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Development and validation of an analytical method for quantification of total flavonoids in *Alternanthera brasiliana* by ultraviolet-visible absorption spectrophotometry

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***Alternanthera brasiliana* is popularly known in Brazil as "penicillin" or "benzetacil" and is used in traditional medicine for the treatment of infections and for the healing of wounds. It is also used as an ornamental plant due to the characteristic purple coloration of its leaves when cultivated in shade. The objective of this study was to develop and validate an analytical methodology by Ultraviolet-Visible absorption spectroscopy to quantify total flavonoids in crude ethanolic extract of *A. brasiliana*. The parameters analyzed in validation were those indicated in resolution 166/2017 of ANVISA, as selectivity/specificity, linearity, accuracy, precision, robustness, limits of detection and quantification, and also by ICH Q2(R1) for analytical validation. The method developed was simple, fast, low cost, linear, selective, precise, accuracy and robust. All parameters analyzed were within the limits recommended by the Brazilian legislation. Thus, this methodology can be useful for quality control of the extract and vegetal derivatives of *A. brasiliana*.**

Keywords: Analytical validation, Amaranthaceae, UV-Vis, natural products, quality control.

INTRODUCTION

The Amaranthaceae family has about 170 genera and 2,000 species, occurring in Brazil 27 genera (6 endemics) and 157 species (74 endemics) (Marchioretto et al., 2010). About 20 genera and 94 species occur in the

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Brazilian Northeast, and 13 genera and 29 species in Pernambuco State (A Flora do Brasil, 2020). Several species of this family present in their composition biologically active compounds such as betalains, ecdysteroids, flavonoids, saponins and triterpenes (Ferreira and Dias, 2000).

Plants of *Alternanthera* genus are known to have antimicrobial and antiviral properties. In some species the inhibition of lymphocyte activity, hepatoprotective, analgesic, antifungal and antidiarrheal activity were reported. However, although the number of species of this genus is significant, the number of studies aiming to determine the chemical composition is still scarce (Delaporte et al., 2002; Ferreira et al., 2003).

Alternanthera brasiliana is used in folk medicine to treat infections and is popularly known as "terramycin", "penicillin" or "benzetacil" (Facundo et al., 2012; Caetano et al., 2002). In relation to its phytochemistry, several constituents have already been isolated or identified. Glycosylated flavonoids (Brochado et al., 2003), oxylipines (Trapp et al., 2015), alkaloids and triterpenoids (Anunciação, 2012) were isolated from the leaves. From the leaves and stalks, flavones, flavonols, steroids, betalains, betacyanins, betaxanthines, hodroxybenzoic acid derivatives and hydroxycinnamic acids has already been reported (Deladino et al., 2017).

Some studies conducted with *A. brasiliana* sought to validate their ethnopharmacological uses, such as the antimicrobial activity, proven for chloroform, ethyl acetate and methanolic fractions of their aerial parts (Silva et al., 2011). The analgesic effect of its crude ethanolic extract has also been reported, being its response more effective than the drugs used as standards (acetylsalicylic acid, dipyrone and indomethacin) (Souza et al., 1998). The anti-inflammatory effect was also studied and was mainly attributed to the glycosylated flavonoids present in its composition (Brochado et al., 2003). The wound healing effect of *A. brasilaina* has also been reported in both immunocompromised rats and aged rats, showing retraction of the wound halos larger than the standard drugs used (Enechi et al., 2013; Barua et al., 2009, 2012a, b).

Flavonoids are the main biologically active compounds present in *A. brasiliana*, so they were selected in this study to develop and validate analytical methodology for their quantification in samples of this species (Deladino et al., 2017). The importance of validating procedures for analytical safety and obtaining reliable results is known. Thus, for the development of an analytical method, adaptation or implementation of a known method involves an evaluation process that estimates its efficiency in the routine of the laboratory and this process is the validation (Brito et al., 2003). For validation of analytical methodologies some parameters must be analyzed such as, selectivity/specificity, linearity, robustness, precision, accuracy, limit of detection and limit of quantification and all of this is of fundamental importance in product quality control, being the validation part of the good manufacturing practices and control (Brasil, 2017). Therefore, the objective of this work was to perform an adaptation and validation of an analytical methodology for the quantification of total flavonoids in *A. brasiliana* by ultraviolet-visible (UV-Vis) absorption spectroscopy.

