

Evaluation of the Folin-Ciocalteu Method and Quantification of Total Tannins in Stem Barks and Pods from *Libidibia ferrea* (Mart. ex Tul) L. P. Queiroz

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ABSTRACT

The stem bark and pods of “jucá” are widely used in Brazilian folk medicine and, despite their therapeutic relevancies, there are insufficient reports about their quality control. Both herbal drugs from the species are rich in tannins, which showed a lot of biological and economic interest. Thus, the purpose of this study was to evaluate the method for quantification of tannins in the stem bark and pods from *L. ferrea*. The performance of the method was tested against the influence of the most relevant parameters (reaction time; amount of drug; type and amount of precipitation agents; and, concentration of the reagents). The procedure was validated according to the literature. After optimization, the experimental conditions were established as: 30 min of reaction; λ of 760 nm; 1.0 mL of Folin-Ciocalteu; 29% of Na₂CO₃ (w/v); and, skin powder as complexion agent. The method validation showed that the instrumental response was linear and sensitive to the investigated analyte. The method presented the necessary precision (RSD < 2.19%) and accuracy (96.84%-109.13%), as recommended by the official codex. Moreover, the deliberate introduction of experimental variations showed the stability of the procedure error against external sources (RSD < 2.42%). In conclusion, the data demonstrated the suitability of the method and can be used as an appropriate analytical tool for quality control of herbal drugs from *L. ferrea*.

Keywords: *Libidibia ferrea*, Quality control, Spectrophotometry, Tannins.



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INTRODUCTION

Libidibia ferrea (Mart. ex Tul.) L. P. Queiroz is a tree that belongs to the Fabaceae family and is presented as basionym *Caesalpinia ferrea* Martius. The species is widely known as "jucá" or "pau ferro" and can be found on the "Caatinga" region extending to the northeast and north of Brazil^{1,2}. Moreover, the species is used in the north of Brazil for urban arborization and landscape design³. The stem bark and pods are the parts of *L. ferrea* most commonly used in popular medicine, and is mainly used as an anti-inflammatory and anti-asthmatic aid. In addition, the literature reports the relationship between the tannins of this species and its several biological properties, these include: antimicrobial, anti-inflammatory and hypoglycemic properties⁴⁻⁹. Regarding the chemical composition of *L. ferrea*, the presence of flavonoids, coumarins, steroids and other phenolic compounds have already been reported^{6,9,10}. Notwithstanding the relevance of this species in traditional practices, there are not enough data/parameters of quality to establish specifications for use of this species in folk medicine or as raw material for industrial production of herbal medicines^{11,12}. In this context, the quantification of marker compounds (analytical or active) plays an important role on the evaluation and prediction of the quality of herbal drugs and herbal medicines¹³. What is more, the quantitative determination of tannins becomes a valid strategy for the chemical analysis and initial quality specifications of herbal materials from this species.

Despite the reports of selective techniques for the quantification of tannins, such as high performance liquid chromatography¹⁴⁻¹⁶, which includes the pods from *L. ferrea*¹⁰, the procedure is used only to quantify the monomers of tannins (gallic acid, ellagic acid or catechin) due to the complexity, variability and stability of hydrolysable or condensed tannins¹⁷⁻¹⁹. However, the quantification of tannins by spectrophotometry allows for the estimation of the total content including the polymers (hydrolyzable or condensed derivatives), avoiding the underestimation of the analytical response, a common fact in certain selective methods due to the unavailability of reference standards and/or difficulty of structural elucidation of these macromolecules. Additionally, the spectrophotometric method is still the most used due to its simplicity, low cost and availability in analytical laboratories^{20,21}. This approach also ensures the principle of the synergistic effect attributed to medicinal plants, especially for species whose active compounds have not yet been reported, such as *L. ferrea*²².

In order to quantify tannins by UV-spectrophotometry, the colorimetric Folin-Ciocalteu method shows the reactional stability able to ensure the necessary reproducibility for the validation study required by international guidelines and health authorities²³⁻²⁵.

The reaction product from Folin-Ciocalteu is result of unspecific oxidation with polyphenols. Thus, the coloration and intensity observed after reaction does not correspond exclusively with tannins. Therefore, the complexation of tannin is indispensable to attribute specificity to the method. Then, the tannins are removed by filtration or centrifugation after formation of insoluble complexes with macromolecules. After this, the content of tannin is established by the difference between the reaction product of total polyphenols and the reaction product of the fraction of non-adsorbed polyphenols²⁶⁻²⁹.

In spite of reports in the literature, the adoption of spectrophotometric methods for qualitative analysis of plant drugs is highly dependent on the composition of each matrix and critical factors such as reagents, reaction medium and time. Consequently, the performance of analytical methods must be subjected to a series of tests with the purpose of proving their efficiency in the uses that are being proposed and the

applicability of these in the laboratory routine^{20,23}. Thus, the main purpose of this study was to evaluate the analytical critical variables and to validate the Folin-Ciocalteu method for quantitative determination of total tannins in stem bark and pods of *L. ferrea*.

MATERIAL AND METHODS

Herbal Material

The stem bark and pods of *L. ferrea* were collected in Limoeiro (Pernambuco) and the identification was performed by the Agronomic Institute of Pernambuco (IPA), the registration was deposited under number 88145. The samples were submitted to drying in a circulating air oven (Luca-82/480, Lucadema[®]) at 40 °C, for 96 h, and then ground in a Willey knife mill (Tecnal[®]).

Reagents and Glassware

Calibrated glassware and analytical grade reagents were used: anhydrous sodium carbonate (FMaia[®]), Folin-Ciocalteu (Dinâmica[®]), casein (Sigma[®]), skin powder (Merck[®]), polyvinylpyrrolidone - PVP (Sigma[®]), gallic acid (Vetec[®]), catechin (Sigma[®]), and pyrogallol (Fluka[®]).

