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VALIDATION OF THE ANALYTICAL METHOD BY HPLC FOR DETERMINATION OF CATECHIN IN *EUGENIA DYSENTERICA* DC. DRY AQUEOUS EXTRACT: A BRAZILIAN SAVANNAH NATIVE PLANT.

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Abstract

The *Eugenia dysenterica* DC., popularly known as cagaita, is a fruit tree belonging to Myrtaceae family, native to the Brazilian Cerrado. In general, its leaves are used as antidiarrheal as its fruits have laxative properties, according to popular use. However, in the literature, little has been reported concerning the chemical and biological activities of this plant. Preliminary analysis indicated the presence of catechin and epicatechin, catechin being the largest concentration. Thus, the present study aimed at the development and validation of an analytical method by high performance liquid chromatography (HPLC) for the determination of catechin in a standardized dry aqueous extract of *E. dysenterica*. The powdered plant material was extracted by infusion and then lyophilized for analysis by HPLC. The detection and quantification limits of catechin were 0.57 and 1.54 µg/mL, respectively. The proposed method has demonstrated good linearity with a correlation coefficient (r) of 0.9956. It has shown to be selective to detect catechin before degradation products generated by acidic and basic hydrolysis of the extract. For the used concentrations of 13.6, 17.0 and 20.4 µg/mL, the accuracy of the proposed method was 90.7, 98 and 97%, respectively. There was also robustness against flow rate, temperature and wavelength variations.

Keywords: *Eugenia dysenterica,* catechin, analytical method validation, standardized dry extract.

Introduction

The Cerrado (savannas of central Brazil) is characterized by a mosaic of vegetation ranging from grasslands to forests, presenting trees and shrubs up to 8-20 m tall. Fire and seasonal distribution of rainfall are common in this biome, which provides highly weathered and nutrient-poor soils, thereby influencing the selection of species with high nutrient retention and chemical diversity present in its vegetation [1,2]. It is estimated that the Cerrado has 11,000 species of native plants, of which approximately 45 % are endemic species of this biome and have a high therapeutic value with some biological activities confirmed by scientific studies [3-5]. Within the Cerrado biodiversity, Myrtaceae stands out as one the most prominent families in the Brazilian flora, with 129 genera and 4,620 species [6,7]. *Eugenia dysenterica* DC., known as cagaita or cagaiteira, is a representative of this family; it is a fruit tree species widely used by local population as food (fruits) and treatment (flowers and fruits) of various diseases as dysentery, diabetes, jaundice, kidney and bladder infections and as a laxative [8,9]. However, there are not many pharmacological research reports or chemical characterization of this species. Studies have shown, *in vitro* antifungal activity of the essential oil from leaves of cagaiteira on *Cryptococcus neoformans* [10,11]. Ethanolic and aqueous extracts of pulp, seeds and peel of *E. dysenterica* were evaluated to determine the potential to scavenge free radicals in DPPH assay, showing antioxidant potential, especially of ethanolic fraction of leaves [12]. The presence of provitamin A carotenoids (α and β-carotene) and vitamin C content in the cagaita's fruit pulp determined was [13]. A recent research studied the methanolic extract of *Eugenia pollicina*, other species of the Myrtaceae family and found catechin contents [14]. Studies examined the content of catechin in eight species of the Myrtaceae family, applying extraction by maceration, using acetone/water (70/30 v/v) as solvent. These authors found catechin in all species, being the *Syzygium mauritianum* the species with higher contents of catechin [15].

Considering these studies and based on preliminary analyzes performed by our research group, where the flavonoids catechin and epicatechin were identified in an aqueous extract of *E. dysenterica* DC., catechin being the major chemical (data not showed) [16]. Therefore, the aim of the present work was to develop and validate a method by analytical high performance liquid chromatography (HPLC) to quantify catechin in aqueous extract of E. dysenterica DC. Catechin and epicatechin are plant secondary metabolites and are derived from the shikimate pathway. The shikimate pathway provides aromatic aminoacids that are the precursors of natural products as pigments and alkaloids [16]. Catechin was first isolated from the extract of *Acacia catechu*, from which it is named after [17]. Studies show that catechins have antioxidant, antimutagenic, hypocholesterolemic, antidiabetic, antibacterial and anti-inflammatory activity [17,18].