MATERIALS AND METHODS

Chemicals, glassware, solvents and equipment

All solvents used were analytical grade: sodium nitrite (Sigma-Aldrich®), sodium hydroxide (Alphatec®), aluminum chloride (Vetec®), methanol (MeOH, Synth®, AppliChem®). Phox® glassware was used. As standard for flavonoids, hydrated rutin (Sigma-Aldrich®), purity ≥ 94% was used. The equipment used was EVEN® analytical balance (FA-2204B model, Brazil); Cristófoli® ultrasonic bath; Ethik Technology® stove with air circulation (420-6TD model, Brazil); Solab® knife mill (SL-31 model, Brazil); EVEN® UV-Vis spectrophotometer (IL-592 model, Brazil); Nova Instruments® UV-Vis Spectrophotometer (NI-1600 model, Brazil).

Plant material and extraction process

The aerial parts of *A. brasiliana* (L.) KUNTZE growling wild were collected from Campus of Agricultural Sciences of Federal University of San Francisco Valley (UNIVASF). A botanist from Centro de Recuperação de Áreas Degradadas da Caatinga (CRAD) identified the sample by comparison with a voucher (number 19072) already deposited in Herbarium Vale do São Francisco (HVASF). The harvested material was oven dried with air circulation at 40°C (62.80% of humidity) and then pulverized in a knife mill (20 mesh) (SOLAB, SL31 model, Brazil). The dried and pulverized material was macerated with 95% ethanol in a stainless steel vessel. Three extractions were performed, replacing the solvent every 72 h. Then, the extractive solution was concentrated in a rotary evaporator under reduced pressure at 50°C, obtaining the *A. brasiliana* crude ethanolic extract (Ab-CEE) (13.14% yield) (Silva et al., 2014).

Experimental procedures and adaptation of the analytical method

The development of analytical method for quantification of total flavonoids by UV-Vis was made using the method proposed by Silva et al., (2014) for *A. brasiliana*, with the necessary adaptations. Initially an extract stock solution of 5 mg/ml was prepared using 10% methanol in distilled water. A dilution was performed to obtain a concentration of 1 mg/ml by adjusting the final volume with distilled water. The analyzes were performed in UV-Vis spectrophotometer (EVEN®, model IL-592), using quartz cuvettes with 1 cm of optical path.

The methodology used by Silva et al., (2014) as the starting point was as follows. The volumes of 1.5 ml of distilled water, 300 µl of the 1 mg/ml extract solution and 90 µl of 5% (w/v) sodium nitrite solution ($NaNO_2$) were initially added and waited 6 min. Then 180 µl of 10% (w/v) aluminum chloride ($AlCl_3$) was added and waited 5 min. Finally, 600 µl of 1 M sodium hydroxide (NaOH) was added and the final volume was adjusted to 3 ml with 330 µl of distilled water. The spectrophotometric reading was performed at 520 nm.

A UV-Vis scan of 300 to 600 nm was performed for the procedures with and without the complexing agent, in order to verify the bathochromic effect provided by the complexing agent, which

was the function of $AlCl_3$, and also with in order to verify the wavelength that the maximum absorption of the sample occurs. Thus, the procedure was performed as previously described for the procedure with complexation (PWC) and for the procedure without complexation (PNC), instead of adding 180 µl of $AlCl_3$, the same volume of distilled water was added. After the maximum absorption wavelength of the sample was verified, the methodology was developed and adapted to the chosen wavelength (370 nm).

Then, it was decided to check if there was a need to wait the methodology times (6 min after adding $NaNO_2$ and 5 min after adding $AlCl_3$), because in literature consulted it was found that the complexation with $AlCl_3$ can occur immediately (Sampaio et al., 2018a). In this way, the analyzes were performed with the times determined by the initial methodology and without the predetermined times, in which case a reagent added immediately after the previous one, preceded only by homogenization of the contents, and therefore, two types of procedure: the immediate procedure and the procedure with the times predetermined by the methodology. In addition, for each of these procedures, the influence of the reading time of the analysis at 0, 10, 20 and 30 min times was verified. It was also verified whether the volume of complexing agent could be relevant for the quantification of total flavonoids in the methodology. Thus, at the time of adding the complexing agent, different volumes of $AlCl_3$ were added (140, 160, 180 and 200 µl).

