on their flavonoid fingerprint (Figure 1 and 2). Although previous phytochemical analyses report the presence of flavonoids in these species,[9,10,16] there are no comparative studies

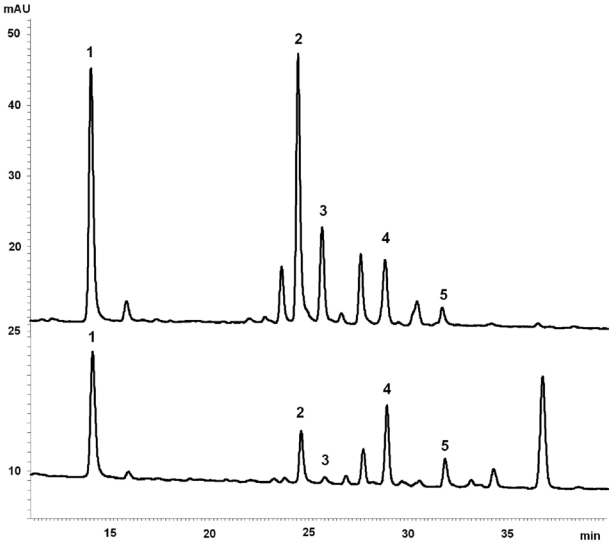


Figure 1. HPLC chromatograms of crude aqueous extracts (1,000 µg mL^{-1}) of *C. pachystachya* (up) and *C. glaziovii* (down) leaves with diode array detection at 340 nm. 1. chlorogenic acid; 2. isoorientin; 3. orientin; 4. isovitexin; 5. isoquercitrin. For chromatographic conditions, see Experimental section.

concerning the chemical profiles between *Cecropia* species, which could increase the knowledge of their differentiation.

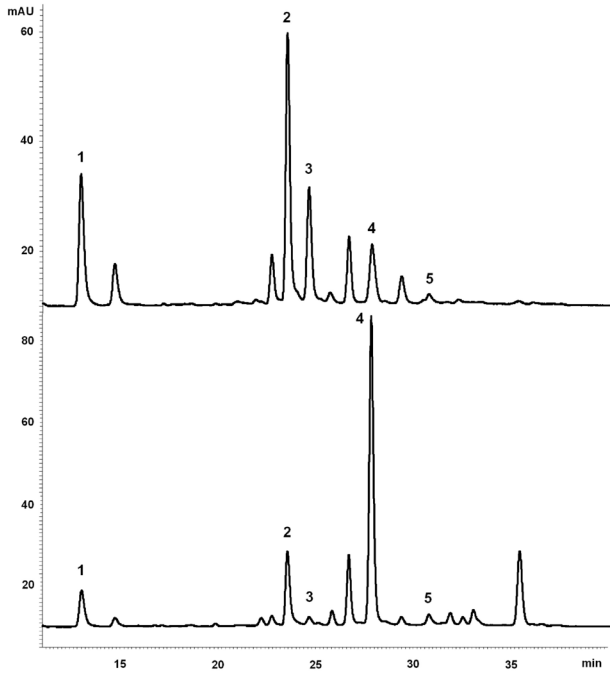


Figure 2. HPLC chromatograms of MeOH fractions (500 μg mL^{-1}) of *C. pachystachya* (up) and *C. glaziovii* (down) with diode array detection at 340 nm. 1. chlorogenic acid; 2. isoorientin; 3. orientin; 4. isovitexin; 5. isoquercitrin. For chromatographic conditions, see Experimental section.

Both extracts were fractionated, and three flavonoids were isolated from *C. pachystachya*, codified as **2** (36.4 mg), **3** (40.8 mg), **4** (3.3 mg). **2** was identified as isoorientin, **3** as orientin and **4** as isovitexin, by co-chromatographic analysis with standard *C*-glycosylflavonoids and UV spectra/shift reagents. Additionally, these compounds were submitted to ^{1}H NMR spectrometry analysis in order to confirm their identity.

From *C. glaziovii*, two flavonoids were isolated, codified as **4** (9.0 mg) and **2** (9.0 mg). TLC and HPLC-DAD co-chromatography with compounds previously isolated from *C. pachystachya*, together with UV spectra/shift reagents, led to the identification of **4** as isovitexin and **2** as isoorientin.