Methods

Reagents and Standards

All solvents were HPLC grade (Tedia, Fairfield, Ohio, USA) and were degassed by helium gas. The water was purified using a Milli-Q system (Millipore, Massachusetts, USA). All solutions were filtered through 0.45 µm membrane (Millipore, Massachusetts, USA). The standard catechin was purchased from Sigma-Aldrich, USA.

Plant material

Eugenia dysenterica DC. leaves were collected at the University of Brasilia, Campus Darcy Ribeiro, Brasilia. Botanical identification was performed by Professor Sueli Maria Gomes. A voucher specimen was deposited at Herbarium of the University of Brasilia (UB 914). All necessary permits were obtained for the described field studies.

Preparation of Eugenia dysenterica DC. aqueous extract (EAE)

The plant material was dried at room temperature and powdered in a knife mill. The aqueous extract from 100 g of plant material was obtained by infusion, using distillated water (0.5 L) at 70 °C, approximately. Aqueous extracts were prepared by infusion with water pure 70 °C, and then filtered. The extract was kept in an amber flask and cooled at -30 °C.

Dry extract

The dry extract from the *E. dysenterica* DC. leaves was obtained through the lyophilization technique, using a Lyophilizer Advantage Plus XL-70 (SP Scientific). The yield was calculated by the ratio of the mass of dry extract obtained x 100, divided by the total mass of the botanical material originally used in the extraction**.** Using the following equation:

$$\text{Yield} = \frac{massf}{massi} x100$$

where *massf* = final mass, after lyophilization and *massi* = initial mass of leaves added in the process.

The total solids content was measured by gravimetric method. After lyophilization, the crude aqueous extract and moisture content were evaluated according to the gravimetric method.

Reproducibility of process to obtain Eugenia dysenterica DC. aqueous extract

For the reproducibility of the extraction process, seven different extractions were carried out following the pre-established conditions. In this step, the following parameter settings were evaluated: total solids before lyophilization of the extract, humidity after lyophilization and concentration of catechin by HPLC.

HPLC analysis

Eugenia dysenterica DC. aqueous extracts were analyzed using Dionex UHPLC Thermo Scientific TM Dionex Ultimate TM 3000 BioRS (Dionex, Idstein, Alemanha) liquid chromatograph equipped with L2130 pump, L2200 auto-sampler; L2300 column oven was set at 25 °C and L2455 DAD detector (Hitachi, Tokyo, Japan). The detector was set at 280 nm. Separation was performed on Purospher Star reverse phase C18 column (5 µm, 150 x 4,6 mm, Merck, Germany) in combination with an appropriate guard column (4 x 4 mm; 5,0 µm particle size, Merck, Germany). The eluents were a linear solvent gradient system consisting of phosphoric acid (1%) (A) and methanol (B), at a flow rate of 1.3 mL/min. Data acquisition was performed using Chromeleon software (version 7.1.2.1541). The gradient employed was: 10% A and 90% B for 0 min, 30% A and 90% B for 18 min, 50% A and 90% B for 19 min, 10% A and 90% B for 20 min, up to 25 min. The sample solution was prepared diluting 5.0 mg of extract into 5.0 mL MeOH. The solution obtained was then filtered. The compounds present in the extract were characterized according to their UV–Vis spectra and identify by their retention times in comparison with those of commercial standards.

Sample preparation

The dry extract was diluted at 1:5 in the mobile phase and filtered through a 0.45 µm cellulose regenerated membrane filter.

Standard solution

Catechin (5.0 mg) was dissolved in 5.0 mL of mobile phase by sonication during 5 min to prepare a freshly working solution, which was used to validate the method.

Methodology validation

Validation was performed following the ICH guidelines and Brazil's legislation [19,20]. The method was validated considering the parameters linearity, accuracy, precision, limit of detection, limit of quantitation and robustness.