Validation of the analytical method

To validate the analytical method, the standards established by the Brazilian National Health Surveillance Agency (ANVISA) were used, in accordance with Resolution N° 166/2017, which defines what should be considered during the validation of analytical methods and non-chromatographic methods, such as UV-Vis spectrophotometry. The ICH Harmonised Tripartite Guideline for Validation of Analytical Procedures Q2(R1) was also consulted for validation. The following parameters were evaluated: selectivity, linearity (working range), precision (repeatability, intermediate precision and reproducibility), limit of detection (LOD), limit of quantification (LOQ), accuracy and robustness. All analyzes were performed in triplicate and the reliability of the parameters was verified by the relative standard deviation (RSD%) (Fernandes et al., 2015; Hollands et al., 2017). Only the procedure with complexation was validated considering that the procedure without complexation does not satisfactorily quantify the total flavonoids in *A. brasiliana* extract.

Selectivity

The selectivity of the method was demonstrated by the overlapping of the standard spectra used (the flavonoid rutin) and the Ab-CEE sample of the PWC, with the extract solution (1 mg/ml) and the rutin (0.25 mg/ml), obtained by the scanning curve in the range of 300 to 600 nm.

Linearity

The linearity (working range) of the extract sample was evaluated from the mean of the analyzes of three curves with five concentration levels (0.5, 0.75, 1.0, 1.5 and 2.0 mg/ml) for the PWC. The calibration curves were obtained from the mean absorbance as a function of concentration. For quantification of flavonoids in rutin equivalents, three calibration curves were obtained for rutin analytical standard in five concentration levels (0.05, 0.1, 0.25, 0.5 and 0.75 mg/ml) for the PWC. The results were statistically treated by linear regression, to determine the straight line equation and the coefficient of determination (r), with the minimum acceptable value being > 0.990 for the analytical standard rutin, and > 0.980 for the extract solution.

Precision

Precision was evaluated in terms of repeatability (intra-run precision) and intermediate precision (inter-run precision). Intra-run precision was obtained from three stock solutions at the concentration of 1 mg/ml, the analyzes being carried out in six-fold, by one analyst on the same day, giving a total of 18 determinations. The inter-run precision was performed in the same way, in six-fold of each of the three stock solutions, and the analyzes were done by two analysts on two different days, totaling 18 analyzes for each analyst. Also, the reproducibility test of the method was carried out in another laboratory, in this case, varying the entire physical infrastructure.

Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ were calculated (mg/ml) from the respective calibration curves for the PWC, according to their formulas, where SDa is the standard deviation of the intercept with the y axis, obtained from the average of the three linearity curves, and S is the slope of the line of the respective calibration curves. The LOD and LOQ can be calculated by Equations 1 and 2:

$$LOD = SDa \times 3/S \quad (1)$$

$$LOQ = SDa \times 10/S \quad (2)$$

Accuracy

Accuracy was assessed by the rate of recovery, from the addition of a known amount of rutin, a flavonoid present in Ab-CEE. In the analyzes for PWC, the same procedures were performed as previously described, but now adding at the same time with the sample 100 µl of analytical standard rutin (200 µg/ml). The result of the recovery was obtained by Equation 3:

$$R\ (\%) = TFC - CFE / CFP \times 100 \quad (3)$$

Where R is the percent recovery, TFC corresponds to total flavonoid concentration (rutin) added to Ab-CEE solution, CFE corresponds to concentration of rutin in Ab-CEE and CFP corresponds to rutin concentration.

Robustness

This parameter was performed by performing small changes in the wavelength of the analyzes (370 by 380 nm), as well as by modifying the label of the solvent (methanol) that was used to prepare the stock solution (exchange of the Synth® laboratory by AppliChem®).

Statistical analysis

All analyzes were performed in triplicate and the reliability of the parameters was verified by the relative standard deviation (RSD%). The results were analyzed statistically by analysis of variance (ANOVA), One-Way or Two-Way, when applicable, being considered statistically significant F calculated less than tabulated F ($p > 0.05$). The statistical treatment was obtained by the software

