Development and validation of analytical method by spectrophotometry UV-Vis for quantification of flavonoids in leaves of Senna occidentalis Link

Desenvolvimento e validação de método analítico por espectrofotometria UV-Vis para quantificação de flavonóides em folhas de Senna occidentalis Link

Desarrollo y validación de método analítico por espectrofotometría UV-Vis para cuantificación de flavonoides en hojas de Senna occidentalis Link

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Abstract

Senna occidentalis Link (Fabaceae), known as “fedegoso”, is used in folk medicine due to its anti-inflammatory, antioxidant, muscle relaxant and inhibiting lipid peroxidation imputed to flavonoids contained in its composition. The species is also a constituent of processed products are commercialized in various parts of the world including Brazil, although there are few reports in the literature about the development of an analytical method for quantification of flavonoids in it. The main purpose of this research was the evaluation of an analytical methodology to determine the content of total flavonoids in leaves of S. occidentalis, for quality control. The parameters evaluated were: drug: solvent ratio, concentration of aluminum chloride and reaction time. The quantification of total flavonoids was obtained after reaction with aluminum chloride by UV/Vis spectrophotometry. The results revealed a maximum absorption peak (λ = 392 nm) was the amount of 0.5 g of a plant raw in 100 mL of solvent and there were no significant influences between the concentrations of aluminum chloride or hydroalcoholic solutions analyzed. The evaluation of reaction time allowed to characterize the kinetics as slow, stabilizing from 60 min, choosing this as reading time. The method was specific, linear, precise, accurate and robust, according to the specifications set in RDC n. 166/2017. Finally, the results of the study showed that the measured methodology is simple and accurate and can be applied for quality assessment of the species S. occidentalis.

Keywords: Senna occidentalis; Total flavonoids; Ultraviolet-Visible spectrophotometry; Validation.

Resumo

Senna occidentalis Link (Fabaceae), conhecido como “fedegoso”, é utilizado na medicina popular por ser anti-inflamatório, antioxidante, relaxante muscular e inibidor da peroxidação lipídica imputada aos flavonoides contidos em sua composição. A espécie também é constituinte de produtos processados, sendo comercializados em várias partes do mundo inclusive no Brasil, embora existam poucos relatos na literatura sobre o desenvolvimento de um método analítico para quantificação de flavonoides na mesma. O objetivo principal desta pesquisa foi a avaliação de uma metodologia analítica para determinação do teor de flavonoides totais em folhas de S. occidentalis, para controle de qualidade. Os parâmetros avaliados foram: relação fármaco: solvente, concentração de cloreto de alumínio e tempo de reação. A quantificação dos flavonoides totais foi obtida após reação com cloreto de alumínio por espectrofotometria UV/Vis. Os resultados revelaram que um pico máximo de absorção (λ = 392 nm) foi a quantidade de 0,5 g de uma planta crua em 100 mL de solvente e não houve influências significativas entre as concentrações de cloreto de alumínio ou soluções hidroalcohólicas analisadas. A avaliação do tempo de reação permitiu caracterizar a
1. Introduction

*Senna occidentalis* Link (Fabaceae), popularly known as fedeogoso, mata pasto, manjerioba, or café negro, is a native species of the Neotropics, can be found in almost all Brazilian territory, being a frequent invader of pasture areas, orchards, roadsides and cultivated soils, especially with soybean (Ish et al., 2019; Scheidegger & Rando, 2020).

Phytochemical and pharmacological studies performed with leaves and fruits of *S. occidentalis* proved that this species stands out as the majority source of anthraquinones and flavonoids compounds, with anti-inflammatory activity, muscle relaxant, and inhibiting lipid peroxidation associated with the presence of flavonoids (Issa et al., 2021). Although there are studies in the literature related to the research of anthraquinones in this species, in the case of flavonoids, references about the analytical development are still scarce, judging by the importance of this metabolite in the composition of the plant.