Chromatographic Profile by TLC of Tannins from Stem Bark and Pods of *L. Ferrea*

The samples were obtained by reflux, at 10% (w/v) with methanol as solvent. Pre-coated TLC silica gel 60 F₂₅₄ aluminum plates (10 cm × 10 cm; 250 μm thickness; Merck[®], Germany) were used and the mobile phase constituted of the mixture of ethyl acetate: formic acid: water (90:5:5, v:v:v). The sample applicator using 100 μL syringe (Hamilton[®], Schweiz) connected to compressed air and winCATS[®] software (Camag[®], Switzerland). The plate was developed in a twin trough vertical glass chamber (Camag[®]) with 10 x 10 cm dimensions and was used to develop the chromatograms after 1 hour of saturation with the mobile phase. The following were applied on the plate, 10 μL of each sample (stem bark or pods) and 5 μL of standard solution of gallic acid (0.5 mg/mL). After development, the plate was dried and the components were visualized by ultraviolet (UV) irradiation at 254 nm and then derivatized with solution of ferric chloride 1% (w/v).

General Procedure for Quantification of Total Tannin Content

Stock Solution

About 0.75 g of the ground herbal material was transferred to a 250 mL round bottom flask and 150.0 mL of distilled water was added. The mixture was refluxed in a water bath (Lucadema[®]) for 30 minutes at 85 °C, cooled to room temperature and then quantitatively transferred to a 250 mL volumetric flask. After that, the round-bottomed flask was rinsed and the washings collected in the volumetric flask, then diluted to 250 mL with water, and finally filtered through filter paper, discarding the first 50 mL. The filtrate was the stock solution (SS - 0.002 g/mL).

Sample Solution for Total Polyphenols

An aliquot of 5.0 mL of the **SS** was taken and diluted with distilled water in a 25 mL volumetric flask. Then, 2.0 mL of this solution, 1.0 mL of Folin-Ciocalteu reagent and 10 mL of distilled water were transferred to a 25 mL volumetric flask and the volume was filled with a solution of anhydrous sodium carbonate (Na_2CO_3) at 29% (w/v). After 30 minutes, the absorbance corresponding to total polyphenols was measured at 760 nm (A_1) using distilled water for zero adjustment.

Sample solution for non-adsorbed polyphenols

An aliquot of 10.0 mL of the **SS** was added to the complexing agent (skin powder, casein or polyvinylpyrrolidone) and subjected to 125 mL erlenmeyer agitation for 60 minutes. The resulting solution was filtered through filter paper and 5.0 mL of filtrate diluted with distilled water in a 25 mL volumetric flask. Then, 2.0 mL of this solution, 1.0 mL of Folin-Ciocalteu reagent and 10 mL of distilled water were transferred to a 25 mL volumetric flask and the volume was filled with a solution of Na_2CO_3 at 29% (w/v). After 30 minutes, absorbance corresponding to the non-adsorbed polyphenols was measured at 760 nm (A_2) using distilled water for zero adjustment.

Standard Solution

50.0 mg of each reference standard (gallic acid, catechin or pyrogallol) were dissolved in 100 mL of distilled water. Then, 5.0 mL of the standard solution was transferred to a 100 mL volumetric flask and the volume filled with the same solvent. Finally, 2.0 mL of this solution, 1.0 mL of Folin-Ciocalteu reagent and 10.0 mL of distilled water were transferred to a 25 mL volumetric flask and the volume was filled with a solution of Na_2CO_3 at 29% (w/v). After 30 minutes, the absorbance of the standard solution was measured at 760 nm (A_3) using distilled water for zero adjustment.

The total tannin content was calculated as percentage of tannins (dry drug), expressed in g% of the standard, according to Equation 1:

$$TT = \frac{62.5 \times (A_1 - A_2) \times m_2}{A_3 \times m_1} \text{ (Eq. 1)}$$

Where: TT = total tannin content in g% of standard; A_1 = absorbance of the sample solution for total polyphenols; A_2 = absorbance of the sample solution for non-adsorbed polyphenols; A_3 = absorbance of standard solution; m_1 = mass of the sample used in the test, in grams, considering the determination of water; m_2 = mass of the standard, in grams, considering purity.

Evaluation of Operational and Reactive Parameters of the Folin-Ciocalteu Method

Determination of Wavelength and Reaction Time

The wavelength for analyses was evaluated for samples and standards by spectrums in the region from 400 to 900 nm, after 30 minutes of addition of the last reagent described in the general procedure for preparation of samples for total polyphenols. The spectral data were used to choose the maximums and for evaluation of the samples dilutions. The reaction time was studied from the reaction kinetics

(absorbance *versus* reaction times) for each analyte (samples or standards). The measurements were initiated 5 minutes after the addition of the last reagent (Na_2CO_3 solution, 29%, w/v), and repeated at each 5 minutes interval during 60 minutes.

Determination of the Reference Standard

Calibration curves of reference substances (gallic acid, catechin or pyrogallol) were constructed using five concentration levels (1.00 to 8.00 $\mu\text{g/mL}$) for choice of reference standard. The determination was carried out in a spectrophotometer after the execution of the reaction procedures to determine total polyphenols. The results were analyzed by linear regression using the least squares method, and the coefficient of determination (R^2) was calculated. The selection of the reference standard for the methodology was performed according to Bueno (2012)³⁰, evaluating the average angular coefficient of three curves and the molar absorptivity of each substance calculated by the equation: $A = \varepsilon \cdot b \cdot c$, where A = absorbance (A.U.); ε = molar absorptivity (mol/L); b = optical path of the cell (cm); c = sample concentration ($\text{g}/100 \text{ mL}$)^{30,31}.