Linearity

To determine the linear relationship between peak areas and concentration of catechin, six solutions with concentrations at 1; 2; 5; 10; 25 and 50 µg/mL of the catechin standard, were analyzed in triplicate and all solutions were injected three times. The linearity was evaluated by adding increasing concentrations of the standard in a fixed concentration of the sample solution. The linearity equations were calculated by linear regression analysis, using GraphPad Prism Version 5.0. The follow parameters were determinated: coefficient of correlation (r), y-axis intercept, slope of the line and relative standard deviation.

Precision

Precision was expressed as relative standard deviation (RSD %) and was determined by repeatability (intra-day) and intermediate precision (inter-day). Repeatability was evaluated by analysis of six replicates of the same aqueous extract (1.0 mg/L) of *E. dysenterica* DC. in the same day. The intermediate precision was determined in triplicate analyzed it in three different days with the same analyst and equipment. Every sample was injected once.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

Both limit of detection (LOD) and limit of quantitation (LOQ) were determined on the basis of the standard deviation of the response and the slope of the constructed calibration curve. The LOD was expressed as (3.3 x σ)/S and the LOQ was expressed as (10 x σ)/S, where σ is the standard deviation of the response and S is the slope of the calibration curve.

Accuracy

The accuracy of the analytical method was verified by the standard addition method. Different amounts of standard (catechin) were added to 80% of the crude extract until a total concentration of 100% and 120% of catechin (Table 1). The catechin concentrations were obtained from an initial solution

(200 µg/mL), taking 25.5, 42.5 e 59.5 µL and adding methanol to complete 500 µL. To such solution, it was added 500 µL of 100% extract (17 µg/mL). After the addition of the standard to the samples, the solutions were filtered, and 10 µL were injected in liquid chromatography, following the analytical conditions previously described. The solutions on the different concentration levels were prepared in triplicate, and each solution was injected once.

Robustness

Robustness was examined by evaluating the influence of small variation in the experimental parameters on the analytical performance of the proposed method. The studied parameters were: variation of wavelength (280-354 nm), flow (1.0-1.5 mL/min.) and temperature (20-30 °C). The effects on the parameters retention time and peak area were observed.

Statistical analysis

All the statistical analyses were accomplished using the computer software GraphPad Prism Version 5.0. All experiments were carried out in triplicate, and data are expressed as mean ± SD (Standard Deviation).

Results

The chromatographic profile obtained by HPLC of aqueous extract of *E. dysenterica* showed six major peaks, which reveals a wide chemical diversity. By comparing the retention time of the standard, it was shown the presence of catechin [21] among the substances present in the extract (Figure 1). To quantify catechin in samples, values of peak areas corresponding to the retention time on the elution of catechin were used. To execute the calculations was employed the equation y = 0.01109x - 0.2187 (*r = 0.9996*), obtained by relating the peak area *versus* the concentration of the catechin standard. The content of catechin found in the extract was 47.51 mg/g of extract (relative standard deviation = 7.08%).

Analytical validation

Linearity

The area of the peak *versus* the concentration of catechin showed a linear response in a wide concentration range showing the linear correlation. Coefficient (r), the intercept, slope and relative standard deviations are shown in Table 2. For the linearity evaluation, the equation y = 0.0784x + 0.0438 was obtained by linear regression studies, between the concentration of catechin and their responses, furnishing a determination coefficient of 0.9956. These values can be characterized as linear and suitable for such use, in accordance with the Brazilian legislation [19] that considers 0.99 as the minimum acceptable criteria of the correlation coefficient. The linearity study of the analytical method was performed using analysis of variance, verifying the significant linear regression and the non-significant deviation from linearity ($p < 0.05$) with injection concentrations ranging between 1.0 and 50.0 µg/mL in triplicate.

Precision

For the evaluation of the repeatability, separate six samples were analyzed on one day, with a relative standard deviation (%RSD) of 3.52%. The inter-day study was determined through triplicate analysis of the sample on three different days and showed a variation of 3.07%. The value of RSD below 5% (established by ICH Q2B) shows the high precision of the method for the closeness of the results obtained in a series of measures using a multiple sampling HPLC, of the same sample.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The limits of detection and limit of quantification of catechin found for the proposed method were 0.57 µg/mL and 1.74 µg/mL, respectively.

