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Comparative assessment of kaempferitrin from medicinal extracts of *Bauhinia forficata* Link

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Abstract

The leaves of the pantropical genus *Bauhinia* (Fabaceae) are popularly known as cow's-paw or cow's hoof due to their unique characteristic bilobed aspect. The species *Bauhinia forficata* (Brazilian Orchid-tree) is widely used in folk medicine as an antidiabetic. This article deals with the quantitative analysis of kaempferitrin from *B. forficata* medicinal extract (aqueous and hydro alcoholic) using the LC method, and the comparison of kaempferitrin content in leaves collected from two different regions in the south Brazil. The total flavonoid content assessed by LC was also compared with the classical spectrophotometric determination. Kaempferitrin was found in different amounts, in samples from two geographical areas (Telêmaco Borba/PR and Itajaí/SC), for aqueous (368.68 and 77.91 μg/mL) and hydro alcoholic extracts (1952.59 and 211.61 μg/mL), respectively. The method was subjected to recovery assay, to determine its accuracy. A marked difference in total flavonoid concentration was observed in relation to kaempferitrin content: 2759.95 and 2188.20 μg/mL for the fluidextract and 863.35 and 856.77 μg/mL for the aqueous extract (Telêmaco Borba/PR and Itajaí/SC). The spectrophotometric assay overestimated the total flavonoid content (3620 μg/mL) in relation to the LC assay.

Keywords: *Bauhinia forficata*; Liquid chromatography; Spectroscopy; Flavonoids; Kaempferitrin; Extraction

1. Introduction

The leaves of the pantropical genus *Bauhinia* (Fabaceae) are popularly known as cow's-paw or cow's hoof (in Portuguese: pata-de-vaca or unha-de-vaca) due to their unique characteristic bilobed aspect. The neotropical species *Bauhinia forficata* (Brazilian Orchid-tree) is an evergreen tree with white flowers, which is widely used in folk medicine as an antidiabetic [1].

The leaves of *Bauhinia forficata* Link are considered by many to be more effective for medicinal purposes than other species of *Bauhinia*. A chemical survey of the genus afforded mainly glycosil flavonoids [2–7], other phenolic derivatives [8,9] and cyano glicosides [10]. Many pharmacological experiments have been carried out with the leaf extracts (aqueous and alcoholic), in the search for antidiabetic activity [11–17], and the majority of phytochemical investigations have lead to the isolation of compounds from *Bauhinia* roots, bark and stems.

The volatile oil from the leaves of *B. forficata* has been characterized [18], and only kaempferol and quercetin *O*-glycosides have been isolated [2–4]. Some attempts have been made to attribute the antidiabetic effects of the leaf extracts to the major flavonoid derivative (kaempferitrin) [19,20]. This paper deals with a quantitative analysis of kaempferitrin from *B. forficata* and also the fingerprint evaluation of its medicinal extracts (aqueous and hydro alcoholic) using the LC/PDA method, comparing the profile and the kaempferitrin content in extracts from plants collected from two different regions in the south Brazil. The total flavonoid content, assessed by LC, was also compared

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with the classical spectrophotometric determination, and the equivalence in terms of total flavonoids observed in dosage forms studied were analysed and discussed.

2. Experimental

2.1. Plant material

Leaves of *B. forficata* were collected in January 2002, at Praia Brava beach, Itajaí, Santa Catarina, Brazil and at Telêmaco Borba, Paraná, Brazil. Voucher specimens were deposited at the Herbário Barbosa Rodrigues [HBR, M. Biavatti no. 13 (10 April 2002)] in Itajaí (SC), and identified by Professor Ademir Reis (Botany Department, Universidade Federal de Santa Catarina (UFSC), Florianópolis, SC).

2.2. Preparation of extracts

The dried and powdered *B. forficata* leaves (500 g, 1.40 mm/μm) were percolated according to the Brazilian Pharmacopeia method [21], using ethanol–water (1:2) to obtain a fluidextract (1:1, w/v). The extracts were stored in a refrigerator until analysis.

The aqueous extracts were prepared on the day of analysis, using 1 g (1.40 mm/μm) of leaves for 100 mL of boiling water, and allowing them to stand for 10 min.