Evaluation of the Drug:Solvent Ratio

The samples were prepared using different amounts (0.50, 0.75 or 1.00 g) of the herbal material (stem bark or pods), following the procedures for obtaining the stock solution and total polyphenol determination³².

Evaluation of the Influence of Folin-Ciocalteu Reagent and Na_2CO_3 Solution

The influence of the reaction conditions on the total tannin content was evaluated through a factorial design 2^2 using Folin-Ciocalteu reagent (1 and 3 mL) and Na_2CO_3 solution (10 and 29%, w/v) as independent variables). The matrix was added with three central points (Folin = 2 mL, Na_2CO_3 = 15%, w/v), and the experiments were performed in triplicate³³. The dependent variable evaluated in the study was the total tannin content (TT) expressed in g% of reference standards, from the absorbance read after 30 minutes of addition of the last reagent. Statistical analysis of the data was performed by ANOVA and the data were used to calculate response surfaces using the Microsoft Excel[®] and STATISTICA[®] 6.0 programs (StatSoft, USA).

Evaluation of the Complexing Agent

In this step, different types (casein, PVP and skin powder) and amounts (100, 150 and 200 mg) of complexing agents were evaluated to verify the influence on total tannin content^{28,34}. The total tannin content was calculated and the results expressed as mean, standard deviation and relative standard deviation (RSD%).

Validation of Analytical Methods

The method was validated according to the parameters recommended by the regulatory agencies: National Agency of Sanitary Surveillance (Anvisa)²³ and International Conference Harmonization (ICH)²⁴, using as parameters: linearity, specificity, limits of detection and quantification, accuracy and robustness. The statistical reliability of the results was established from the relative standard deviation (RSD%). Some of the results were treated by analysis of variance (ANOVA) *One-Way* or *Two-Way* and *t*-student test, in order to verify significant differences between means or variances resulting from the assays.

Linearity

The stem bark or pod samples were prepared as described above, and after appropriate dilutions, had a concentration range of 16.0-48.0 µg/mL for stem bark and 8.0-40.0 µg/mL for pods. In addition, standard solutions were obtained, with concentrations of between 1.0 and 8.0 µg/mL. The results were analyzed by linear regression using the least squares method to calculate the coefficient of determination (R^2) and other parameters of the mathematical model.

Specificity

Specificity was evaluated by the method of addition of the standard, where the solutions used for the linearity test were contaminated with defined amounts of reference standards (25 mg of pyrogallol, 50 mg of gallic acid or catechin) to elaborate the specificity curves. Finally, a comparison was made between the slopes obtained for the linearity curves and the specificity of the method^{30,35}.

Limits of Detection and Quantification

The limits of detection (LOD) and quantification (LOQ) were estimated using the equations below available in Specific Resolution (RE 899)²³:

$$LOD = \frac{S \times 3}{I} \quad LOQ = \frac{S \times 10}{I}$$

Where, S is the standard deviation of the intercept with the y-axis of at least 3 calibration curves constructed for the substance of interest and I is the mean slope of the 3 authentic calibration curves. All results were expressed in µg/mL.

Precision

The precision was evaluated at two levels: repeatability, with analysis of 6 samples at the same concentration (100%), on the same day and by the same operator; and, intermediate precision, by analyzing 3 samples at the same concentration (100%), by two analysts and on two different days. The results were expressed as g% of standard.

Accuracy

Accuracy was evaluated by recovery assay, through which extractive solution samples from stem barks or pods were fortified with the standard at three concentration levels. The percent recovery was calculated from the ratio between the experimentally determined mean concentrations and the expected theoretical concentrations for the fortified solution. Extracts were used in concentrations of 16.0 µg/mL of stem barks and 32.0 µg/mL of pods, and to these were added three quantities of standard (25.0, 50.0 and 75.0 µg), then the usual procedure was performed to determine the absorbance.

Robustness

To evaluate the robustness, the parameters of stability of the reaction product to the luminosity (absence or presence), stability of the extractive solutions (0 h and 6 h) and different equipment for the reading of the samples were considered (Evolution 60S, Thermo Scientific® and AJX 1900, Micronal®).

RESULTS

The chromatographic analysis by TLC of the samples evidenced the presence of hydrolyzable tannins from stem bark and pods of *L. ferrea* (Figure 1). In addition to the typical bands for hydrolyzable tannins (gray bluish), both samples showed a band with the same color and R_f value (0.85) than the spot observed for the standard of gallic acid.

