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Comparative evaluation of UV/VIS and HPLC analytical methodologies applied for quantification of flavonoids from leaves of *Bauhinia forficata*

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Abstract: Spectrophotometric determinations (direct dilution or after acid hydrolysis) of flavonoid content in leaves of *Bauhinia forficata* Link, Fabaceae, from two different regions from Brazil were compared with contents of kaempferitrin quantified by HPLC analysis. The absence of kaempferitrin peak in one sample denotes the risk of LC-method failure. On the other hand, the both spectrophotometric assays showed satisfactory performance and do not underwent influence from absence of kaempferitrin. Since several flavonoids are present in the herbal material, different content of flavonoids were observed for each analytical procedure. However, a strong positive association could be detected among methods (R^2>0.99). Although a conversion factor should be adopted to compare procedures, the data showed that the spectrophotometric methods remain as an important tool for analysis of complex matrices such herbal drugs, notably when there aren't any pharmacological or chemical marker established for the species. Moreover, it's possible to suggest that the techniques studied in this work exhibit similar performance under the conditions employed. Nevertheless, before the adoption of kaempferitrin as an analytical marker by HPLC for Bauhinia species, the pharmacological knowledge should be better developed. In this cases, the use of spectrophotometric assay provide higher assurance of the reproducibility of the efficacy and safety.

Article

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Introduction

Flavonoids, an important class of polyphenols from secondary metabolism in plants, are chemical and pharmacological markers of great importance for the quality control of medicinal plants and phytopharmaceuticals (Petry et al., 2001; Soares et al., 2003; Xu et al., 2005; Silva et al., 2009; Fernandes et al., 2012; Marques et al., 2012). A variety of analytical methods can be used to quantify these compounds, however UV/Vis spectroscopy and high performance liquid chromatography (HPLC) are the most prominent techniques (Komarova et al., 2009; Luo et al., 2011; Obmann et al., 2012).

The UV/Vis spectrophotometric determination is one of the most widely used methods for quantification of total flavonoids in raw plant materials due to its simplicity, low cost of implementation and wide availability in laboratories for quality control (Lombard et al., 2002; Popova et al., 2004; Chabariberi et al., 2009; Komarova et al., 2009; Silva et al., 2009; Luo et al., 2011). On the other hand, the HPLC analysis is an analytical procedure more sensitive and selective in the area of natural products to quantify isolated substances and is widely used for all classes of flavonoids (Lombard et al., 2002; César et al., 2007; Komarova et al., 2009; Luo et al., 2011).

Despite the wide applicability of the both techniques mentioned above, some questions about the specificity and comparability of their results have been done in view of the broad structural variability presented by the flavonoid compounds, as well as limitations inherent in each methodology. Accordingly, several scientific studies conducted with both procedures have shown conflicting results (Popova et al., 2004; Chabariberi et al., 2009; Müller et al., 2005; Pinheiro et al., 2006).

Moreover, the choice to quantify a set of compounds or isolated compounds in biological matrices such as herbal materials is a very controversial point in the analysis and quality control. This is because, unlike

synthetic drugs, the pharmacological activity from herbal drugs or phytopharmaceuticals is a result of joint action of a group of substances (Popova et al., 2004; Pinheiro et al., 2006; Chen et al., 2007).

Furthermore, there is great difficulty to relate the concentration of a single or a few substances to the activity of herbal drugs. Several scientific studies make clear that many natural products exhibit higher biological activity than their isolated active components (Popova et al., 2004; Chen et al., 2007; Wagner, 2011; Weerakkody et al., 2011).

Faced with these analytical obstacles, the aim of this paper is to evaluate comparatively, the analytical performance of procedures by HPLC and spectrophotometry, for the quantification of flavonoids from leaves of *Bauhinia forficata* Link, Fabaceae. Widely known in Brazilian traditional medicine as "pata-de-vaca" (cow's hoof), the leaves of the species are used as antidiabetic agent which property has been attribute to the flavonol glycosides from quercetin and kaemperol, especially the 3,7-di-*O*-α-L-ramnopiranosilkanferol (kaempferitrin), reported in the literature as chemical marker for this herbal drug (Jorge et al., 2004; Pinheiro et al., 2006; Da Cunha et al., 2010).