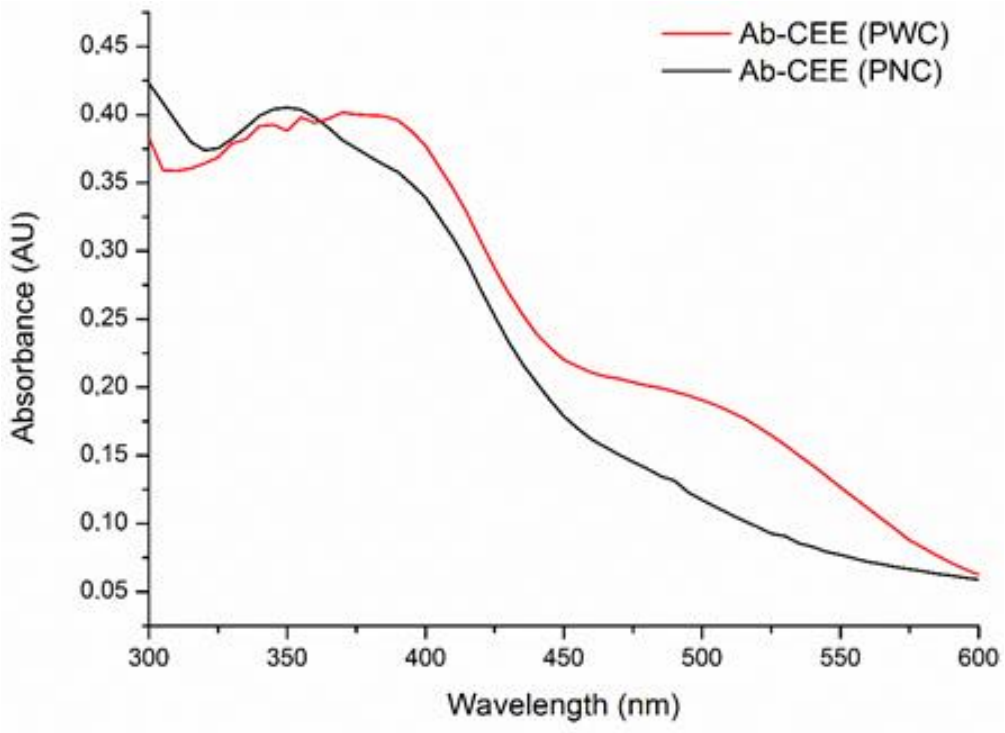

Figure 1. Superposition of scanning graphs of the procedure with complexation (PWC) and without complexation (PNC).

OriginPro® 8 (OriginLab).

RESULTS AND DISCUSSION

For the development and adaptation of the method proposed by Silva et al., (2014) was initially scanned from 300 to 600 nm for procedures with and without the complexing agent, in order to verify the bathochromic effect provided by $AlCl_3$, and to verify the wavelength where the maximum absorption of the sample occurs. Figure 1 shows the curves superposition of the solutions with complex agent and without complexation (1 mg/ml). Still in Figure 1, one can perceive the maximum absorption wavelength at 370 nm for the PWC sample.

Next it was verified whether there was a need to wait the predetermined times for the methodology between the addition of each reagent. From the analysis and application of specific statistical test (unpaired Student t test), the results obtained (in absorbance) were 0.421 ± 0.008 for the procedure with the predetermined times and 0.399 ± 0.009 for the procedure performed immediately. So that, it was possible to conclude that, for the study sample, it is necessary to wait the predetermined times, since the time of complexation interferes in the quantification.

The influence of the reading time of the analysis was verified, being this realized in times 0, 10, 20 and 30 min. The results obtained (in absorbance) were 0.421 ± 0.008 in time 0 min (T0) and 0.417 ± 0.006 in time 30 min (T30) for the procedure with the predetermined times. For the procedure performed immediately, the results were 0.399 ± 0.009 in T0 and 0.391 ± 0.009 in T30. The results showed that the analysis can be performed in both time 0 and 30 min times, since there is no statistically significant difference in T0 and T30 (unpaired Student t test). In this way, time 0 was selected to perform all the analyzes. Comparing the results in T0 for the procedure with and without the predetermined times, there is significant difference between the analysis, showing that it is necessary to wait for the times between the addition of the reagents, otherwise it will interfere with the quantification.

