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Short communication

LC and UV determination of flavonoids from *Passiflora alata* medicinal extracts and leaves

Simony D. Müller, Sonia B. Vasconcelos, Michelly Coelho, Maique W. Biavatti*

Núcleo de Investigações Químico-Farmacêuticas (Niqfar), Curso de Farmácia, CCS, University of Vale do Itajaí (UNIVALI), Rua: Uruguai 458, Caixa Postal 360, Itajaí, Santa Catarina (SC) 88302-202, Brazil

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Abstract

The leaves of *Passiflora alata* Curtis (an official species in the Brazilian Pharmacopoeia) are used to treat anxiety, as antispasmodics and sedatives. The development of new analytical methods for analysing flavonoids in *Passiflora* species is needed in order to improve the quality assurance of the plant and derived extracts and phytomedicines. A survey on the profile of flavonoids and content of isovitexin in the leaves and fluidextract of *P. alata* through LC was carried out, comparing its chromatographic profiles with a commercial *P. incarnata* fluidextract. Also, the concentration of the total flavonoids of extracts and leaves according to phamarcopoeial photometrical methods was determined and discussed. The fluidextract of *P. alata* was produced in accordance with the Pharmacopoeia Helvetica method; the presence of isovitexin in both species (which have distinct chromatographic profiles) was evidenced, this being the major flavonoid compound in the *P. incarnata* (1.198 g%), but not in *P. alata* (0.018 g%) fluidextract. Only traces of vitexin could be observed in the *P. alata*, as well as the absence of the other tested flavonoids: orientin and swertisin (*P. incarnata* markers), hyperoside, rutin, hesperidin and clorogenic acid. The LC developed method was shown to be appropriated for the qualitative and quantitative analyses and for differentiate species, suitable to be applied to commercial sample analysis. The spectrophotometrical results of three different methods described for *P. incarnata* were not comparable, the best performance being the British Pharmacopoeia method.

Keywords: *Passiflora alata*; Liquid chromatography; Spectrophotometric determination; Flavonoids; Isovitexin

1. Introduction

Leaves from several *Passiflora* (maracujá) lianous species are widely used in folk medicine as anxiolytics and sedatives, and since 1867, have formed part of classical medicine as sedatives in cases of sleeplessness and irritability [1]. The leaves of *Passiflora alata* Curtis (vernacular name: maracujá-doce) is comprised in three editions of the Brazilian Pharmacopoeia, however, without a valuable quantitative analytical method, that allows reliable evaluation of the drug. Despite the official nature of this species, studies focusing on its chemistry and pharmacological activity are scarce, making the standardisation of this traditional drug difficult.

The principal bioactive compounds described in this genus are *C*-glycosyl derivatives of apigenin and luteolin (vitexin, isovitexin, orientin, isoorientin, schaftoside, among others), constituting the actual best option as quality markers, as same as for *P. edulis* and *P. incarnata* [2,3].

Although several in vivo experiments have been carried out in order to precise the pharmacological activity of *Passiflora*, is not possible yet to attribute the overall effects of this species to any single class of compounds or entity [4–6]. Previously, the possible concerted action of several alkaloids and flavonoids for producing therapeutic effects of the *Passiflora* drug was emphasised [7].

Until now, the attributed harmane β-carbolinic alkaloids have not been found in the *Passiflora* analysed by high efficiency liquid chromatography [8] or not at the previous preconised quantities [9].

* Corresponding author. Tel.: +55 47 341 7835; fax: +55 47 341 7835.
E-mail address: maique@univali.br (M.W. Biavatti).

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On the other hand, anxiolytic action has been demonstrated for some isolated flavonoids without inducing sedation or muscular relaxation, such as chrysin isolated from *P. coerulea* (1 mg/kg) [10], which was also characterised in *P. incarnata* extracts [11]. A benzoflavone isolated from *P. incarnata* prevented abstinence syndrome induced by naloxone in the swimming endurance test (20 mg/kg) [12].

Some pharmacological activities have also being attributed to the flavonoids vitexin and isovitexin, such as hypotensive, antinflamatory, antispasmodic [13] antimicrobial [14], antioxidant/free radical scavenging [15,16] and radioprotective effects [17].