2.3. Chromatographic analysis

LC separations were performed using a Waters 600 (Millford, USA) pump, and a NovaPak C18 (4 μm) (3.5 mm × 150 mm i.d.) column from Waters (Millford, USA), termostatized to 30 °C. A Waters 2996 photodiode array detector (monitoring 340 nm) and a Rheodyne manual injector model 7725i (loop 20 μL) were used for sample injection (Rohnert Park, CA, USA). All the reagents used were LC grade and filtered over regenerated cellulose membrane [0.45 μm pore diameter (Schleicher&Schuell, Dassel, Germany)] and degassed through an in line degasser AF from Waters (Millford, USA).

2.4. Chromatographic conditions

The analysis of glycosil-flavonoids 3,7-di-*O*-α-L-rhamnopyranosylkaempferol (kaempferitrin) and 3,7-di-*O*-α-L-rhamnopyranosylquercetin (previously obtained from *B. forficata*, according to Pizzolatti et al. [3]), kaempferol (from Spectrum) and quercetin (from Sigma) were performed in a gradient elution mode with a 0.8 mL/min flow: 1–23 min 10–40% solvent B (ACN) in A (H_2O–H_3PO_4, 100:0.05, pH 2.88). An equilibration period of 10 min was used between runs.

Kaempferitrin in the plant material was determined by the external standard method, diluted (MeOH 50%) in triplicate to 12.5, 25.0, 50.0, 100.0 and 200.0 μg/mL. The software Millenium Empower (Waters) was used to fit the regression curve and to calculate the corresponding correlation coefficient. All the samples were analysed in triplicate.

The accuracy of the method was determined through an analyte recovery test [22], adding known standard concentrations from 30, 60 and 90 μg/mL to the matrix sample, appropriately diluted in triplicate, to determine 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.

2.5. Spectrophotometric analysis

The methodology used was based on the pharmacopeial *Calendula* monograph [23] (measure of kaempferol and quercetin derivatives), with slight modification: 1 g of the powdered drug (1.40 mm/μm) was used.

UV–vis spectra were collected with a UV–vis double-array spectrophotometer Shimadzu UV-1601.

2.6. Sample preparation

For the LC analysis, the fluidextracts were diluted in MeOH 50% (1:10, v/v), and the aqueous extract was injected without dilution. All samples and standards were filtered over a regenerated cellulose membrane [0.45 μm pore diameter (Schleicher&Schuell, Dassel, Germany)] and injected in triplicate.

3. Results and discussion

Medicinal plant extracts and phytopharmaceuticals products are complex mixtures of hundreds of primary and secondary metabolites, with inherent biological variation due to differences in growth environment, harvesting season, drying, storage, and extraction conditions. The concentration of chemical constituents may vary naturally from batch to batch. It is therefore desirable to standardize cultivation and processing conditions, in order to achieve uniformity in the pharmaceutical preparations, and enhance the quality of the products.

Generally, one or two markers (whether pharmacologically active components or not) in plant extracts are normally employed for assessing the quantitative herbal composition. In this study, just one marker was possible to quantify. This kind of determination, however, does not give a complete picture of a phytopharmaceutical product, because multiple constituents are usually responsible for its therapeutic effects. These effects are based on the synergic response of its constituents, which can hardly be separated into active parts.

The chromatographic fingerprinting technique has been considered and accepted by the WHO [24] as a more meaningful way of controlling the quality of herbal products, based on the systematic characterization of the composition of medicinal extracts, and focusing on the qualitative identification and evaluation of peaks. Using this technique, the full extract can be regarded as the active 'compound', facilitating the reliability and repeatability of pharmacological and clinical research, the understanding of their bioactivities and the possible side effects of active compounds together [25,26].