The last step in the adaptation of the methodology was to verify the $AlCl_3$ volume in the quantification, so different volumes of complexing agent (140, 160, 180 and 200 µl) were tested. The volumes of 140, 160 and 180 µl had no significant statistical difference. The volume of 200 µl made it impossible to read the analysis because it provided a cloudy solution, and its reading was inadequate. In this way, the volume of 140 µl was selected, being the smallest one used in the development in order to use the minimum of reagents. The changes that occurred from the predetermined methodology for the methodology adapted in this work was the reading wavelength of the analysis, which went from 520 to 370

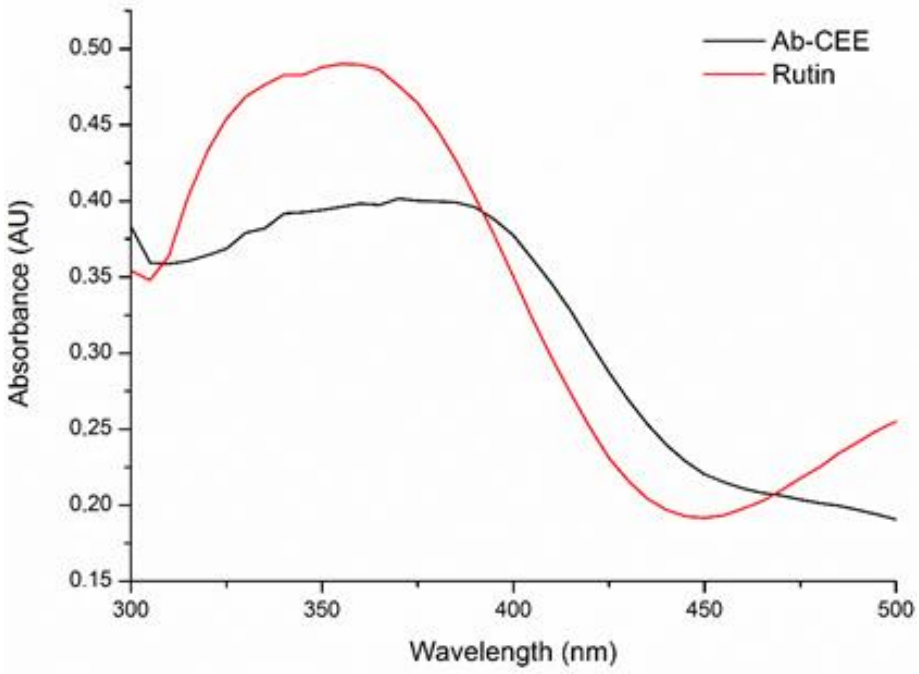

Figure 2. Selectivity of the method, constructed with the analytical standard rutin and Ab-CEE.

nm, and the volume of $AlCl_3$ added as a complexing agent dropped from 180 to 140 µl.

Validation of the analytical method

The selectivity was analyzed from the evaluation of different scanning spectra. The evaluation demonstrated the efficiency of the method for this assay, showing that other components do not interfere in the reading of the solutions, since they do not absorb in the region of the wavelength (370 nm) used for the quantitative analysis of the extract. It is possible to observe that there is similarity between the spectra of the analytical standard rutin and the extract, as can be observed in Figure 2.

Linearity corresponds to the ability of the method to provide a response directly proportional to concentration of analyte of interest present in the sample. The correlation coefficient (R^2) allows an estimate the quality of the obtained curve, so the closer to 1.0, the less the dispersion of the set of experimental points and the less the uncertainty of the estimated regression coefficients (Sampaio et al., 2018b). To verify the linearity, it was observed the linear equation and the correlation coefficient, which can be verified in Figure 3 (linearity of the Ab-CEE) and Figure 4 (linearity of the analytical standard rutin). The result of the regression obtained for R^2 was 0.99, proving that more than 99% of the method showed satisfactory linearity between the increase of the analyte concentration and the spectrophotometric response, in the concentration range chosen and the analysis of the mean residuals showed homoscedasticity. The working range of the method for Ab-CEE was determined to be 0.5 to 2 mg/ml. After calculating the total flavonoids, it was obtained as a result 0.3387 ± 0.0054 µg of rutin equivalents per mg of extract, which is equivalent to approximately 33.87% of total flavonoids in *A. brasiliana* crude ethanolic extract.

The LOD and LOQ were calculated from the equation line. LOD and LOQ values were 18.04 and 60.15 µg/ml, respectively. This shows that the method provides spectrophotometric responses with high sensitivity to detect and quantify flavonoids in extracts of *A. brasiliana* in very low concentrations (Hollands et al., 2017).