One of the quantification methods for flavonoids frequently used is spectrophotometry, that after acid hydrolysis the flavonoids are complexed with aluminium chloride [18]. The *C*-glycosyl flavonoids resist acid hydrolysis and consequently are not determined through classical spectrophotometric methods [3,19]. There is a great demand for the development of appropriate methods for the quality control of phytomedicines contending maracujá. In the present work, LC methods were developed for qualitative and quantitative analysis of *C*-glycosyl flavonoids in the leaves of *P. alata* and the results obtained were compared to the spectrophotometric methodologies described for the *Passiflora incarnata* [18,20,3]. The results obtained from the developed methodology for *P. alata* were also compared to a commercial medicinal extract of *P. incarnata*.

2. Experimental

2.1. Plant material

The leaves of *P. alata* were collected in Ilhota, Santa Catarina, Brazil (January and August, 2001) and voucher specimens were deposited at the Department of Botany Herbarium (UPCB No. 43.414), Universidade Federal do Paraná, PR, Brazil, identified by Professor Armando Cervi.

2.2. Preparation of extracts

The dried and powdered *P. alata* leaves (500 g, 1.40 mm/μm) collected in summer (January) and winter (August) were percolated according to the PHARMACOPOEA HELVETICA method [18] to obtain a fluidextract (1:1, w/v). The extracts were stored in a refrigerator until analysis.

A commercial fluidextract of *P. incarnata* was obtained from "Chemische Fabrik Dr. Hetterich®", Fürth, Germany.

2.3. Chromatographic analysis

LC separations were performed using a Shimadzu LC-10AD (Tokyo, Japan) pump, a Luna RPC18 (5 μm), (4.6 mm × 250 mm i.d.) column from Phenomenex (Torrance, CA, USA), a Shidmadzu SPD-M10A photodiode array detector (monitoring 340 nm) and a Shimadzu CTO-10A column oven fit to 30 °C. A Rheodyne manual injector model 7725i was used for sample injection (Rohnert Park, CA, USA). All reagents used were LC grade and filtered over regenerated cellulose membrane [0.45 μm pore diameter (Schleicher and Schuell, Dassel, Germany)].

The accuracy of the method was determined through an analyte recovery test (ICH, 2000), adding known standard concentrations from 5, 6 and 10 μg/mL to the matrix sample, appropriately diluted in triplicate, regarding the linearity of the method. The analyte recovery in the presence and absence of the extract matrix was compared, in order to analyse the specificity of the method.

2.4. Chromatographic conditions

The qualitative analysis of the flavonoids: orientin, swertisin, vitexin, isovitexin, hyperoside [provided by Dr. Valdir Cechinel Filho (UNIVALI) and Dr. Franco Delle Monache (CNR/Rome, Italy)], rutin, hesperidin and clorogenic acid (from Sigma) was performed in a isocratic elution mode (CAN–H_2O–HOAc 18:82:0.5) with a 1 mL/min flow.

Isovitexin was determined in plant material by the external standard method, diluted (MeOH 50%) in triplicate to 4.0, 8.0, 12.0, 16.0 and 20.0 μg/mL. A gradient elution mode with a 1 mL/min flow was employed: 1–20 min 10% solvent B (MeOH) and C (ACN) in A (H_2O–HOAc, 100:0.5, pH 2.88), 20–30 min 15% B and C in A, 30–35 min 20% B and C in A. An equilibration period was employed between runs. The software Shimadzu Class-VP 5.03 was used to fit the regression curve and to calculate the corresponding correlation coefficient. All samples were analysed in triplicate.

2.5. Spectrophotometric analysis

The employed spectrophotometric methodologies are described for the *Passiflora incarnata* [18,20,3] and the pharmacopoeial extracts prepared for the spectrophotometric analysis were also evaluated and quantified by LC.

UV–vis spectra were collected with a UV–vis double-array spectrophotometer SHIMADZU UV-601.