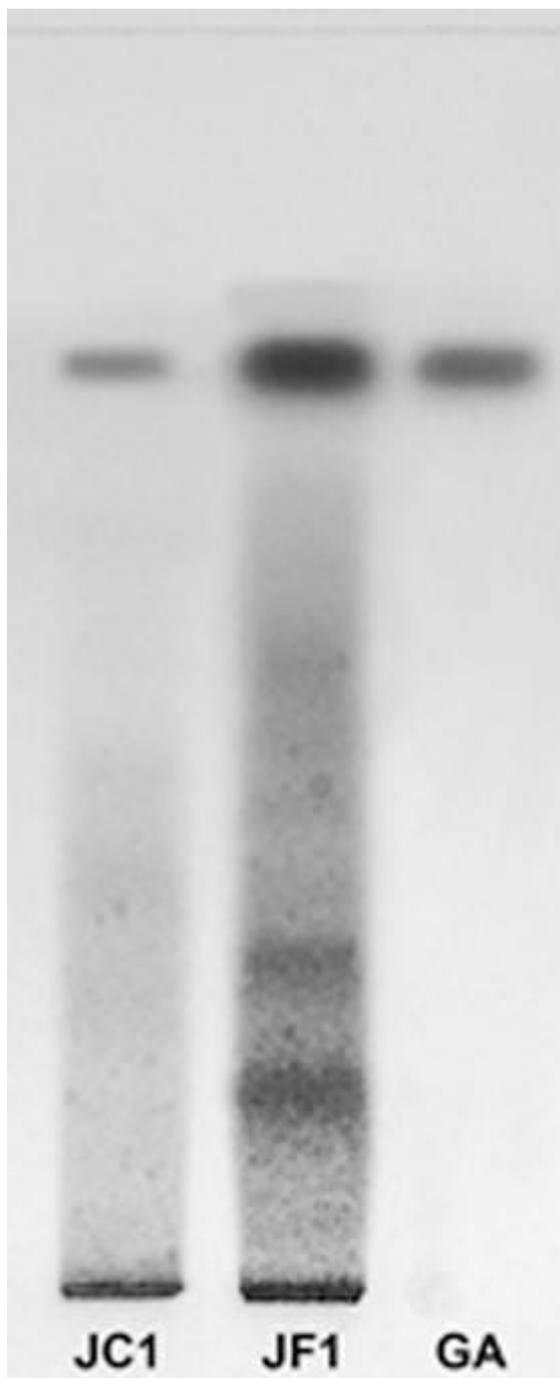


Figure 1. Chromatogram obtained by TLC indicating the presence of gallic acid (GA) in the samples of stem bark (JC1) and pods (JF1) *L. ferrea* after derivatization with ferric chloride (1% ethanol).

Evaluation of the Analytical Parameters of Folin-Ciocalteu Method

In order to study the most appropriate reference standard, calibration curves were constructed for each chemical standard (gallic acid, catechin and pyrogallol) and the coefficients of molar absorptivity (ϵ) and angular coefficients (α) were calculated. The results are presented in Table 1 and show a similar behavior for gallic acid and catechin, while the analytical procedure was more sensitive to pyrogallol showing higher values for both coefficients.

Table 1. Results of linearity, limits of detection (LOD) and quantification (LOQ) and molar absorptivity, from calibrations curves for samples (stem bark and pods) and standards (pyrogallol, catechin and gallic acid).

Parameters	Herbal drugs		Standards		
	Stem bark	Pods	Pyrogallol	Catechin	Gallic acid
Concentration ($\mu\text{g/mL}$)	16.0-48.0	8.0-40.0	1.0-5.0	2.0-7.0	2.0-8.0
Slope (a)	0.0134	0.0204	154.0	101.94	102.52
Interception (b)	0.1115	0.0772	-0.0060	0.0401	0.0072
R^2	0.9994	0.9988	0.9996	0.9994	0.9999
LOD ($\mu\text{g/mL}$)	1.5302	1.0425	-	-	-
LOQ ($\mu\text{g/mL}$)	5.1007	3.4751	-	-	-
Molar absorptivity (ϵ)	-	-	1462.2	1113.3	1045.0

All the scanning spectrums obtained for extractive solutions and standards showed a similar maximum at $\lambda = 760$ nm, after the Folin-Ciocalteu reaction. The reaction kinetics at this wavelength showed that the absorbance increased during the initial 30 minutes, then stabilization was achieved and was followed by a gradual decrease in the absorbance intensities. In regard to the amount of herbal material, 0.50 g of drug material was enough to provide the highest experimental responses, independent of the part of plant evaluated (stem bark or pods). The data were calculated as total polyphenols content and expressed in g% of each of the standards (Fig. 2A and 2B).

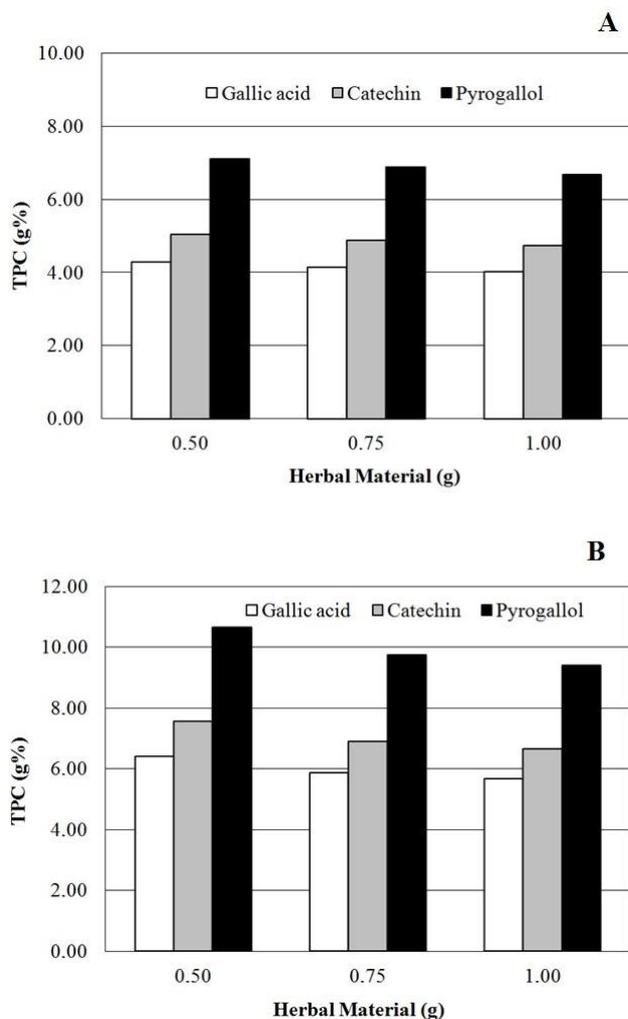


Figure 2. Effect of drug amount on total polyphenol content (TPC, g%) of stem bark (A) and pods (B) from *L. ferrea*.