Precision represents the dispersion of results between independent and repeated assays of the same sample, similar samples or standards, under defined conditions (Padilha et al., 2017). For the validation, the precision was considered in three distinct levels: repetitiveness (intra-run), intermediate precision (inter-run) and reproducibility (inter-laboratory). For precision assays, the results of repeatability and intermediate precision (Table 1) showed RSD values below 5%, which is the maximum value recommended. In the repeatability, the value of the coefficient of variation (CV) was 0.008%. For the intermediate precision, the calculated F was lower than the table F ($p > 0.05$), that is, no significant statistical difference was observed when the same analyst evaluated the method on different days, and when different analysts evaluated on different days. In the

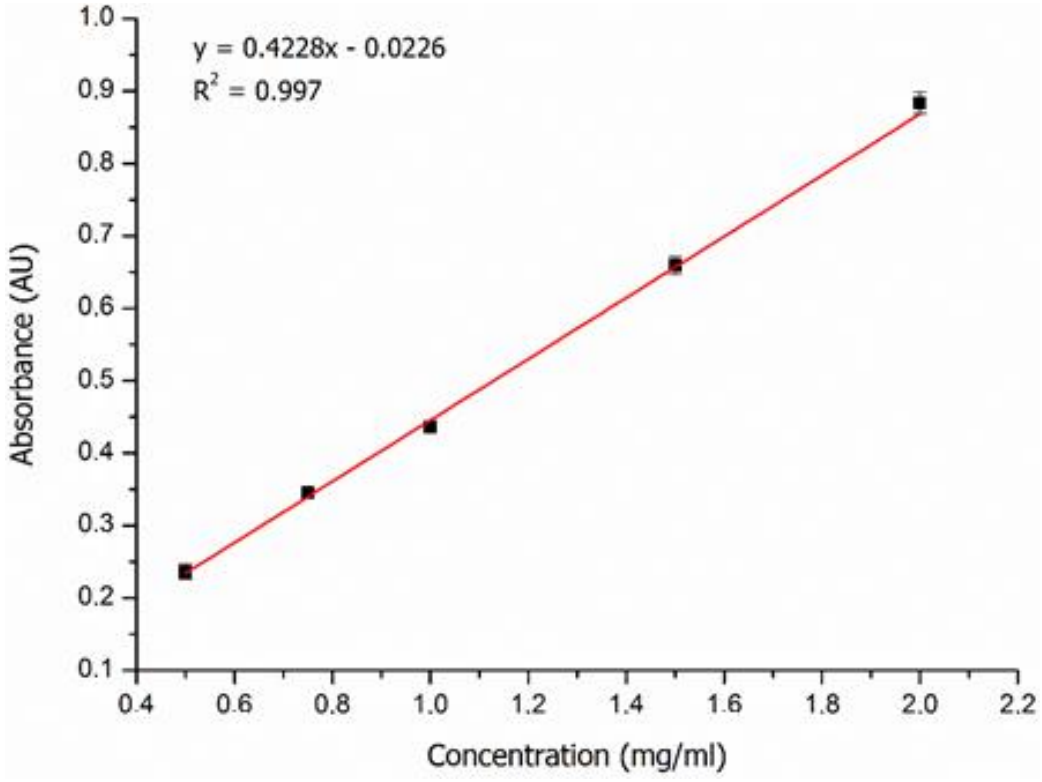

Figure 3. Linearity of the Ab-CEE.