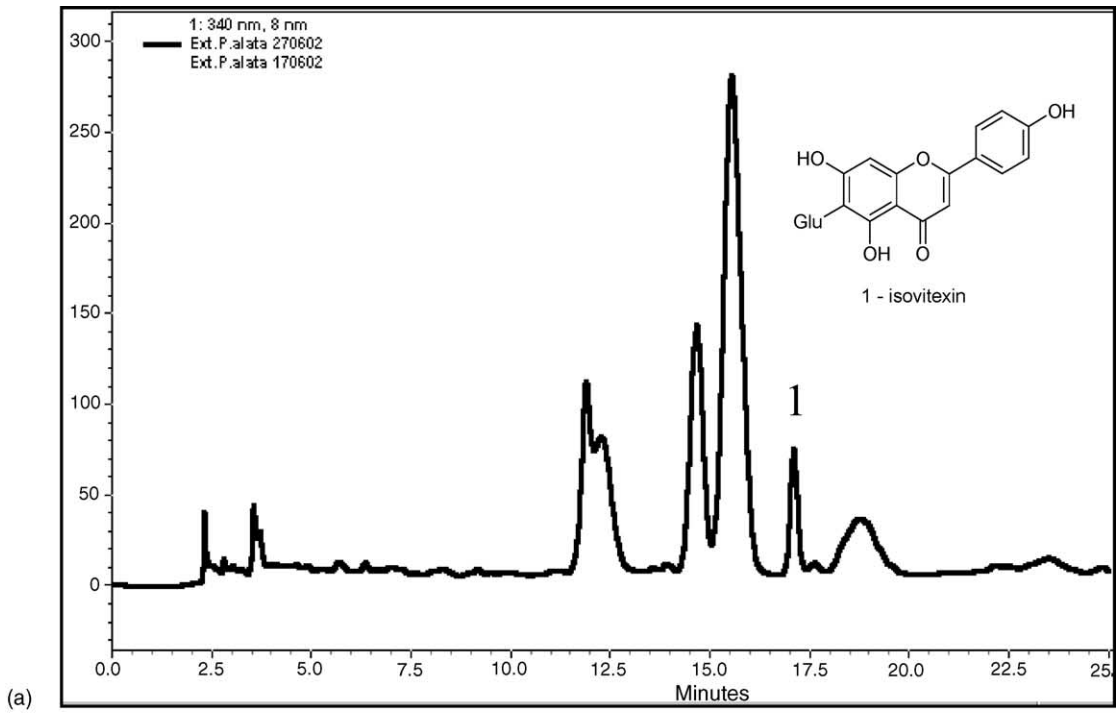
2.6. Sample preparation

For the LC analysis all extracts and standards were diluted in MeOH 50% (1:10 v/v), filtered over regenerated cellulose membrane [0.45 μm pore diameter (Schleicher and Schuell, Dassel, Germany)] and injected (20 μl) in triplicate.

For the analysis of the leaves, the pharmacopoeial refluxed extracts prepared for the spectrophotometric analysis were evaluated and quantified by LC, filtered as cited above.

3. Results and discussion

The qualitative evaluation of the *P. alata* flavonoid contents was made by comparison of retention times and the UV