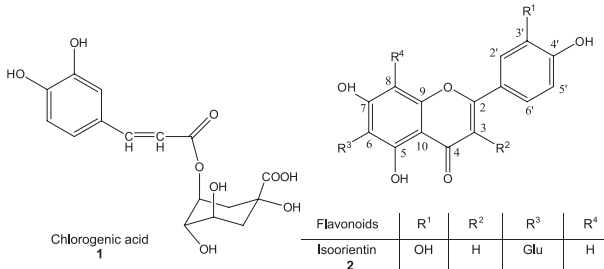
These flavonoids were already reported for these species.[9,10,17] Besides these compounds, two other previously reported phenolic compounds[9,10] were identified for these species: chlorogenic acid (3-*O*-caffeoylquinic acid) and isoquercitrin. Another significant substance was observed in *C. glaziovii* extract (HPLC Rt > 35 min), which was present just in traces in the *C. pachystachya* extract. Data about the UV profile of this peak suggests a flavones skeleton, although this compound was not isolated, identified or quantified.

HPLC analysis

Our preliminary investigations testing HPLC systems previously reported in the literature for *Cecropia* species[9,10,17] resulted in poor resolution of the compounds analyzed, or a long run time. The chromatographic system that showed the most promising results was achieved using a reverse-phase column (C_{18}), with acetonitrile and acidified water (with acetic acid, 1% v/v, pH 3.0) as the mobile phase. The use of acetonitrile instead of methanol improved the resolution, resulting in sharp and symmetrical peaks, with a good baseline level and minimal tailing, thus facilitating the accurate measurement of the peak area ratio.

Quantification and validation procedures

Standard solutions of chlorogenic acid, isoorientin, orientin and isovitexin were prepared, at a concentration range of 0.5-30 μg mL^{-1}, and the quantification showed a good linear relationship between peak area and concentration ($r^2 > 0.995$) for all standard solutions (Table 1). The limit of quantification (LOQ) and limit of detection (LOD) were defined by relative standard deviation (RSD > 5%) and by a signal:noise ratio of 3:1, respectively. The contents of the four phenolic compounds in these two species are shown in Table 2. The chromatographic analysis showed a distinct HPLC profile for these two *Cecropia* species, in which isovitexin is the major *C*-glycosylflavonoid for *C. glaziovii*, while isoorientin and orientin are the main *C*-glycosylflavonoids for *C. pachystachya*.



Flavonoids	R^1	R^2	R^3	R^4
Isoorientin **2**	OH	H	Glu	H
Orientin **3**	OH	H	H	Glu
Isovitexin **4**	H	H	Glu	H
Isoquercetrin **5**	OH	*O*-Glu	H	H

Figure 3. Compounds identified in the leaves of *C. glaziovii* and *C. pachystachya*.

There are no reports in the literature concerning the quantification of *C*-glycosylflavonoids in *Cecropia* species, in spite of several studies concerning the quantification of these compounds in other species.[18,19,20] In the *Cecropia* genus, quantitative analysis of chlorogenic acid has been reported only for *C. obtusifolia*. The contents of

Table 1. Calibration data of phenolic standards

Compound	Linearity range (μg mL^{-1})	Calibration equation[a]	Correlation factor (r^2)	LOD[b] (μg mL^{-1})	LOQ[b] (μg mL^{-1})
Chlorogenic acid	2.5 – 30.0	y = 20674x – 14755	0.997	0.1580	1.25
Isoorientin	0.8 – 20.0	y = 29806x – 4715,4	0.995	0.0625	0.50
Orientin	0.7 – 20.0	y = 10059x + 2484,5	0.999	0.0875	0.35
Isovitexin	0.5 – 15.0	y = 22875x + 5112,9	0.998	0.0625	0.25

[a] Six data points (n = 3). [b] LOD = limit of detection; LOQ = limit of quantification.

Table 2. Phenolic acid and *C*-glycosylflavonoids content in *Cecropia* extracts[a]

Cecropia species	Chlorogenic acid (Rt: 14.0 min)	Isoorientin (Rt: 24.5 min)	Orientin (Rt: 25.7 min)	Isovitexin (Rt: 28.8 min)
C. glaziovii	11.1 ± 0.42	2.7 ± 0.06	0.8 ± 0.03	4.6 ± 0.11
C. pachystachya	27.2 ± 0.94	17.3 ± 0.59	17.2 ± 0.36	5.9 ± 0.27

[a] Expressed as mg g^{-1} of extract ± SD (n = 3).