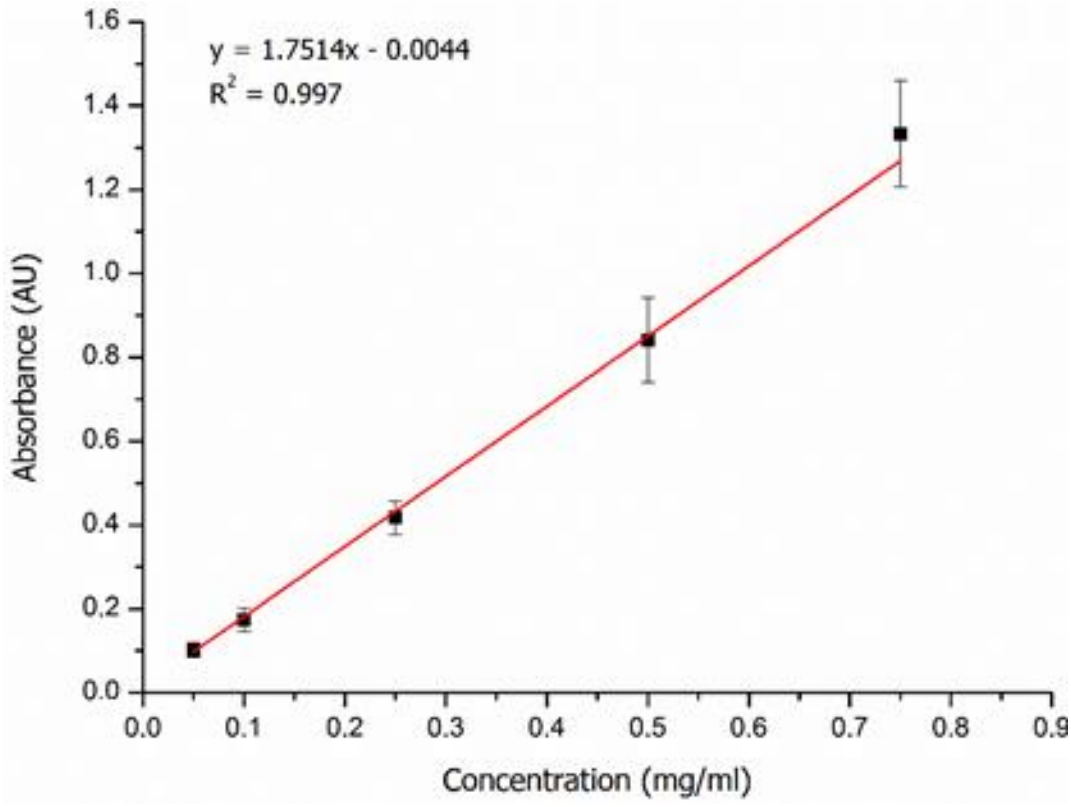

Figure 4. Linearity of the analytical standard rutin.

Table 1. Results obtained in intermediate precision analysis of the method.

Analist	Day 1 (mean of absorbance)	Day 2 (mean of absorbance)	F values
Analist 1	0.4366	0.4364	F *cal* 0.0956
Analist 2	0.4288	0.4322	F *tab* 0.3236

Table 2. Results obtained in reproducibility analysis of the method.

Parameter	Variable	Mean (absorbance) ± RSD%	F values
Spectrophotometer UV-Vis	EVEN® (IL-592 model, Brazil)	0.455 ± 0.093	F *cal* 0.4885
	Nova Instruments® (NI-1600 model, Brazil)	0.408 ± 0.003	F *tab* 0.6551

Table 3. Robustness test result for the evaluated method.

Parameter	Variable	Mean (absorbance) ± RSD%	F values
Wavelength	370 nm	0.446 ± 0.028	F *cal* 0.0082
	380 nm	0.417 ± 0.010	F *tab* 0.0645
Solvent brand	Synth®	0.442 ± 0.012	F *cal* 0.1180
	AppliChem®	0.487 ± 0.015	F *tab* 0.9882

reproducibility (Table 2), the calculated F was lower than the table F, that is, no statistical difference was observed, and the method was reproducible.

Regarding the parameter accuracy, the average recovery of the rutin was 93.46% ± 0.87 (CV = 1.03%), an acceptable value for natural products. These values show that the analytical method developed is sufficiently accurate. The recovery represents the degree of agreement between the individual results found in a given test and a reference value accepted as true (Fernandes et al., 2015).

Robustness is the measure of the method's ability to withstand small and deliberate variations in analytical parameters, such as the sample analysis wavelength, the pH of the solution used as the solvent or eluent, and the brand of the solvents (Brasil, 2017). The data obtained showed that the procedure was robust in terms of the analyzed parameters (difference in analysis wavelength and solvent brand used), since the calculated F values were lower than those of tabulated F (one-way ANOVA), as can be seen in Table 3.

Conclusions

This study aimed to adapt and validate an analytical method for the quantification of total flavonoids in *A. brasiliana* extract, a medicinal plant with strong pharmacological and technological potential. The method developed proved to be simple, fast, low cost, linear, selective, precise, accuracy and robust. Therefore, this methodology can be useful for quality control of the extract, plant drugs and herbal medicines obtained from *A. brasiliana* and to quantify the total flavonoids in this species.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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