Material and Methods

Plant material

The leaves of *Bauhinia forficata* Link, Fabaceae, were obtained from two different regions of Brazil: Viçosa-MG and Telêmaco Borba-PR. The drug material was collected in Viçosa in December/2009 at Vila Gianetti, whose identification was performed by Professor José Fernandes Martins and a voucher specimen was deposited at Herbarium VIC, Department of Herbal Biology, Federal University of Viçosa, under registration nº. 32513. Regarding the plant material originated from Telêmaco Borba, it was collected at Monte Alegre Farm in April/2010 and kindly provided by Klabin Paraná Forest Products Ltd., whose identification and deposit of voucher specimen were performed at Herbarium of State University of Maringá, under registration nº. 150. The identities of both samples were confirmed in the Botanical Garden of Rio de Janeiro-RJ, Brazil, by the expert in the genus *Bauhinia,* Angela Vaz. Both sample of the raw herbal materials were dried under 45 °C for 72 h and then pulverized in knife mill.

Reagents and glassware

All reagents were of analytical grade: ethanol, aluminum chloride (Vetec Fine Chemicals®, Rio de Janeiro, Brazil), acetone, hydrochloric acid, ethyl acetate, methanol, acetic acid (Contemporary Chemical Dynamics®, São Paulo, Brazil) and methenamine (Merck®, Darmstadt, Germany). For analysis by liquid chromatography acetonitrile chromatographic grade was employed (Merck®, Darmstadt, Germany), ultrapurified water obtained by Milli-Q Millipore® (Milli-Q System, Massachusetts, USA), phosphoric acid (Chemical Dynamics® Contemporary, São Paulo, Brazil) and kaempferitrin standard, kindly supplied by Prof. Dr. Maique Weber Biavatti (Federal University of Santa Catarina, Florianópolis, Brazil). Calibrated volumetric flasks were used with calibration certificate from Satelit®.

Spectrophotometric determination of total flavonoids content in leaves of B. forficata

The total flavonoid contents (TFC) in the leaves of *B. forficata* were determinated by two spectrophotometric methods: direct dilution (DDM) or after acid hydrolysis of flavonoid glycosides (AHM), according methodologies developed and validated by Marques et al (2012).

HPLC analysis of kaempferitrin from leaves of B. forficata

Extractive solution

The extractive solution was obtained by extraction of drug material provide from Telêmaco Borba-PR under reflux with hydroalcoholic solution, in according to the procedure described by Petry et al (1998). Thus, the dried and powdered leaves (1.0 g) were extracted with 30 mL of ethanol:water 40% (v/v), under reflux on a water bath for 30 min. The extract was cooled to room temperature and filtered through cotton. The residue (cotton and drug material) was re-extracted (10 min) twice. After cooling, the extracts were filtered and the volume adjusted to 100.0 mL volumetric flask with ethanol: water 40% (v/v), obtaining the extractive solution (10 mg/mL; drug/solvent). The hydroalcoholic extractive solution was injected without dilution.

Reference standard

An ethanolic solution containing 0.05 mg/mL of kaempferitrin was prepared. Aliquots of this solution were diluted in ethanol to obtain concentrations corresponding to 0.003; 0.005; 0.007; 0.01 and 0.013 mg/mL.

Chromatographic conditions

Chromatographic analysis to quantify kaempferitrin in the extractive solution were conducted on a Shimadzu® liquid chromatography (UFLC, Japan) controlled by the LC Software Solution 1.0 and with LC-20 AT pump, degasser DGU-20A5, Sil-20A autosampler

and diode array detector (DAD) SPD-M20A. A Restek® C18 column (250 mm x 4.6 mm, 5μm) was used under 40 °C and protected by a pre-column Restek C18® (10 mm × 4 mm, 5 mm). The standards and samples were eluted using a gradient mobile phase consisting of phosphoric acid: water 0.02% (pH 2.5) (A) and acetonitrile (B). The gradient conditions were: 0-5 min 85% A, 5-30 min 70% A, 30-32 min 80% A, 32-35 min 85% A, followed by wash up with 100% of B for 15 min. The flow was 1.0 mL/min and injection volume of 20.0 μL. The kaempferitrin in plant material was determined by external standard method for 0.003; 0.005; 0.007; 0.01 and 0.013 mg/mL. The computer program Excel (Microsoft®) was used to adjust the regression curve and calculate the corresponding determination coefficient. All samples and standards were filtered through membranes with porosity of 0.22 μm (Millipore®) and injected in triplicate.

Validation of liquid chromatographic method

The LC-method was validated according to the parameters required by the specific resolution nº 899/03 of the National Agency for Sanitary Surveillance (Anvisa, 2003) and recommended by the International Conference on Harmonization (ICH Q2B, 1995): linearity, precision (repeatability and intermediate precision) and accuracy. All tests were performed in triplicate.