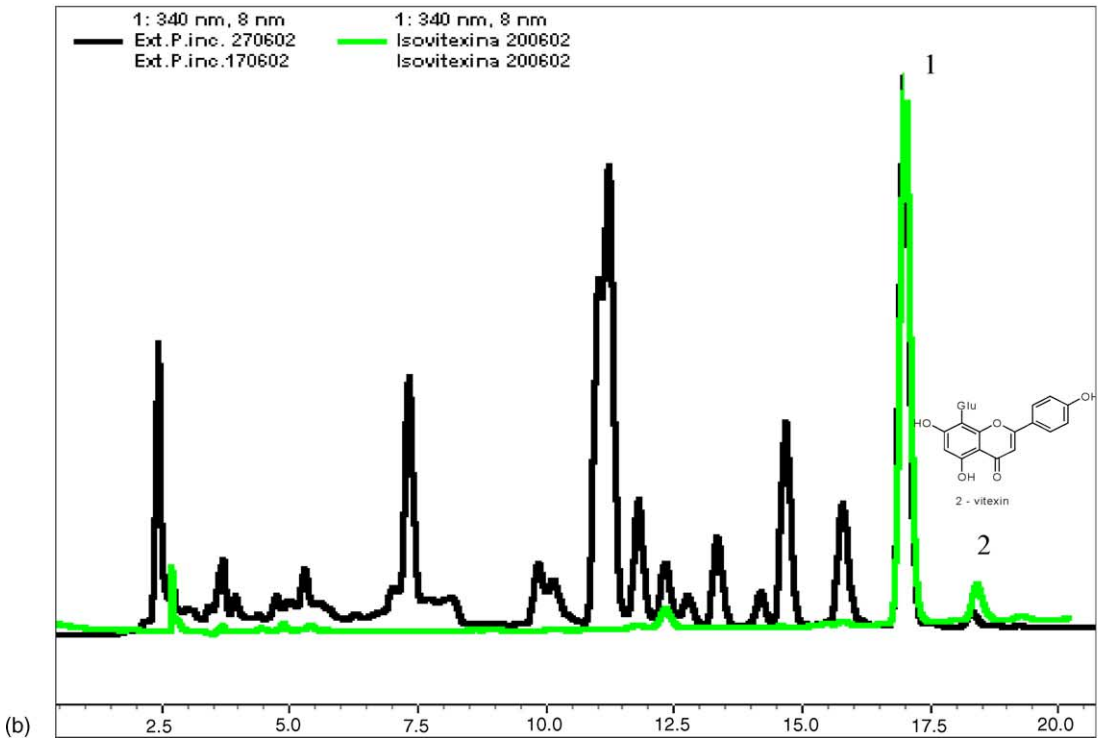


Fig. 1. Chromatographic profile of (a) *P. alata* and (b) *Passiflora incarnata*, and the standard isovitexin (*rt* = 17.3 min), detection at 340 nm. For chromatographic conditions, see Section 2.

spectra from PDA detector of standards and the extracts under the same chromatographic conditions. The chromatograms shown a good separation profile (Fig. 1) and only vitexin (trace amounts) and isovitexin were identified in the extracts, which are not the majority flavonoid compounds. The isocratic condition of analysis used in this work was modified based on a previous paper [21] being suitable for quick comparative analysis of *Passiflora* extracts. Orientin, swertisin, rutin, hyperoside and clorogenic acid were not found in the analysed samples. There were no qualitative changes in the

Table 1
Comparative results of LC (isovitexin) and UV (total flavonoids) analysis for *P. alata* (leaves and medicinal extracts) and *P. incarnata* commercial extract

Sample	LC isovitexin (g%), λ = 340 nm		UV[a] hyperoside (g%), λ = 422 nm		UV[b] vitexin (g%), λ = 401 nm		UV[c] apigenin (g%), λ = 397 nm	
	Mean[d]	S.D.[e]	Mean[d]	S.D.[e]	Mean[d]	S.D.[e]	Mean[d]	S.D.[e]
Summer leaves, *P. alata*	1.137	0.002	0.775	0.001	0.470	0.001	0.087	0.0005
Summer fluidextract, *P. alata*	0.018	0.001	0.116	0.0005	0.256	0.069	0.054	0.001
Winter leaves, *P. alata*	0.018	0.006	0.405	0.001	0.395	0.0005	0.061	0.007
Winter fluidextract, *P. alata*	0.007	0.0007	0.084	0.0005	0.204	0.001	0.033	0.005
Commercial fluidextract, *P. incarnata*	1.198	0.009	0.032	0.0009	0.164	0.00	0.023	0.003

[a] Pharmacopoeia Helvetica method.
[b] British Pharmacopoeia method.
[c] Petry et al. adapted method [3].
[d] Average of three determinations.
[e] Standard deviation.

chromatographic profile of the samples collected in winter or summer, only in the overall quantity of the analysed flavonoids, with a decrease in extracts produced with winter leaves (Table 1). The presence of vitexin and isovitexin by LC in alcoholic extracts of *P. alata* and *P. edulis* was previously verified [22] and the flavonoids 2″-xylosylvitexin, vitexin, isoorientin and orientin were also isolated from *P. alata* by preparative LC [23].

Fig. 1 presents the chromatographic profile of standards (vitexin/isovitexin) and the extract material. No interference of current components was observed in the elution window corresponding to the target compounds.

The calibration curves using the standard isovitexin showed a good linearity of the detector over the tested range (4–20 μg/mL), as shown by the correlation coefficient of the regression line ($R^2 = 0.999414$, $y = 2.11816e-008x - 0.000543441$). To evaluate the accuracy of the method, a recovery experiment was carried out, spiking the standard isovitexin in the appropriated diluted matrix extracts, regarding the linearity of the method. Table 2 shows the results of the isovitexin recovery. The recovery average indicated the accuracy of the method, which was 98.7%. In the target level (5 μg/mL) the recovery was 99.2%, close to the desirable 100 ± 2.0% [24]. Comparing the recovery experiments in the presence and absence of matrix, no interference of the extract matrix on the isovitexin determination was observed, demonstrating the specificity of the method. The coefficient variation was less than 5.0%. The limit of quantification (LQ) of the method was 1.29 μg/mL, showing good sensitivity [25].