The fingerprint evaluation of the contents of *B. forficata* flavonoid was carried out by comparing the retention times

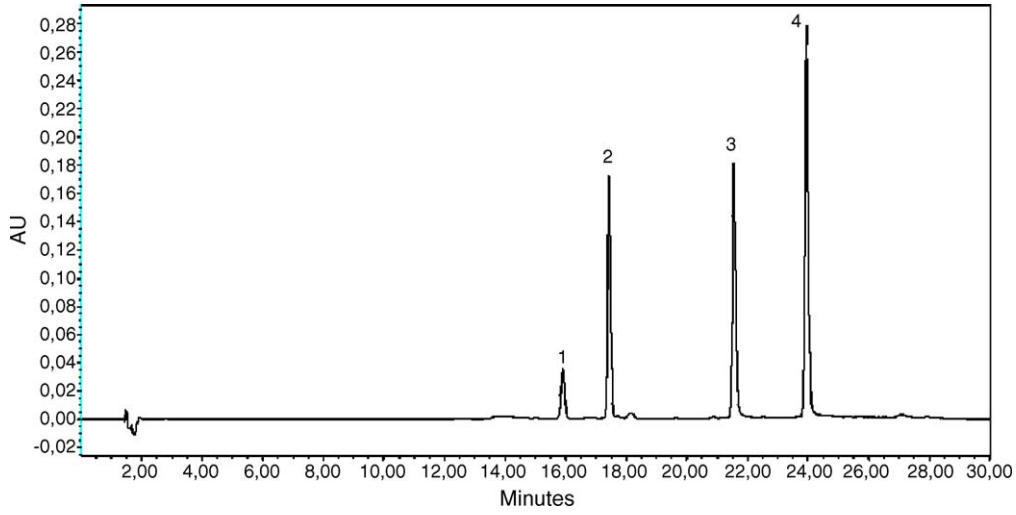

Fig. 1. Chromatographic profile of standard flavonoids. For chromatographic conditions, see Section 2: (1) 3,7-di-*O*-α-L-rhamnopyranosylquercetin, (2) kaempferitrin, (3) quercetin and (4) kaempferol.

and the UV spectra from the PDA detector of standards with extracts under the same chromatographic conditions. The chromatograms showed a good separation profile for the standards (Fig. 1) and samples (Figs. 2–5). The chromatographic parameters obtained in this work are shown in Table 1. High retention factors may be necessary when analysing complex samples; the selectivity (α) and resolution (Rs) obtained for glycoflavonoids and aglycons are close to the desirable levels. Comparing the fingerprint obtained for the aqueous extracts prepared with *B. forficata* from two geographical regions, harvested in the same season period (summer), it is possible to observe major differences in the global profile: the marker flavonoids are in different concentrations (Table 2). The PDA detector allows every resolved peak of the chromatogram to be analyzed, enabling the researcher to determine which one is a flavonoid derivative. In the samples analysed, flavonoid aglycons were not found, but abundant peaks corresponding to glycosilated flavonoids, can be observed at 2–8 min of retention, mainly in the aqueous extracts. The *Itajaí* sample was obtained at an altitude of 0 m, close to the beach (500 m from the sea), and presented a major flavonoid kaempferitrin. The *Telemaco Borba* sample was collected from the forest at an altitude of 700 m and 350 km from the sea, and presented rich flavonoid content, with no major constituent.

The fluidextracts samples from both geographical regions of origin presented a pattern of peaks which was similar to that observed for aqueous extracts.

The calibration curve, using the standard kaempferitrin, showed a good linearity of the detector over the tested range (12–200 μg/mL), as shown by the correlation coefficient of the regression line ($r = 0.999794$, $y = 2.39e+004x + 6.37e+003$).

Table 2 presents the quantitative analysis of the samples, and it is possible to observe the variation in kaempferitrin content. In order to estimate the total flavonoid content of the samples, peak areas with a UV profile typical of flavonoids were added