Flavonoids, an important group within the polyphenolic compounds derived from the secondary metabolism of plants, chemical and pharmacological markers are of great importance for the quality control of medicinal plants and herbal products (Petry et al., 2001; Soares et al., 2003; Ramos et al., 2017). Several techniques can be employed for the determination of flavonoids in herbal material, and the ultraviolet-visible spectrophotometry (UV/Vis) of great applicability due to its operational simplicity, speed, low cost of implementation and wide availability in control laboratories quality (Luo et al., 2011; Lozada-Ramírez et al., 2021).

Some procedures were developed to decrease the amount of interferences that may compromise the absorption of the compounds of interest to the study, among which the use of aluminum chloride (AlCl₃) (Ramos et al., 2017; de Araújo et al., 2021). Harborne in 1954, suggested the use of this reagent for recognizing groups in ortho-dihydroxyl flavonoids. The aluminum cations form complexes with the labile acid with the flavonoids in methanol, occurring in the spectrophotometric analysis a bathochromic displacement to longer wavelength and an increasing of absorption (Mabry et al., 1970).

The chemical standardization of herbal medicines and ensuring its effectiveness and safety require suitable analytical methods for the detection and quantification of active principles (Balekundi & Mannur, 2020). Of considerable applicability in the fields of food and pharmaceutical industry, especially regarding quality control of herbal drugs, the techniques used to
quantify the levels of flavonoids have been widely studied, due to the scarcity of monographs in the official compendiums. For this, it is necessary to develop simple and effective analytical methods for the quantification of these compounds (Ramos et al., 2017; Barbosa et al., 2020).

The development of new methodologies and optimization of existing ones is of significance for the launch of herbal products in the market, since the Resolution - RDC 14 (Brasil, 2010a), official legislation regulating the registration of herbal establishes all requirements required to its grant, based primarily on quality assurance. The permanence or entry of these products are related to the development of scientific studies aiming at obtaining raw materials controlled, the development of appropriate technologies for obtaining plant extracts and especially the conduct of clinical trials (Abubakar & Haque, 2020).

According to Resolution No. 17 of 16 April 2010 (Brasil, 2010b), which comes to Good Manufacturing Practice for Medicinal Products, analytical methods unofficial should be validated before being inserted in the routine laboratory, considering the physical and technological conditions of the same. Validation studies are a requirement of the RDC 14 (Brasil, 2010a) and ensure, through experimental studies, that the method meets the requirements of analytical applications, ensuring the reliability of the results (Brasil, 2017).

In this context, the aim of this work was the development and validation of a methodology by UV/Vis spectrophotometric able to quantify the total content of flavonoids in the leaves *Senna occidentalis*.

**2. Methodology**

This work consists of experimental analyzes and quantitative data research, which involve several steps, which are described in the next topics.

**2.1 Plant Material**

Leaves of *Senna occidentalis* were collected in Recife, Pernambuco, Brazil (08°04'03" S, 34°55'00" O). A voucher specimen was deposited in the herbarium of the Instituto Agronômico de Pernambuco - IPA, with number 87.030 (IPA-PE). After that, the leaves were dried at 40 °C for 96 hours and then were pulverized in a mill (Tecnal®).

**2.2 Solvents, reagents and glassware**

The solvents and reagents used in the preparation and extraction of the sample were of analytical purity: Aluminum chloride (Vetec®), ethanol (Cinética®; Carlo Erba®). The standard used was vitexin (USP). The volumetric glassware used were properly calibrated with calibration certificate for manufacturer lot Satelit®, Pyrex® and Premier®.