this compound in the leaves of this species are in the range of 3.0-13.2 mg g^{-1} of dry weight.[21,22] Other works report a content of 0.2 mg g^{-1} aqueous extract.[23,24] In our analysis, higher chlorogenic acid contents were found for *C. glaziovii* (11.1 ± 0.42 mg g^{-1} extract) and *C. pachystachya* (27.2 ± 0.94 mg g^{-1} extract) than those reported for *C. obtusifolia*, according to Revilla-Monsalve and co-workers.[23]

It is important to emphasize that the methodology developed herein allowed the analysis of two *Cecropia* species and the quantification of four substances from two classes of phenolic compounds (phenolic acids and *C*-glycosylflavonoids) in a single run. On the other hand, the analysis showed a relative long run time (40 min) considering only *C*-glycosylflavonoids, but bearing in mind that different classes of secondary metabolites could be analyzed with good separation and baseline resolution between all peaks, the methodology represents an improvement on the simultaneous quantitative assay of these phenolic compounds, compared with other reports in the literature.[25,26,27] Besides the fact that *C*-glycosylflavonoids isomer pairs, especially orientin/isoorientin, are usually difficult to separate with good resolution,[28,29] the 40 min analysis is acceptable, since our method is suitable for this separation.

The precision was determined by repeatability (intra-day assay) and intermediate precision (inter-day assay) (Table 3).[30] The intra-day assay was performed by triplicate analysis of three different concentrations of standard solutions, and expressed as relative standard deviation. Good repeatability was obtained from lower, medium and higher concentrations of the curve, with an RSD < 3.5% for all standard analyses. The inter-day assay was determined by the analysis of a medium concentration in the curve, three times a day, on three different days. Like the other parameters of precision, the RSD value did not exceeded the limits recommended in the literature.[14,15] In relation to accuracy, good recovery was observed for all the standards in both extracts, which was determined by spiking samples with the standard solutions of chlorogenic acid, isoorientin, orientin or isovitexin (1:1 v/v). The concentrations of samples and standard solutions, as well as the average recovery values, are shown in Table 4.

Table 3. Repeatability and intermediate precision data of phenolic standards

Compound	Repeatability[a]		Intermediate precision[a]	
	Concentration (μg mL^{-1})	R.S.D. (%)	Concentration (μg mL^{-1})	R.S.D. (%)
Chlorogenic acid	10.0	2.9	15.0	0.4
	15.0	0.5		
	25.0	0.5		
Isoorientin	5.0	2.3	7.0	0.9
	7.0	0.9		
	15.0	0.3		
Orientin	1.0	2.7	7.0	4.3
	7.0	1.0		
	10.0	1.4		
Isovitexin	2.0	3.4	7.0	1.0
	7.0	0.7		
	10.0	0.7		

[a] Limits: R.S.D. < 5%.

Conclusions

Five phenolic compounds were identified in *C. glaziovii* and *C. pachystachya* and three of them (orientin, isoorientin

Table 4. Accuracy data of phenolic compounds

Species	Compound	Recovery[a]	
		Mean (%)	R.S.D. (%)
C. glaziovii (1,500 μg mL^{-1})	Chlorogenic acid (15.0 μg mL^{-1})	98.9	0.2
	Isoorientin (7.0 μg mL^{-1})	101.1	1.7
	Orientin (3.0 μg mL^{-1})	101.6	3.8
	Isovitexin (7.0 μg mL^{-1})	104.9	0.9
C. pachystachya (700 μg mL^{-1})	Chlorogenic acid (15.0 μg mL^{-1})	100.0	0.7
	Isoorientin (15.0 μg mL^{-1})	101.4	0.4
	Orientin (10.0 μg mL^{-1})	104.3	0.5
	Isovitexin (5.0 μg mL^{-1})	101.4	1.6

[a] Recovery was determined by injection of spiked samples, in triplicate, with standard solution.

and isovitexin) were isolated. Furthermore, a precise, accurate and reproducible HPLC-DAD method has been developed. The *C*-glycosylflavonoids contents reported here suggest that it is possible to use isovitexin as a phytochemical marker for *C. glaziovii*, while isoorientin could be used as a phytochemical marker for *C. pachystachya*.

Acknowledgements

This project was supported by the CNPq (Grant: 475826/2008-6) and the FAPESC (Grant: 05798/2007-3). The authors E. P. Schenkel and F. H. Reginatto are also grateful to CNPq for their research fellowships. The authors also thank Prof. Dr. Ângela Campos for her collaboration and Prof. Dr. Lilian A. Mentz and Prof. Dr. Daniel Falkenberg for their collection and identification of plant material.

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Submitted: April 7, 2010
Published online: February 17, 2011