Regarding the influence of the complexing agent, Fig. 3 shows the performance of each macromolecule (casein, PVP or skin powder) as precipitants of tannin in stem bark (A) and pods (B) from *L. ferrea*. Regarding the analysis of tannin from stem bark, the complex profiles observed for casein and PVP showed a similar behavior, improving the analytical response when the amount of macromolecules was increased (Fig. 3A). On the other hand, the use of skin powder as an agent provides higher interaction with tannin and the maximum could be observed at a lower concentration of the agent (100 mg). Casein and PVP also achieved maximum interaction, but this occurs only at a higher concentration (200 mg). Thus, total tannin in stem bark was calculated and was found to range from 10 to 12 g%.

Concerning the evaluation of tannin content in pods from *L. ferrea*, the use of PVP or skin powder provides the maximum yield from the lowest concentration (Fig. 3B). However, the content was lower than that observed for stem bark, at about 8 g%. Additionally, the casein showed less ability to interact with the tannin than the other macromolecules.

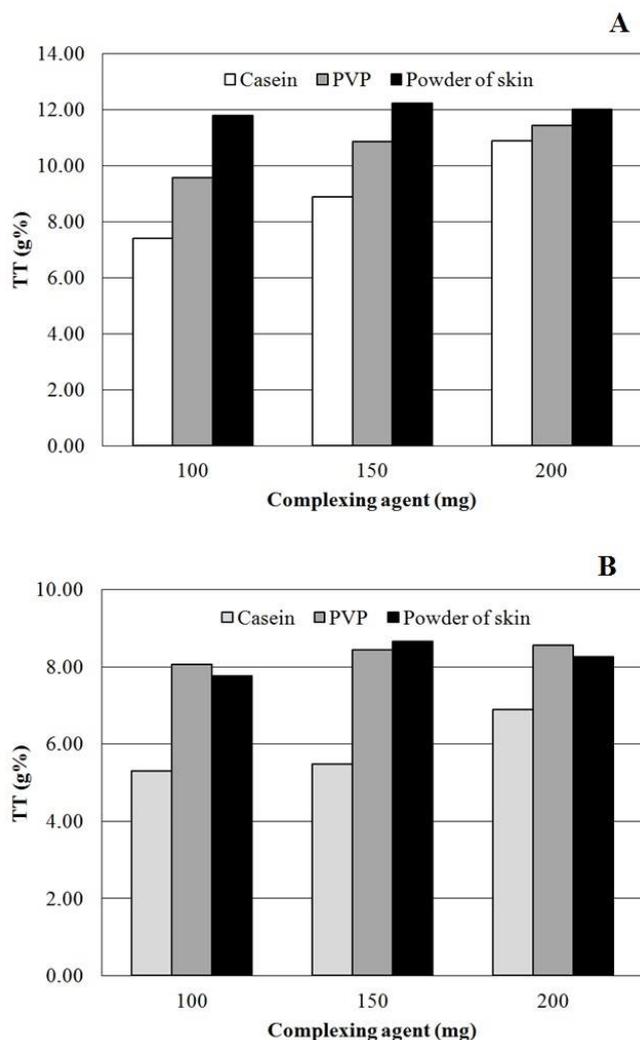


Figure 3. The effect of complex agent on the total tannin (TT, g%) of stem bark (A) and pods (B) from *L. ferrea*.

The influences of the Folin-Ciocalteu reagent and the Na_2CO_3 solution on the response of the analytical method were studied by response surface methodology. The mathematical models used to generate the surfaces were evaluated by ANOVA and the data are presented in Table 2. According to the results, the proportion of the Folin-Ciocalteu reagent caused a negative effect on the analytical response reducing the total tannins content. While the concentration of Na_2CO_3 presented a positive influence, favoring the responses in all the conditions studied. In this way, the maximization of the responses could be observed when using lower Folin-Ciocalteu reagent volume and higher Na_2CO_3 concentration (Fig. 4).

Table 2. Statistical analysis of the experimental design.

Factors	Stem bark						Pods					
	Cathechin		Gallic acid		Pyrogallol		Cathechin		Gallic acid		Pyrogallol	
	<i>F</i> -test	<i>t</i> -test										
Folin (Linear)	59.50*	-7.71*	46.91*	-6.85*	23.21*	-4.82*	54.58*	-7.39*	42.99*	-6.56*	22.12*	-4.70*
Folin (Quadratic)	1.63	1.28	7.30*	2.70*	15.42*	3.93*	19.34*	4.40*	0.29	0.54	0.06	0.25
Na ₂ CO ₃ (Linear)	49.22*	7.01*	112.98*	10.63*	48.46*	6.96*	45.61*	6.75*	104.73*	10.23*	47.13*	6.87*
Interaction (Folin x Na ₂ CO ₃)	6.27*	-2.50	0.53*	0.73	0.21	-0.46	6.04*	-2.46*	0.42*	0.65	0.27	-0.52
LOF	0.21		0.22		1.67		0.20		0.21		1.61	