**2.3 Preparation of extraction solution**

For the determination of flavonoids by direct dilution technique, the extraction solution was obtained by extracting hydroalcoholic solution under reflux with 40% (v/v), based on the method developed and validated by Ramos et al. (2017). The extraction was performed in a bottom flask, to which 0.5 g of leaves of *S. occidentalis* were extracted with 30 mL of hydroalcoholic solution for 30 min. The extract was cooled to ambient temperature (25 °C) and filtered through cotton and the residue (cotton and herbal material) re-extracted, twice for 10 min with more 30 mL of hydroalcoholic solution each time. The filtered fractions were collected in a volumetric flask and the volume adjusted to 100 mL with hydroalcoholic solution.
2.4 Sample preparation

Aliquots of the extractive solution were transferred to volumetric flasks of 25 mL, to which was added 2 mL of ethanolic solution of aluminum chloride (AlCl₃) 5% (w/v). The volume was completed with hydroalcoholic solution (40%, v/v) and the absorbance of the samples were measured in the UV-VIS spectrophotometer (Model 60S Evolution, Thermo Scientific®) by scanning (200-500 nm), using the same sample solution without aluminum chloride as blank.

2.5 Optimization of the spectrophotometric method

Several samples, diluted from aliquots of increasing extractive solution, were subjected to scanning spectrophotometer in the range 200-500 nm 60 min after addition of ethanolic solution of AlCl₃ to the sample, to identification of the dilution and wavelength whose value of absorbance was showed better suited for the method.

Were evaluated the extractive solutions prepared with the hydroalcoholic solvent at three different concentrations (30, 40 and 50% v/v).

Reaction time was determined by scanning the range from 200 to 500 nm conducted in intervals of 5 min during 120 min after addition of ethanolic solution of AlCl₃.

For the evaluation of the presented methodology, extractive solutions were obtained by varying the quantity of raw material (0.25, 0.5, 0.75, 1.0, 1.25 and 1.5 g). The proportion of drug-solvent was evaluated by the ratio between the concentration of the six extractive solutions and their total flavonoid content, expressed by the average of three determinations.

Were also evaluated the influence of the concentration of the ethanolic solution of AlCl₃ on the response method. For this purpose, three solutions were prepared at different concentrations (2.5, 5.0 and 7.5%) of hexahydrate AlCl₃ (MW = 241.33) using as solvent 5 mL of water, with ethanol to final volume of 100 mL.

The total flavonoid content (TFC) expressed in mg/mL of vitexin, in accordance with the specific absorbance of the standard vitexin (628).

2.6 Validation of analytical method

The analytical method was validated for specificity/selectivity, linearity, precision (intermediate and repeatability), accuracy and robustness, according to the standards set by RDC 166/2017 of the National Agency for Sanitary Surveillance - ANVISA (Brasil, 2017). The tests were run in triplicate and the reliability of the parameters was checked by the coefficient of variation (CV%) and were not accepted CV values exceeding 5%.

Specificity

The specificity assay was conducted by overlapping of spectrum of the standard and extractive samples with and without the addition of AlCl₃ solution (w/v), obtained in the range of 200 to 500 nm. Vitexin was used as standard.

Linearity

For evaluation of linearity was used the average of three authentic curves constructed with samples of the extraction solution in six concentration levels ranging from 0.2 to 0.7 mg/mL. The curve was constructed by the representation of the mean values of absorbance versus concentration. The results were statistically analyzed by linear regression by the least squares method and the homoscedasticity of the data was also evaluated.
Precision

The parameter precision was evaluated by repeatability, which were examined in a single day, six individual determinations for samples 100% of test concentration, obtained from different extractive solutions, and the intermediate precision which was determined by two operators on two consecutive days for samples also at a concentration of 100%.

Accuracy

The accuracy was assessed using the parameter of the recovery, by adding known amounts of the extraction solution to samples at 100% concentration test. The recovery values, expressed as percentages, were determined by the ratio between the average concentrations determined experimentally and the corresponding theoretical concentrations. To perform the test, increasing amounts (1.0, 2.0 and 3.0 mL) of extractive solution (5.0 mg/mL) were added to the sample solution, resulting in three solutions (0.7, 0.9 and 1.1 mg/mL of final concentration of the extraction solution) with three replications each.