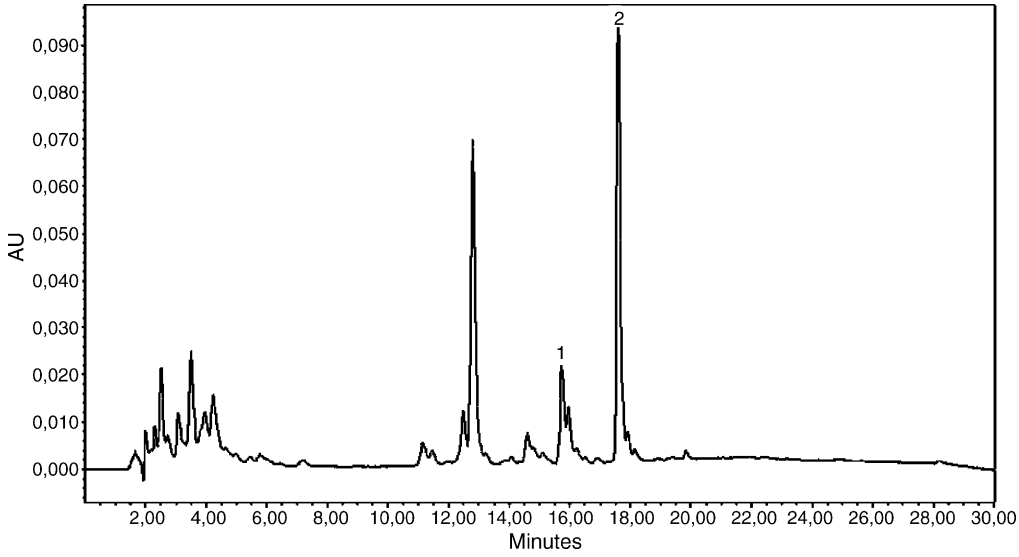

Fig. 2. Chromatographic profile of *Itajaí* aqueous extract. For chromatographic conditions, see Section 2: (1) 3,7-di-*O*-α-L-rhamnopyranosylquercetin and (2) kaempferitrin.

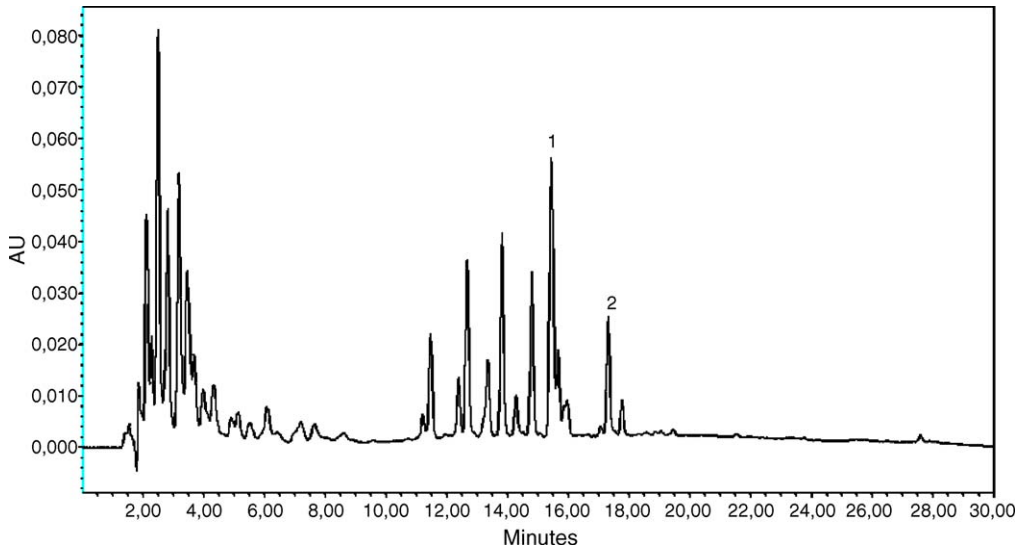

Fig. 3. Chromatographic profile of *Telêmaco Borba* aqueous extract. For chromatographic conditions, see Section 2: (1) 3,7-di-*O*-α-L-rhamnopyranosylquercetin and (2) kaempferitrin.