*Significant for $\alpha = 0.05$; LOF = lack-of-fit

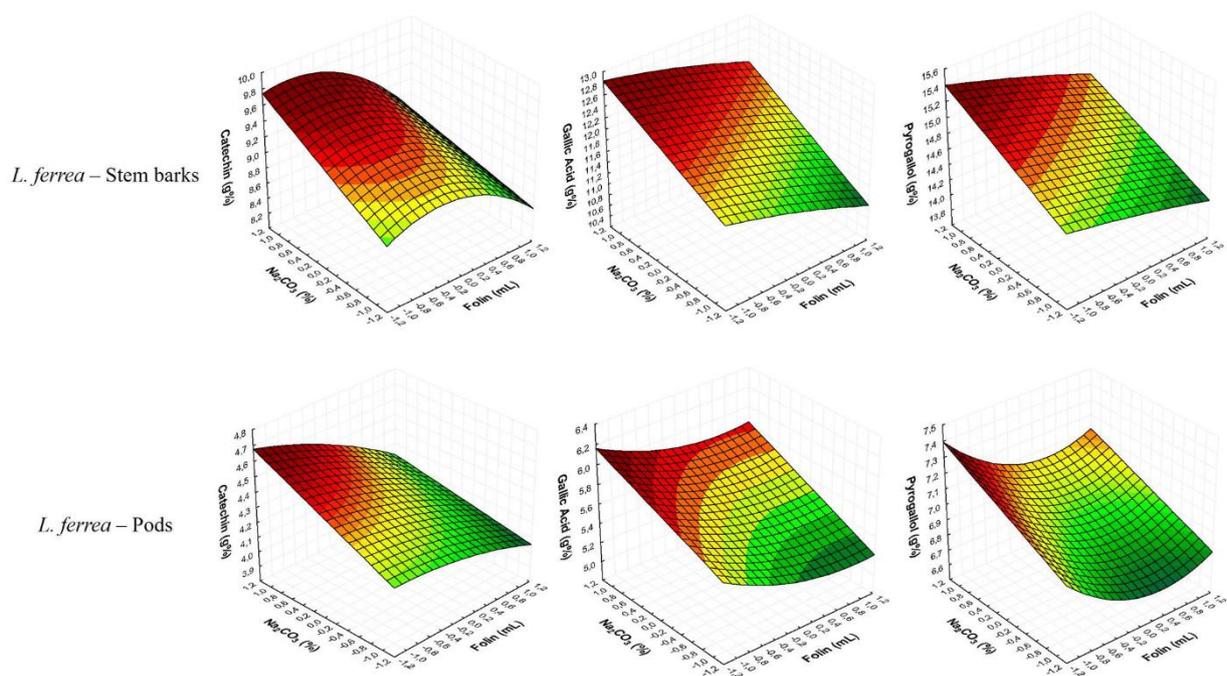


Figure 4. Response Surfaces of stem bark and pods, for evaluation of Folin-Ciocalteu reagent and sodium carbonate on the levels of tannins.

Validation

Curves of Linearity, Limits of Detection (LOD) and Quantification (LOQ)

To evaluate linearity curves of the samples, we adopted the absorbance data resulting from the difference between total polyphenols and non-adsorbed fraction. Stem bark and pods were evaluated in the concentration ranges presented in Table 1, and the curves showed determination coefficients (R^2) equal to 0.9999 and 0.9988 for stem barks and pods, respectively. This data confirm the linear dependence of analytical response and the analyte concentration for both samples under study. The limits of detection (LOD) and quantification (LOQ) were calculated through the calibration curves obtained for the solutions of the standards.

Specificity

Figure 5 presents the average linearity curves (total polyphenols solution for stem bark and pods) and the mean specificity curves obtained with the addition of reference substances. The statistical analysis of the mathematical models obtained by linear regression indicates that there was no significant difference between the angular coefficients of curves (Table 2). Thus, the response behavior of sample curves (stem bark and pods) can be explained satisfactorily by any of the three reference standards evaluated.

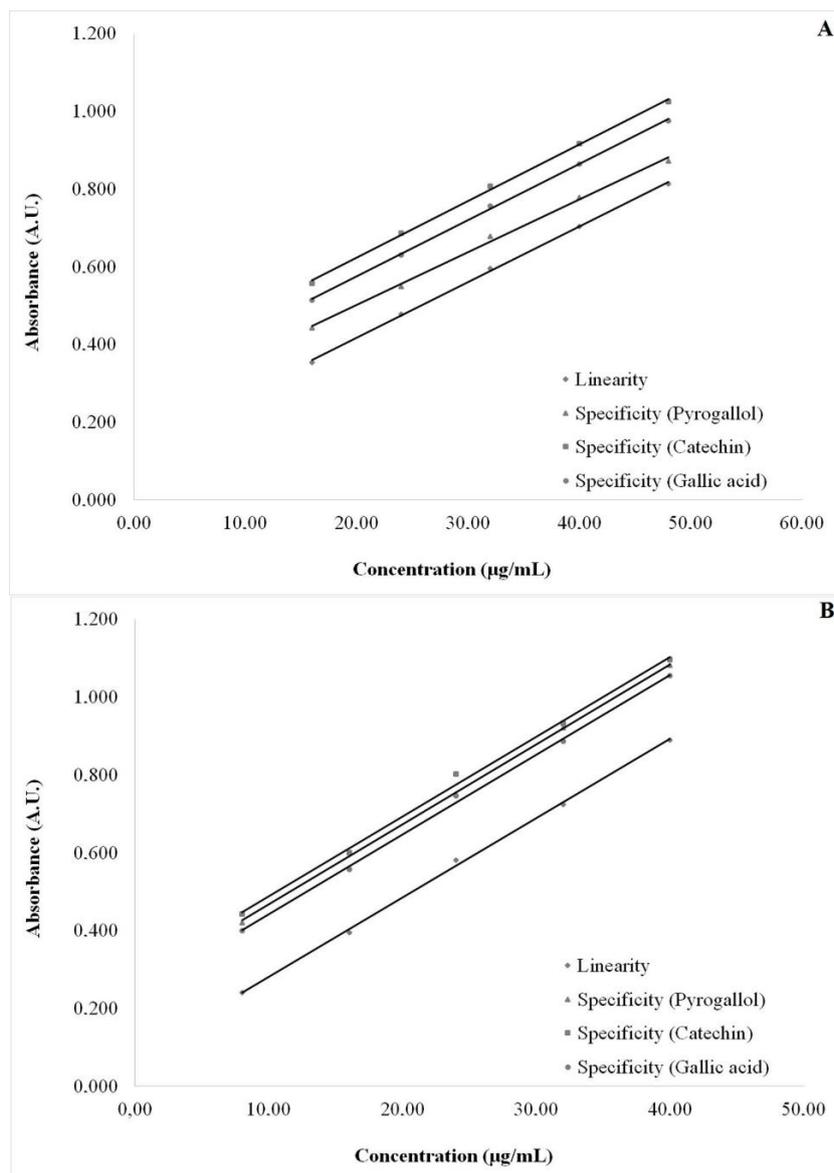


Figure 5. Curves of linearity and specificity from stem bark (A) and pods (B) of *L. ferrea*.