Robustness

The factors to be considered in the analysis of robustness were influence of light (presence and absence of light) and several manufacturers of solvent ethanol (Brand A and Brand B) being in accordance with the recommendations by the legislation.

3. Results and Discussion

3.1 Optimization of the spectrophotometric method

The wavelength at which the maximum of absorbance after complexation reaction with AlCl₃ was observed was 392 nm. Considering the dilution of extractive solutions tested, the one that presented the absorbance value more appropriate for the procedure was to 2.5: 25.0 mL, with absorbance value equal to 0.469 U.A., whose final concentration was 0.5 mg/mL.

In the evaluation of extractive solutions prepared with three concentrations of the solvent (30, 40 and 50%) were obtained TFC expressed in vitexin, 2.86 ± 0.009 (0.34%), 3.03 ± 0.037 (1.23%) and 2.75 ± 0.016 (0.61%) mg/mL, respectively. Thus, the concentration of hydroalcoholic solution as the solvent employed for the tests was 40% because have better response to the extraction process.

Several studies have shown that the most common method for determination of flavonoids by UV/Vis is using aluminum chloride reagent. This is because the aluminum cation complexing can be stably with flavonoids, to provide a displacement for longer wavelengths and a consequent enhancement of absorption. Thus, it becomes possible to determine the amount of flavonoids with the least possible interference from other phenolic compounds. The reaction time required for formation of Al³⁺-flavonoid complex exerts important function in the response of the method and depends on factors such as the substitution pattern of the aglycone and aglycone-Al³⁺ ratio. To S. occidentalis, the reaction kinetics with time up to 120 min from the addition of 5% AlCl₃ allowed characterizing the complexation as slow kinetics. There is a tendency after the stabilization time of 60 min at maximum absorbance after the addition of the reagent (Figure 1) and therefore defaulted to reading time.
The results of the influence of drug-solvent ratio for obtaining the extractive solution showed that the condition in which a greater response was obtained was with 0.5 g of herbal drug in 100 mL of 40% ethanol (Figure 3), with statistically significant difference between the amounts of drugs evaluated ($F_{cal} = 217.6$ and $F_{crit} = 3.10$). The apparent decrease in the content of flavonoids with an increase in the proportion of plant has been explained in studies of List and Schmidt (1989), which can be explained by saturation of the solvent and decreased efficiency of extraction of flavonoids in the sample.

In the evaluation of different concentrations of aluminum chloride, the values of total flavonoids obtained were $2.90 \pm 0.057$ mg/mL (1.98%) to 2.5% concentration, $2.98 \pm 0.045$ mg/mL (1.54%) to 5.0% and $2.90 \pm 0.023$ mg/mL (0.79%) to the concentration of 7.5%. The statistical analysis by one-way ANOVA possible to observe that there was no statistically
significant influence on the concentration of the aluminum chloride reagent on the response of the method. Thus, the intermediate concentration to 5.0% by presenting best response was selected for development testing ($F_{cal} = 2.33; F_{cri} = 4.45$).

3.2 Validation of analytical method

The specificity is defined as the capacity to distinguish a particular method for one analyte or the presence of other components in the matrix. Typically, these components can include impurities and degradation products of the matrix components (Brasil, 2017). Figure 3 illustrates the overlapping spectra obtained for the extract of *S. occidentalis* and the standard vitexin, in the range from 200 to 500 nm. After addition of AlCl$_3$, there was an approximate peak of the sample in relation to the standard in which first had its maximum absorbance at 392 nm, while for the second, the response was observed at 390 nm.

**Figure 3.** Specificity of the method constructed with the standard vitexin and extractive solution of *S. occidentalis* (with and without AlCl$_3$) in the range from 200-500 nm.