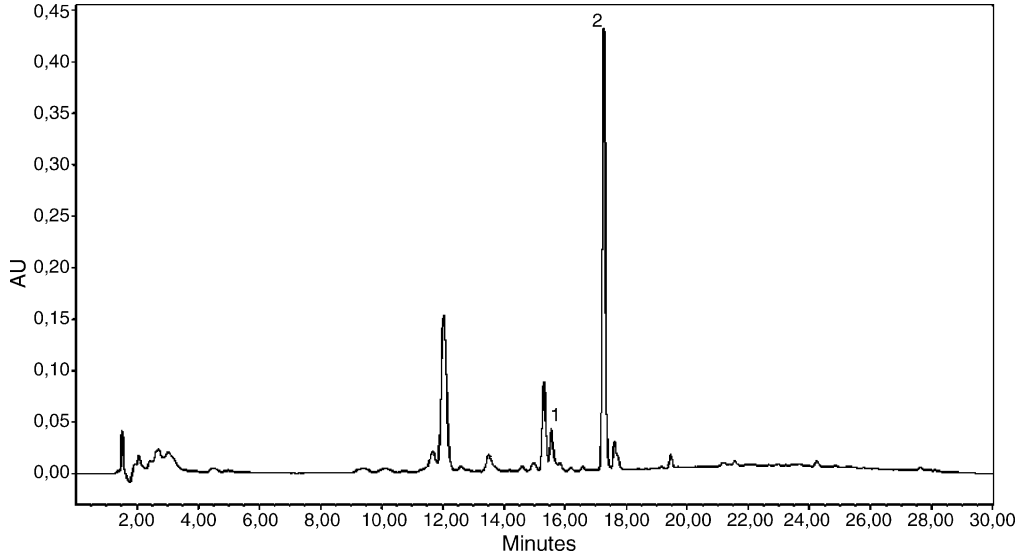

Fig. 4. Chromatographic profile of *Itajaí* fluidextract. For chromatographic conditions, see Section 2: (1) 3,7-di-*O*-α-L-rhamnopyranosylquercetin and (2) kaempferitrin.

together and calculated in terms of kaempferitrin, assuming its molar absortivity to all peaks. Using this method, the equivalence in terms of total flavonoids was observed in the dosage forms analysed. Despite the lower flavonoid concentration (mL) in the aqueous extract, the total intake per day (usually three cups) is equivalent to single dose of the fluidextract.

Although these findings can aid recognition of the complex therapeutic action of a phytopharmaceutical product, it is not always easy to attribute the overall bioactivity to a single constituent [27]. Some synergistic profiles of flavonoids have been described [28].

Table 1
Chromatographic and UV parameters of the flavonoids standards

Flavonoids	Rt (min)	*k*	UV_{max} (nm)	*α*	Rs
3,7-Di-*O*-α-L-rhamnopyranosylquercetin	15.64	18.58	255.0, 349.9	1.15	3.09
Kaempferitrin	17.20	21.19	264.4, 341.6		
Quercetin	21.25	26.42	255.0, 372.4	1.23	3.94
Kaempferol	23.63	30.33	265.6, 366.4		

Table 2
Total flavonoids and kaempferitrin content of *B. forficata* samples by LC

Sample	Kaempferitrin (μg/mL ± S.D.)	CV (%)	Total flavonoids (μg/mL ± S.D.)	CV (%)
Fluidextracts				
Itajaí	1952.59 ± 5.90	3.02	2759.95 ± 0.001	2.82
Telêmaco Borba	211.61 ± 1.64	7.77	2188.20 ± 0.001	1.99
Aqueous extracts				
Itajaí	368.68 ± 0.64	1.72	863.35 ± 0.005	2.13
Telêmaco Borba	77.91 ± 0.47	6.78	856.77 ± 0.003	8.90