Accuracy

The accuracy of the standard in the spiked sample was evaluated at three levels: 13.6; 17.0 e 20.4 µg/mL for catechin, with recoveries of about 90.7%, 98.0% and 106.4%, respectively.

Robustness

No statistically significant differences were seen in the values of the area and retention time of *E. dysenterica* DC. aqueous extract solution when observed variations in wavelength and temperature parameters. These results are shown in Tables 4 and 5. The flow variation of mobile phase was the impacting variable in the method with significant changes, which produced significant changes in peak area and retention time (Table 6). The results showed that this parameter is important in the separation, implying in a great influence on the separation of substances and purity of the peaks.

Preparation of Eugenia dysenterica DC. aqueous extract (EAE)

Yield

The yield of *E. dysenterica* DC. aqueous extract

lyophilized at process end was close to 7.0%. The percentage of total solid waste found in the samples was close to 4.2 %, and relative standard deviation was 6.21%. The lyophilized extracts showed 4.7% of humidity (RSD = 13.89%).

Reproducibility of process to obtain Eugenia dysenterica DC. dry extract

The variation of the parameters discussed above for the seven extractions was not significant (p <0.05). Therefore, the process to obtain *E. dysenterica* DC. crude aqueous extract shows to have good reproducibility.

Discussion

The concentration of catechin found in the extract was approximately five times higher than the content found in a study conducted earlier studies, where the authors studied the methanol extract of *Eugenia pollicina*. The contents of catechin found in this study were 9.7 mg/g of extract [14]. Studies examined the content of catechins in eight species of the Myrtaceae family, obtained from extraction by maceration using acetone/water (70/30 v/v) as solvent. These found levels of catechin lower than the ones found in this study, ranging from 6.15 mg/g to 16.98 mg/g, being *Syzygium mauritianum* the species with highest levels of catechin [15].

Catechin and its derivatives belong to an important class of polyphenols, and study have reported relationships between phenolic contents and antioxidant activity [22]. Recent epidemiological studies suggest that diets rich in polyphenols may have beneficial health effects such as cancer prevention and lowering the risk of cardiovascular diseases [23,24]. In this work, a sensitive and time-saving method was developed for quantification of catechin found in dried aqueous extract of *E. dysenterica* DC. leaves and validated for quantitative analysis. The method showed a satisfactory separation of the peaks, with good resolution within a short space of time using an isocratic method. The similar chromatographic profile was observed for dry extract.

The area of the peak *versus* the concentration of catechin showed a linear response in a wide concentration range showing the linear correlation. These values as the minimum acceptable criteria of the correlation coefficient, in accordance with the Brazilian legislation [19]. From the results obtained in the development of the method, it was concluded that the analytical calibration curve can be used for quantification of experimental values of catechin, whereas the determination coefficient was greater than 0.99, confirming the quality of the curve obtained, for the low dispersion of the set of experimental points [19,20,25]. The present study was able to develop a fast and reliable UPLC technique for determination of the flavonoid catechin in the aqueous extract of *E. dysenterica* DC. leaves. Hence, it was validated according to all parameters defined both nationally and internationally.

The method showed to be linear and precise, besides being accurate and robust, with limits of detection and quantification appropriate for the evaluation of plant extracts. Knowing that catechin, in this study, was detected from a natural product and not from a single sample, it reinforces the idea that the results of the presented method were quite significant in the analyzed parameters. The process of obtaining the extract was shown to have a good reproducibility because the parameters yield, total solids content and humidity showed little variation. Moreover, the content of catechin found was higher than when compared to other studies performed with different extracts of the same *Eugenia* species. Besides that, as far as we know, it is the first report of a standardized dry extract in catechin from *E. dysenterica* DC. leaves with the validation of the analytical method.