This result reflects that at this range of wavelength is possible to quantify specifically the standard for flavonoid and substances of this class contained in the extract even in the presence of other compounds or impurities. The choice of standard vitexin was because published papers have shown the presence of flavonoids with chemical structure C-glycosylated, isolated in *S. occidentalis* (Gupta & Singh, 1991; Hatano et al., 1999). These compounds are more resistant to hydrolysis and less soluble in ethyl acetate than the aglycones of flavones, thereby remaining in the aqueous phase after this reaction (Petri et al., 2001; Matos et al., 2016).

The Resolution (Brasil, 2017) defines linearity as the ability of an analytical methodology to demonstrate that the results (dependent variable $y$) are directly proportional to the concentration of analyte in the sample (independent variable $x$), within a range specified. The mathematical equation that expresses this dependency is called a calibration curve (Barbosa et al., 2020). The regression analysis resulted in a coefficient of determination $R^2 = 0.9993$, implying that 99.93% of the total variation around the mean are explained by the regression and are proving the adequacy of the method to the range evaluated, as well as its conformance to the minimum requirements required ($R^2 > 0.99$). The data showed homoscedasticity, since from the Cochran test the value of $C_{cal}(0.086) < C_{cri}(0.684)$ and the residues were randomly distributed.

The analysis of precision is taken as a measure of proximity of the results obtained in a series of multiple samplings of the same sample being analyzed by the intermediate precision and repeatability, in a way that can be expressed using coefficient of variation (CV) (Brasil, 2017). The precision is considered as one of the most representative analysis, by
revealing the effect of variations related to days, analysts or distinct equipment’s implying in ensuring of the reproducibility of the method (Barbosa et al., 2020). By analyzing the calculation of the coefficient of variation of repeatability, note that the method was accurate at this level, with the average value of total flavonoid content of 2.97 ± 0.070 mg/mL (2.33%). The intermediate precision values showed no statistically significant difference, indicating that the method is necessary for the analyzes performed by different analysts on the same day and on different days (Table 1).

| TFC      | Day 1            | Day 2            | Analyst   | Day    |
|----------|------------------|------------------|-----------|--------|
| Analyst 1| 2.66 ± 0.0015 (0.36%) | 2.68 ± 0.0057 (0.13%) | $F_{\text{cal}} = 0.62$ | $F_{\text{cal}} = 0.69$ |
| Analyst 2| 2.66 ± 0.0023 (0.55%) | 2.76 ± 0.0011 (0.26%) | $F_{\text{cal}} = 161.4$ | $F_{\text{cal}} = 161.4$ |

Results expressed in mg of vitexin by mL of extractive solution, as mean ± standard deviation (coefficient of variation). Source: Authors (2021).

| Parameters          | Variables            | TFC          | $F$          |
|---------------------|----------------------|--------------|--------------|
| Luminosity          | Absence of light     | 3.03 ± 0.0373 (1.23) | $F_{\text{cal}} = 11.56$ |
|                     | Presence of light    | 2.80 ± 0.0452 (1.61) | $F_{\text{cal}} = 39.0$ |
| Manufacturer solvent| Brand A              | 2.82 ± 0.0481 (1.70) | $F_{\text{cal}} = 2.19$ |
|                     | Brand B              | 2.82 ± 0.0331 (1.17) | $F_{\text{cal}} = 39.0$ |

Results expressed in mg of vitexin by mL of extractive solution, as mean ± standard deviation (coefficient of variation). Source: Authors (2021).

4. Conclusion

The analytical procedure evaluated in this study showed adequability to validation parameters established by RDC 166/2017 – ANVISA, being qualified as a technique specific, linear, accurate, precise, and robust for quantitative analysis of total flavonoid in the leaves of *Senna occidentalis*, thus ensuring sufficient reliability for its adoption in quality control routine. Furthermore, this spectrophotometric method proved to be simple to perform and low cost. Thus, the importance of this study aims to contribute to the process of identification, control, and standardization of quality parameters for leaves of *S. occidentalis*. Additionally, it is important to carry out the identification and quantification of species markers by chromatographic techniques.
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