The linearity was determined by the analysis of three authentic curves, constructed with extractive solutions (drug material from Telêmaco Borba), in six levels of concentration ranged from 2.5 to 15.0 mg/mL (drug/solvent). The curves were constructed by plotting the average values of the areas according to the concentration. The results were statistically analyzed by calculating the linear regression by PLS method in order to define the determination coefficient (R^2).

The method precision was evaluated at two levels using extracts of 10 mg/mL (drug/solvent): *precision* (repetitivity or repeatability): performed by six individual extracts examined on a single day; and, *intermediate precision:* performed by two analysts on two consecutive days. The results were expressed as relative standard deviation (RSD%) or standard deviation (SD). Additionally, the results were statistically treated by two-way analysis of variance (two-way ANOVA).

The accuracy was evaluated by recovery experiments by adding known amount of the diluted extractive solution to the samples at 100% of the test concentration, expressed by the ratio of the average concentration determined experimentally and the corresponding theoretical concentration.

For the chromatographic method, 10.0 mL of a spiked extractive solution (12.5 mg/mL) were added to the extractive solution of 10.0 mg/mL, resulting in three replicates with 11.25 mg/mL.

Comparative evaluation of the analytical procedures

For comparative evaluation of the techniques for quantification of flavonoids, extractive solutions were prepared with 0.5; 1.0 and 1.5 g of the powdered leaves of *B. forficata*. The flavonoid content was determined by spectrophotometry (direct dilution and after hydrolysis) and by HPLC. All flavonoid concentrations obtained were converted to the same unit (mg/mL), so if they could be compared by obtainment the linear regression line.

Results and Discussion

The most common procedure to determine the total flavonoid content is a UV/Vis spectrophotometric assay, based on the formation of a complex between the aluminum cation, Al (III), and the carbonyl and hydroxyl groups of the flavonoid (Glasl & Becker, 1984; Popova et al., 2004). The aluminum cation acts forming stable complexes with flavonoids, with bathochromic effect thus avoiding interference from other phenolic compounds (Petry et al., 1998, Souza & Giovani, 2005; Silva et al., 2009). Nevertheless, the use of HPLC is the method of choice for qualitative and quantitative analysis of flavonoid markers, due to its higher sensitivity and accuracy. Highlighting the use of reverse phase columns and gradient elution systems to this purpose (Chabariberi et al., 2009; Obmann et al., 2012).

In this paper, spectrophotometric methods employing direct dilution or after acid hydrolysis and using aluminum chloride as a reagent, were comparatively investigated with a HPLC method for kaempferitrin as chemical marker for leaves of *B. forficata*. The ability of spectrophotometric procedures for the quantification of total flavonoid content in leaves of *B. forficat*a was previously studied and validated (Marques et al., 2012). On the other hand, the HPLC procedure was evaluated before the comparative approach. The resulting data are summarized in Tables 1, 2 and 3.

Linearity pattern was checked by analyzing standard curves of the extractive solution and kaempferitrin. The linear regression analysis by least squares method showed a coefficient of determination (R^2) higher than 0.99 for the both procedures, indicating linearity within the range of concentration. The method also showed accuracy in two analyzed levels: precision (repeatability) and intermediate precision. Regarding the precision parameter, the coefficient of variation was lower than the preconized by guidelines (5%), proving the suitability of the procedure (Anvisa, 2003).

Concerning the intermediate precision, the analytical procedure was evaluated by different analysts on the same day and on different days. The statistically analysis performed by two-way ANOVA showed that the procedure is accurate (Table 2).

Table 1. Results of linearity and precision assays for determination of kaempferitrin by HPLC in extractives from leaves of *Bauhinia forficata* supplied from Telêmaco Borba-PR.

Parameters	Extractive solution
Equation	y = 0.956x – 0.0077
R^2	0.999
Precision (repeatability) mean±SD (µg/mL)	9.67±0.18 1.86

SD: standard deviation; RSD: relative standard deviation.

Table 2. Intermediary precision assay for determination of kaempferitrin by HPLC in *Bauhinia forficata* extractives.

	Day 1 (mg/mL) mean±SD (RSD%)	Day 2 (mg/mL) mean±SD (RSD%)
Analyst 1	0.00978±0.0013 (1.33)	0.00993±0.0005 (0.47)
Analyst 2	0.00980±0.0017 (1.69)	0.00975±0.0009 (0.95)

Results expressed as kaempferitrin. SD: standard deviation; RSD: relative standard deviation.

The accuracy was evaluated by the recovery assay and the result was appropriated in accordance com legal requirements for such matrix (Anvisa, 2003).

Table 3. Recovery result (%) for determination of kaempferitrin by HPLC in *Bauhinia forficata* extractives.