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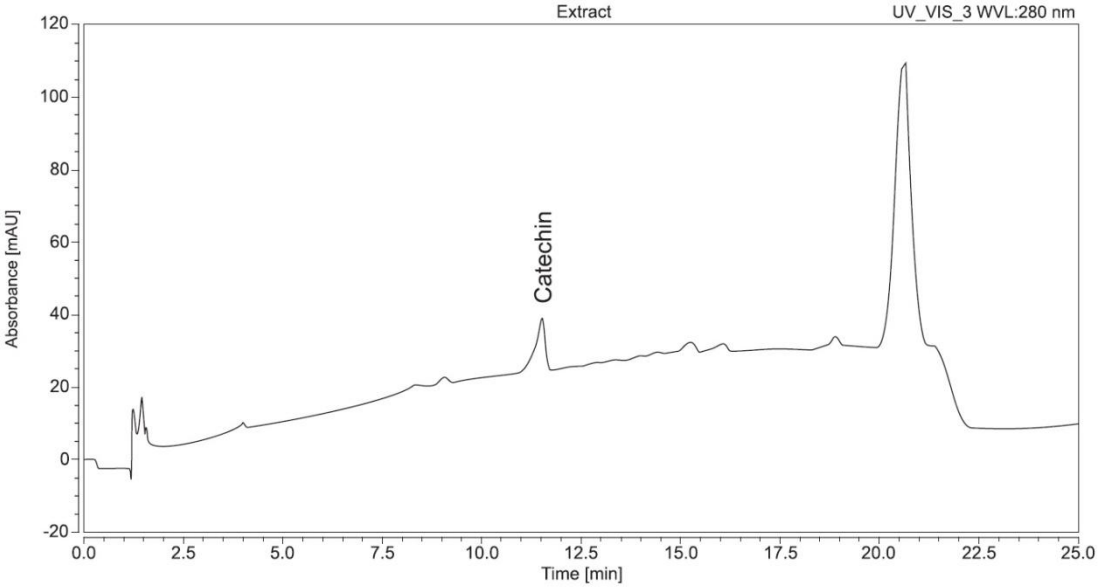

Figure 1. Chromatograms obtained by HPLC of aqueous extract of *E. dysenterica.*

Table 1. Theoretical concentration of catechin of *Eugenia dysenterica* for accuracy analysis.

Catechin Concentration (%)	Extract (μg/mL)	Added Catechin (μg/mL)	Final Concentration (μg/mL)
80	20	5.1	13.6
100	20	8.5	17.0
120	20	11.9	20.4

Table 2. Linearity results of the UPLC method for catechin assay in *Eugenia dysenterica* aqueous extract.

	Catechin (μg/mL)	slope	Intercept	*R*	LD[a] (μg/mL)	LQ[b] (μg/mL)
Catechin	1.00-50.00	0.08±0.01	0,05±0.01	0.9956	0.57	1.74

r – linear correlation coefficient; [a] LD –Detection Limit; [b] LQ –Quantification limit

Table 3. Accuracy results of the UPLC method for catechin assay in *Eugenia dysenterica* aqueous extract.

Concentration	Catechin spiked (μg/mL)	Catechin found (μg/mL)	Recovery (%)
A	13.6	12.3± 0.4	90.4±2.9
B	17.0	16.6±0.4	98.0±2.4
C	20.4	20.2±0.4	99.1±2.1

A: Low concentration (80%), B: Intermediate concentration (100%), C: High concentration (120%).
Data are expressed as mean ± SD.

Table 4. Results of wavelength variation on robustness studies.

Wavelength (nm)	Area (mAU*min)	Retention time (min)
270	0.8298	11.573
280	0.891	11.457
300	*ND*	*ND*

ND: No detected

Table 5. Results of temperature variation on robustness studies.

Temperature °C	Area (mAU*min)	Retention time (min)
20	0.895	12.573
25	0.891	11.457
30	0.732	10.683

Table 6. Results of flow variation on robustness studies.

Flow (mL/min)	Area (mAU*min)	Retention time (min)
1.0	0.963	13.647
1.3	0.899	11.457
1.5	0.688	10.647