Table 2
Recovery of standard isovitexin added to *P. alata* extracts

Isovitexin added (μg/mL)	Isovitexin found[a] (μg/mL) (mean ± S.D.)	Coefficient of variation (%)	Recovery (%)
5	4.96 ± 0.20	4.03	99.2
7	6.93 ± 0.0133	0.19	99.0
10	9.78 ± 0.0156	0.16	97.8
5[b]	5.21 ± 0.0133	0.25	104.2
0[c]	5.38 ± 0.0155	0.29	–

[a] Average of three determinations.
[b] In absence of matrix.
[c] Isovitexin found in the matrix.

Table 1 presents the isovitexin concentration determined by liquid chromatography and the total flavonoid content determined by pharmacopoeal methods [18,20,3] in extracts of *Passiflora alata* collected in summer and winter. It is possible to observe in both methods of quantification that the winter extract and leaves presents less flavonoid, but the chromatographic profile remains unaltered. There was a significant reduction in isovitexin content for the winter leaves, as well as is total flavonoids for all spectrophotometric methods tested. The same reduction can be observed in the isovitexin content in the prepared extracts, independent of the time of collection. This result is very different from the analysis of *P. incarnata* commercial extract, which presented a high content of isovitexin (the major flavonoid in the chromatographic profile, Fig. 1), similar to the isovitexin content in refluxed leaves of *P. alata*, but this flavonoid is not the predominant one for this species (Fig. 1). This fact lead to a discussion of the method used for the preparation of extracts. It is possible that some microbiological degradation of the flavonoids occurred in the *P. alata* extracts, since they were not sterilised and submitted to a concentration step, decreasing the ethanol content. The microbiological degradation of flavonoids for several bacteria [26,27] and fungus [28,29] has been described. Different amounts of isovitexin on *P. incarnata* by LC have been found: 1.332% in a sample of dry extract of *P. incarnata* solubilized in methanol–water (1:1) and 14.19% in a pilular extract [30]. From hydrolysed methanol extract produced from four different commercial samples of *P. incarnata* the following amounts of isovitexin was found: 0.663, 0.761, 1.065 and 0.113% [19].

In a study conducted with 115 samples of *Passiflora* sp. 0.42% and 0.11% of isovitexin was found for two samples of *P. incarnata* among other *C*-glycosyl flavonoids, and for *P. alata* none of the flavonoids tested were found [6]. In a simultaneous LC quantification for *C*-glycosyl flavonoids and harmane alkaloids in *P. incarnata* samples, 500 μg/g of isovitexin of dried herb was found [9]. In this cited work, the quantitative analytical condition employed was similar to the presented here (mobile-phase, flow rate and run time), with

the advantage of analysing two classes of compounds simultaneously, but possibly rendering difficult the separation and differentiation of other flavonoid compounds found in *Passiflora* samples, goal achieved in the present study.

The Helvetica Pharmacopoeia determines total flavonoids of *Passiflora* through acid hydrolysis, extraction with ethyl acetate and complexation with aluminium chloride at $\lambda = 422$ nm, in terms of hyperoside [18]. Several authors consider that this method was not developed for the analysis of *C*-glycosyl flavonoids [2]. In view of this fact, the majority of *Passiflora* flavonoids will remain in the aqueous fraction and not in the analysed organic layer. Despite this, the results obtained for *P. alata* leaves are within the limits preconisated for *P. incarnata* (0.3%).

In the British Pharmacopoeia [20] the drug sample is extracted with ethanol 60% and complexed with oxalo-boric solution and measured at $\lambda = 401$ nm (1.5% expressed in vitexin for *P. incarnata*). Using this method, all the analysed samples were under the lower limit, also the total flavonoid was expressed in apigenin ($\lambda = 397$ nm) (0.55 g%) [3], a method developed for quantifying total flavonoids of *P. alata* without acid hydrolysis and eliminating the lipophilic interference with a C18 cartridge. For all the spectrophotometrical determinations the total flavonoid content appears to have been underestimated, but showed a good correlation between leaves and extracts [31].

4. Conclusions

Extracts of leaves from *Passiflora alata* presented *C*-glycosyl flavonoids isovitexin (0.018 mg/mL) and vitexin (traces) together with other unidentified major flavonoid compounds. The method was subjected to recovery assay, determining its reproducibility. A remarkable difference in flavonoid concentration was observed for leaves collected in different seasons (summer and winter), and its flavonoid profile is different from *P. incarnata*. The three spectrophotometric methods tested to determine the total flavonoid content presented many differences in the values found. Recently, saponins have been described for *P. alata* as additional compounds that can contribute to its pharmacological effects [32].

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