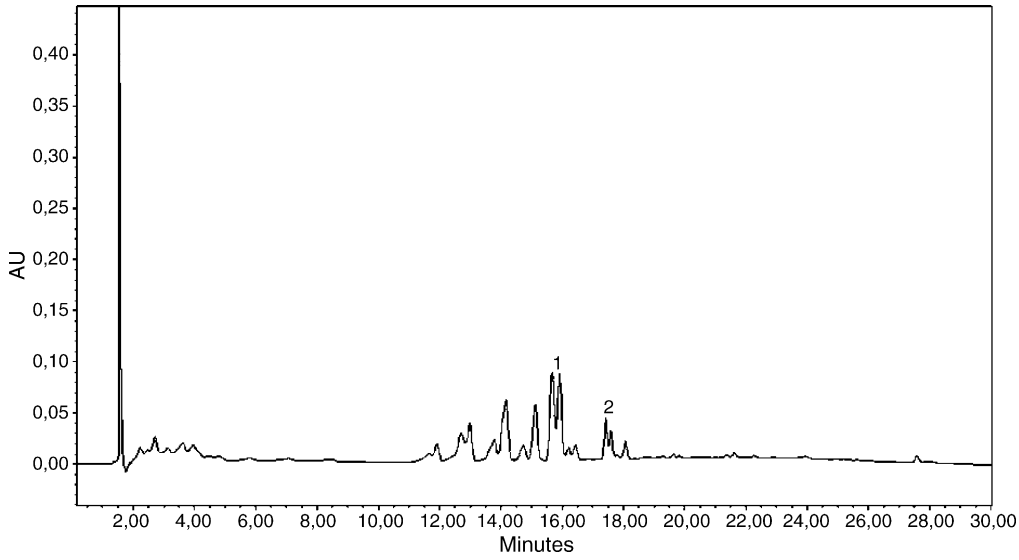

Fig. 5. Chromatographic profile of *Telêmaco Borba* fluidextract. For chromatographic conditions, see Section 2: (1) 3,7-di-*O*-α-L-rhamnopyranosylquercetin and (2) kaempferitrin.

To evaluate the accuracy of the method, a recovery experiment was carried out, spiking the standard kaempferitrin in the appropriated diluted matrix extracts, to determine the linearity of the method. Table 3 shows the results of the kaempferitrin recovery. The recovery average indicated the accuracy of the method, which was 100.6%. In the target level (60 μg/mL) the recovery was 96.4%, close to the desirable level of 100 ± 2.0% [22]. Comparing the recovery experiments in the presence and absence of matrix, no interference of the extract matrix on the kaempferitrin determination was observed, demonstrating the specificity of the method. The coefficient of variation was less than 5.0%. The limit of quantification (LQ) of the method was obtained through the calibration curve, calculating the root mean square error (RMSE): 5.31 μg/mL [29].

In view of the fact that spectrophotometrical techniques are more accessible and economic, a comparison was made between the LC total flavonoids and the UV by the pharmacopoeial method, using the leaves of the *Itajaí* sample. The result obtained – 3620 ± 0.026 μg/mL, CV (%) 7.23 – indicates an overestimation of the flavonoid content, which can be attributed to the extraction method and the absorbance of non flavonoid constituents at the specified wavelength (425 nm, after flavonoids complexation with $AlCl_3$).

Table 3
Recovery of standard kaempferitrin added to *B. forficata* extract

Kaempferitrin added (μg/mL)	Kaempferitrin found[a] (μg/mL) (mean ± S.D.)	CV (%)	Recovery (%)
30	95.29 ± 3.89	4.08	103.7
60	120.71 ± 4.12	3.42	96.4
90	155.09 ± 3.67	2.37	102.5
60[b]	59.87 ± 0.46	0.78	99.8
0[c]	62.82 ± 2.42	3.85	–

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

4. Conclusions

The dosage forms hydro alcoholic (fluidextract) and aqueous extracts from *Bauhinia forficata* present *O*-glycosyl flavonoid derivatives of kaempferol and quercetin. The kaempferitrin (to which the antidiabetic effects of the leaf extracts have been attributed) was quantified by LC in the leaves of *B. forficata* harvested in two geographical regions, being found in different amounts in *Itajaí* and *Telêmaco Borba*, for the preparation of aqueous (368.68 and 77.91 μg/mL) and hydro alcoholic extracts (1952.59 and 211.61 μg/mL), respectively. The method was subjected to recovery assay, to determine its accuracy. A marked difference in total flavonoid concentration was observed in relation to the kaempferitrin content −2759.95 and 2188.20 μg/mL for the fluidextract and 863.35 and 856.77 μg/mL for the aqueous extract. Kaempferitrin was found to be the major constituent only in the *Itajaí* samples, pointing out to a large variability in the *O*-glycosyl flavonoids derivatives production depending on the environment conditions. The spectrophotometric assay overestimated the total flavonoid content (3620 μg/mL) in relation to the LC/PDA assay, which is more advantageous because is possible to determine not only the kaempferitrin content but also the overall fingerprint profile of flavonoids in medicinal extracts.

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