Precision, Accuracy and Robustness

The precision data of the methods are summarized in Table 3. In regard to the precision tests, the results showed a maximum relative standard deviation of 1.28% for repeatability and 4.9% for intermediate precision. In the accuracy test, the stem bark presented recovery rates in the range of 96.84% to 103.13%, with a mean of 100.73% (2.14%); while for pods the recovery was between 104.12% and 109.13%, with an average of 106.07% (2.53%). Finally, in the robustness test (Table 4), RSD% values higher than 5% were not obtained for the means calculated in the different experimental conditions.

Table 3. Results of precision (repeatability and intermediate precision) expressed by total tannin content (TT).

Part of plant	Parameters	TT (g%) (mean \pm SD; RSD%)		<i>F</i> _{calc}
Stem bark	Repeatability	12.12 \pm 0.110 (0.91)		-
	Intermediate precision	Day 1	Day 2	-
	Analyst 1	12.45 \pm 0.071 (0.57)	11.31 \pm 0.243 (2.15)	0.38
	Analyst 2	12.49 \pm 0.082 (0.66)	11.38 \pm 0.094 (0.83)	0.38
Pods	Repeatability	13.90 \pm 0.178 (1.28)		-
	Intermediate precision	Day 1	Day 2	-
	Analyst 1	13.81 \pm 0.136 (0.98)	14.16 \pm 0.081 (0.58)	0.51
	Analyst 2	13.91 \pm 0.080 (0.58)	14.23 \pm 0.081 (0.57)	0.02

g% of pyrogallol; SD: standard deviation; RSD: relative standard deviation.

Table 4. Results of robustness expressed by total tannin content (TT).

Part of plant	Parameters	Variables	TT (g%) mean \pm SD, (RSD%)
Stem bark	Luminosity	Absence	12.18 \pm 0.200 (1.64)
		Presence	12.67 \pm 0.070 (0.55)
	Stability of extractive solution	0 h	12.39 \pm 0.095 (0.77)
		6 h	12.07 \pm 0.199 (1.65)
Equipment	Evolution 60S, Thermo Scientific®	12.39 \pm 0.094 (0.76)	
	AJX 1900, Micronal®	12.67 \pm 0.070 (0.55)	
Pods	Luminosity	Absence	14.06 \pm 0.178 (1.26)
		Presence	14.55 \pm 0.156 (1.08)
	Stability of extractive solution	0 h	14.59 \pm 0.148 (1.02)
		6 h	13.95 \pm 0.027 (0.19)
	Equipment	Evolution 60S, Thermo Scientific®	13.55 \pm 0.047 (0.34)
		AJX 1900, Micronal®	13.64 \pm 0.134 (0.98)

g% of pyrogallol; SD: standard deviation; RSD: relative standard deviation.

DISCUSSION

There are several reports in the literature about certain species of the Fabaceae (Leguminosae) family being a source of polyphenols, these include *Amburana cearensis* (Allemao) A.C. Sm.³⁶, *Hymenaea stigonocarpa* Mart. ex. Hayne³⁷, *Anadenanthera falcata* (Benth.) Speg., and *Caesalpinia bracteosa* Tul.³⁸. When considering the determination of the tannin content described for the species, it is evident that reliable analytical methodologies are fundamental in the evaluation and quantification of the variations in metabolic content due to biological variability and/or their interaction with environmental factors.

In this aspect, prior to the adoption of analytical procedures in new plant matrices, the suitability of the technique must be evaluated for the identification and minimization of possible interferences from different sources, such as the matrix itself or analytical limitations. Thus, the evaluation of variables (dilutions, reagents, time) and the different parts of the plant studied (bark, leaves, pods) are minimum prerequisites for the procedure to be able to detect inter- and intra-individual variations³⁹.

Considering that the spectrophotometric technique presents limitations in the selectivity and specificity, the confirmation of the presence of compounds of interest is fundamental for the establishment of the marker(s). One of the most widely used techniques for establishing chemical profiles of the metabolites present in plant species is thin layer chromatography (TLC). It is notable for its simplicity, low cost and speed, and it is worth noting the ease in which it can qualitatively detect the presence of compounds with the aid of reference standards⁴⁰. To enable a more efficient separation process, the technological advances in the area of chromatography have led to the development of equipment that performs the application in a partially or totally automated way, ensuring volume reproducibility and homogeneity in this application^{41,42}.

The TLC analysis of samples revealed that stem bark and pods from *L. ferrea* presented a band with same coloration and displacement as that observed for the standard solution of gallic acid. However, on evaluation of pods, the band corresponding to the pattern presented a higher intensity in comparison with stem bark, indicating a higher concentration. In addition, other bands detected for pods (Rf value: 0.16; 0.25) suggest the presence of derivatives of hydrolyzable tannin (gallic type). The results obtained corroborate with the literature making it possible to prove the presence of hydrolyzable tannins on the stem bark and pods from *L. ferrea*^{9,10}.

The Folin-Ciocalteu method is described for different plant species in official codes, which includes the Brazilian⁴³ and British Pharmacopoeia⁴⁴ (2014). However, because of the differences related to the specificities of each herbal matrix, the evaluation of reactional and operational parameters should be considered an important step before validation of the methodology, which will ensure the reliability and reproducibility necessary for the specific quantitative evaluation of this herbal drug^{28,30,39}.