	Data
Theoretic concentration (mg/mL)	0.01066
Experimental concentration (mg/mL)	0.01044±0.00004
Recovery (%)	97.89

After validation, both spectrophotometric procedures were employed for the quantification of flavonoids in the samples of leaves of *B. forficata.* The flavonoid contents are presented in Table 4 and the results were comparatively evaluated. According to the results, there were no statistically differences in the performance of each analytical procedure (Table 4). On the other hand, it was observed that the data from obtained by the method after acid hydrolysis were higher for both herbal samples in comparison with the other procedures. This result can be explained by the specificity for flavonoid aglycones. Thus, the procedure allows quantifying free aglycones and aglycones derived from of *O*-glycosyl flavonoids (release after hydrolysis and partition), reducing the interferences from other polyphenolic compounds on the method response, improving the specificity to the group. However, the presence of *C*-glycosyl flavonoids should be take in to account, since they cannot undergo hydrolyses and can be discarded in the aqueous phase at the partition step (Schmidt & Ortega, 1993; Petry et al., 1998; Müller et al., 2005; César et al., 2007).

Table 4. Total Flavonoids content in leaves of *Bauhinia forficata* by spectrophotometric methods.

Sample	DDM (mg/mL) mean±SD	AHM(mg/mL) mean±SD
Viçosa	0.04255±0.017	0.15881±0.00139
Telêmaco Borba	0.04665±0.048	0.15710±0.00096

Result expressed as quercetin; DDM: Direct Dilution Method; AHM: After Hydrolysis Method; SD: standard deviation.

Unlike analytical procedures by spectrophotometry, the LC-methodologies have as major characteristic the ability to separate the substances allowing quantifying each compound individually and therefore they are much more accurate (Lombard et al., 2002; Popova et al., 2004). Furthermore, the use of PDA (photodiode array) detectors provides the UV profile of each compound improving the efficiency on their identification (Popova et al., 2004). Thus, the importance of RP-LC methods for the quality control of plant raw materials either qualitative, through establishment of chemical profiles (fingerprints), or quantitative analysis, is unquestionable. However, the higher selectivity and specificity of LC methods may be considered a negative factor for complex biological matrices such as medicinal plants, particularly for species in which group(s) of active ingredient(s) is still unclear (Popova et al., 2004).

As can be seen in the chromatogram obtained for the samples of *B. forficata* used in this study (Figure 1), the procedure developed was able to separate and detected the peak corresponding to kaempferitrin. Although this flavonoid has been considered by several authors the mainly analytical marker for *B. forficata* (Pinheiro et al., 2006; Da Cunha et al., 2010), our results revealed that it could be observed only to one of the samples studied. Thus, the adoption of such marker for the quality control may undermine the use of HPLC as an analytical tool and lead to mistaken uncorrected conclusions about the quality of the samples.

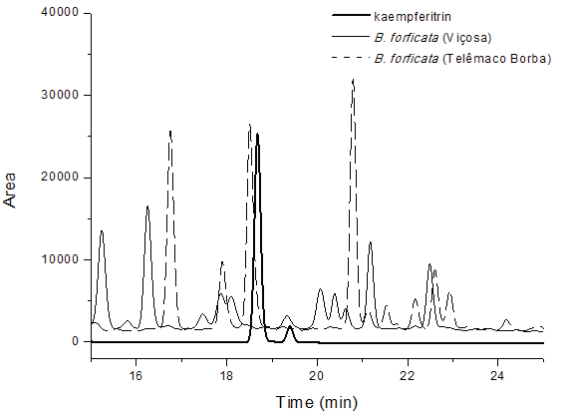

Figure 1. Chromatogram of *Bauhinia forficata* extract from "Viçosa" or "Telêmaco Borba" overlapped with kaempferitrin.

The questioning of the right marker for the specie has been previously reported (Arigony, 2005), and was not the initial objective of this study, but the results of this study highlighted a major challenge for the development of herbal medicine in Brazil, since the chemical variability of samples associated with the absence of an appropriate marker, can be misleading assessment that limit the use of the species. It was evident, therefore, the difficulty to adopt very selective methods for herbal drugs, especially for those who are not clinically investigated as is the case of several Brazilian native species.