In regard to the Folin-Ciocalteu reaction, the presence of hydroxyl groups in the phenolic compounds is a determinant for the oxidation reactions and formation of the reaction product responsible for the coloration proportional to the concentration of the metabolite^{30,39,45}. In addition, the reaction depends on the amount of hydroxyls that are present in the aromatic rings of the polyphenols. This effect can be observed when different molecules are analyzed as markers, and thus an increase in the molar absorptivity value is shown in the following order: pyrogallol > catechin > gallic acid^{30,31,45,46}. In this sense, the selection of the reference standard was based on the analytical responses of the different patterns to the Folin-Ciocalteu reaction and their respective molar absorption coefficients. According to the results presented in Table 1, pyrogallol had a higher coefficient of molar absorptivity ($\epsilon = 1462.2$), a characteristic

that demonstrates greater sensitivity of this compound to the Folin-Ciocalteu method when compared with gallic acid and catechin.

The interaction between tannin and macromolecules is not a simple analytical step and it depends on several factors such as: temperature; pH; [tannin]:[macromolecule]; the molecular weight; solubility and the number of phenolic hydroxyl groups of the tannin^{12,27,28}. Thus, the choice of the reference substance and the wavelength play an important role in the specificity and/or selectivity of the instrumental technique. The bathochromic shift promoted by the Folin-Ciocalteu reagent resulting from the oxyreduction reactions may present λ_{\max} deviation capable of compromising the experimental responses by underestimating the quantitative data. This situation can become more complex due to the structural diversity of polyphenols. In this study, the comparative evaluation of the scanning spectrum (500-900 nm) obtained for the reference substances (pyrogallol, catechin and gallic acid), and for the studied herbal matrices (stem bark and pods), indicated maximum absorbance at the same wavelength ($\lambda_{\max} = 760$ nm). In addition, previous studies for optimization of this method in several species^{21,30,39,45}, reported that pyrogallol was used as reference substance and λ_{\max} of 760 nm was presented.

In relation to the amount of drug, the statistical analysis confirmed the hypothesis of a significant difference between the levels obtained for the different amounts evaluated ($\alpha = 0.05$), and the amount of 0.5 g of drug material from the both herbal parts (bark and pods) showed the maximum extraction calculated as total polyphenol content.

Regarding the factorial design, the data were used to generate second-order models for the calculated tannins expressed in each chemical marker. The coefficients of each significant term and respective statistical analysis are summarized in Table 2. The influences of each independent variable (Folin-Ciocalteu reagent and Na_2CO_3 solution) were significant for both factors, stem bark and for pods. Due to the lack-of-fit (LOF) test not being significant, the experimental variations could be attributed only to a randomized error. Thus, the fitted models provide an adequate approximation of the true values and no violations of the model assumptions occurred¹³.

The statistical data of the coefficients showed an importance in the linear terms of variables concentration of Na_2CO_3 and, mainly, Folin-Ciocalteu reagent. In this way, the conditions elected to use for obtaining higher response of total tannins content were 1 mL of Folin-Ciocalteu and solution of Na_2CO_3 at 29%,

According to the analysis of validation, the calibration curves showed linear behaviors for the analyte in both matrices (stem bark and pods) and the determination coefficients (R^2) in the concentration ranges were higher than 0.99, demonstrating the appropriate sensitivity of the methodology. In addition, the linear regression analysis showed random distribution of residues^{33,47}.

Due to the complexity of the herbal matrices and the impossibility of obtaining the same samples free of interfering substances to the analytical reagents, the assay of specificity is performed by fortifying the samples known concentration of reference solution in order to obtain new calibration curves of total polyphenols. Thus, the constant shifting of the new curves should be proportional to the standard contribution, observed by the parallel behavior (similar slopes), the specificity of which is acceptable^{30,39}. In this study, the specificity of the analytical methodology was verified for both herbal matrices and proved for the three standards.

The analysis of precision showed relative standard deviations lower than 5%, as recommended by the national legislation²³. The spectrophotometric procedure was precise at the two levels: repeatability and intermediate precision; for both herbal matrices (stem bark and pods). In addition, no statistical difference was observed among the data. This test is one of the most important, since it can indicate the effect of the variations that can occur in the laboratory, such as differences between analysts and days, assuring the reproducibility of the methodology.

In regard to the recovery assay, the analytical procedure showed performance within the limits recommended by the literature, for both herbal matrices. Thus, the instrumental responses after fortification with known amounts of the standard solutions (from 80 to 120%) could be attributed to the concentration of analytes⁴⁸.

The measure of robustness is critical to understand the interference of variations from seemingly unimportant procedures such as exposure to day/natural light; the stability of the reaction products (oxidation-reduction of the polyphenols by the Folin-Ciocalteu reagent); stability of the extractive solutions; and/or, the use of equipment from a different manufacturer.

Thus, the robustness can be defined as the ability of the analytical method to withstand small variations from deliberate or random modifications in the standard analytical procedures, complementing the method reliability and availability. The spectrophotometric method for quantification of tannins from the *L. ferrea* matrices showed variability lower than 5 % as required (RSD < 1.65% and RSD < 1.26% for stem bark and pods, respectively). The overall robustness denotes the method stability in the routine analysis of both herbal matrices.

CONCLUSION

This work evaluates the performance of the Folin-Ciocalteu method assay for the quantification of tannins in herbal matrices from *L. ferrea*. The procedure was optimized and the validation was carried out in accordance to the parameters required by the literature and presented linearity, selectivity/specificity, precision, accuracy and robustness. Thus, the experimental data allowed us to conclude that standardized conditions ensure the reproducibility and reliability necessary for the use of this simple methodology in the quantitative determination of tannins from stem bark and pods *Libidibia ferrea*.

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