The lack of studies about the correlation between the chemical composition and the evidence of the therapeutic property of several herbal drugs can lead to mischose chemical markers that have little or no relationship to pharmacological activity. Thus, quality specifications for herbal materials could be established based on these inefficient markers. So, until there is the indisputable active/clinical marker, the variations in the concentration of unique compound, such as kaempferitrin for leaves of *B. forficata*, will be not enough to assure the quality in terms of their use in therapy. In such cases, the quantitative assays of class of compounds seem to be more appropriate since they are much more robust to individual variations in quantitative and qualitative composition of compounds in the mixture. However, regardless the principle of the analytical method, the procedure should not jeopardize the analytical performance. It means that after choosing the chemical marker for the specie, the quantitative performance of the procedures should be similar to the reference substance. In spite of each analytical technique showed responses of different magnitudes, the presence of significant correlation among procedures suggest the possibility of comparison of their results.

Regarding the case of *B. forficata*, the analysis by HPLC showed kaempferitrin in reasonable concentration only for one sample (Figure 1). Although the antidiabetic properties of kampferitrin are related in the literature (Jorge et al., 2004), there are no reports about the activity from other glycosides of kaempferol. Thus, the HPLC usage for quality control remains limited to the species and the method can failure if there are variations in the proportion of the chemical marker in the mixture. Moreover, liquid chromatography plays an important role to evaluate the performance of general assay by spectrophotometry. Since the chemical marker present the physicochemical properties of its group of metabolites, the comparative investigation of selective methods (HPLC) and methods for quantification of groups (UV-VIS), may provide greater reliability to the second techniques if the response of such procedures shows close correlation. In this context, the performances of spectrophotometric methods were compared with the LC-profile of kaempferitrin in. The results are comparable with previously report of Lombard et al. (2002), who also study the correlation between UV-VIS e LC methods for quercetin in onion extracts.

Although the absence of kaempferitrin in one sample does not invalidate the hypothesis of similar performance for the group of flavonoids, the comparative study was carried out by using the drug sample containing kaempferitrin. The procedures were compared to another at three different concentrations levels and analyzed by regression parameter (coefficients of determination, slopes and confidence limits for intersections). The results for the regression parameters are summarized in Table 5.

An important linear correlation was observed for all correlations performed among the three analytical methods. Thus, the concentration of kaempferitrin obtained by HPLC showed a strong correlation with the both spectrophotometric methods either by direct dilution (DDM) or after acid hydrolysis (AHM). Additionally, the high values obtained for the coefficient of determination (R2), indicated that more than 99% of the experimental variance could be explained by linear equations and thus, the responses could be attributed mainly to the variations on the concentration of flavonoids/kaempferitrin (Table 5). In this way, the spectrophotometric responses were appropriated to be use as representative from concentrations of the kaempferitrin.

Regarding the confidence limits for the intersections, a slight shift to higher values could be observed for both spectrophotometric methods in comparison to HPLC. Although these results are typical to presence of constant systematic errors, it can be easily explained by the complex nature of spectrophotometric samples. Thus, the overlapping of spectrums such as kaempferol derivates and similar aglycones, probably increase the spectrophotometric response. Better correlation between spectrophometric and HPLC assays for flavonoids were previously related (Lombard et al., 2002). However, it was only possible when all peaks of flavonoids in the chromatogram were summed. Considering that kaempferitrin represents only one of the compounds among the different flavonoids present in the matrix of *B. forficata*, it is understandable

Table 5. Statistical parameters for comparative analysis of the analytical methods.

	R^2	sd	α	CL_{low}	CL_{up}	t
DDM vs HPLC	0.9990	1.63	4.19	-4.80	19.34	22.62
AHM vs HPLC	0.9997	3.56	13.89	2.28	47.15	40.28
AHM vs MDD	0.9998	3.21	3.31	-19.48	20.99	51.67

DDM: Direct Dilution Method; AHM: After Hydrolysis Method; R^2: coefficient of determination; SD: standard deviation; α: angular coefficient; CL_{low}: lower confidence limit; CL_{up}: upper confidence limit.

that the responses of spectrophotometric methods are far superior to the results observed for HPLC.

Despite the difference expected for both spectrophotometric methods provided by their specificity (Table 5), close correlation was observed and total absence of proportional errors was confirmed by the confidence limits of interception.

Taking into account the discussion above, as well as the advantages of UV-VIS techniques (such as simplicity and low cost of implementation); it could be concluded that the spectrophotometry remains as valid tool for analysis of total flavonoids in herbal materials. This approach becomes more critical due to the high cost or absence of reference substance needed for determination of individual flavonoids (César et al., 2007; Hoffmann-Ribani & Rodriguez-Amaya., 2008).

In spite of the close correlation observed in this study among analytical methods by spectrophotometry and liquid chromatography, before the adoption of this concept to other herbal drugs and/or secondary metabolites, a careful study is required in order to identify and quantify critical steps in the procedure that may compromise the performance.

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