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Article

Validation HPLC-DAD Method for Quantification of Gallic and Ellagic Acid from *Eugenia punicifolia* Leaves, Extracts and Fractions

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Eugenia punicifolia (Kunth) DC, known as "*pedra ume-caá*" or "*pitanguinha do cerrado*", is popularly used to treat infectious diseases like inflammation, and diabetes. Therefore, this study aimed to develop and validate an analytical method by high-performance liquid chromatography with diode array detector (HPLC-DAD) for the seasonal quantification of gallic acid (GA) and ellagic acid (EA), and to co-validate the method for quantification from the ethanol extract and fractions of the *E. punicifolia* leaves. Chromatographic separations were performed with a mobile phase gradient of acetonitrile/methanol/water acidified with 0.2% formic acid, a flow of 0.8 mL min^{-1}, detection at 254 nm, and a C18 column (250 × 4.6 mm, 5 µm). Method validation parameters were determined following Brazilian legislation. The HPLC-DAD analytical method developed for the quantification of GA and EA was validated and demonstrated to be selective, linear, precise, accurate, robust, and without matrix effect. Accordingly, it is helpful for the analysis of these tannins in crude ethanol extracts and fractions from *E. punicifoila* leaves. Thus, our results may be of great help for quality control assessment and standardization of raw materials containing *Eugenia punicifolia*.

Keywords: tannins, seasonality, medicinal plants, Myrtaceae

Introduction

Eugenia punicifolia (Kunth) DC is a plant species from Myrtaceae family known as "*pedra-ume-caá*" or "*pitanguinha do cerrado*".[1] It's leaves are popularly used in the form of decoction or aqueous infusion to treat inflammation, like fever, flu, diabetes; in alcoholic infusions for the treatment of wounds and infectious diseases.[2-4] Scientific studies reported in the literature describe hypoglycemic,[5,6] antioxidant,[7-9] anti-inflammatory, antinociceptives[3,4,10] neuroprotective,[11] gastroprotective,[4,12] vasodilatory[13] and antiproliferative[14,15] activities of *E. punicifolia* leaves.

Galeno *et al*.[9] found in the spray-dried aqueous extract of *E. punicifolia* leaves the content of 21.60 GAE mg g^{-1} (gallic acid equivalent *per* gram of extract) of phenolic compounds and 2.62 EQ mg g^{-1} (quercetin *per* gram of extract) of total flavonoids. Costa *et al*.[10] obtained a hydroalcoholic extract from *E. punicifolia* leaves, contained 74.86 gallic acid (GA) mg g^{-1} of phenolic compounds and 32 EQ mg g^{-1} of flavonoids.

Ramos *et al*.[7] evaluated the methanol extract of freeze-dried *E. punicifolia* fruits at different stages of maturation and verified a higher content of phenolic compounds and more pronounced antioxidant potential by DPPH (2,2-diphenyl-1-picrylhydrazyl) in the yellow pulp (616.2 GAE mg g^{-1}, half-maximal inhibitory concentration (IC_{50}) 89.5) and the green fruit (655.6 GAE mg g^{-1}, IC_{50} 120.5 µg mL^{-1}). Santos *et al*.[14] extracted phenolic compounds and flavonoids compounds from *E. punicifolia* leaves with different solvents (water, ethanol, and methanol) by dynamic maceration, obtaining higher concentrations with 100% ethanol (344.12 mg GA g^{-1} and 128.46 mg quercetin (Q) g^{-1}) and 100% methanol (330.33 mg GA g^{-1} and 137.43 mg Q g^{-1}), respectively. In the literature, there are reports of gallic acid (GA)

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and ellagic acid (EA) detection in different extracts and fractions of *E. punicifolia* leaves and fruits by other analytical techniques,[6,7,9,10,14,16] however, there are no reports of isolation and/or quantification of these compounds.

Although there is some research on the biological activities and chemical constitution of *E. punicifolia*, the development of simple and validated methodologies for quantifying marker compounds is necessary to improve quality control. One of the most common techniques for analyzing plants is high-performance liquid chromatography (HPLC).[17,18]

For that reason, this study aimed to develop and validate an analytical method by high-performance liquid chromatography with diode array detector (HPLC-DAD) for the seasonal quantification of gallic acid (GA) and ellagic acid (EA), and to co-validate the method for quantification from the ethanol extract and fractions of the *E. punicifolia* leaves.

Experimental

Botanical material

Leaf samples of *E. punicifolia* were collected in Hidrolândia, Goiás (GO) (786 m, 16º53'59"S and 49º13'29"W), in the entire first week of each month, from September 2016 to August 2017, in the morning period. Dr José Realino de Paula performed the botanic identification, and a voucher specimen was deposited in the Herbarium of the Federal University of Goiás (UFG) under the number UFG-48579. The leaf samples were dried in an air circulation oven (38 ± 2 ºC, INOVA model 171, Jaraguá do Sul, Santa Catarina, Brazil), and ground in an industrial blender Poli® (model LS-08MBR-N, Santa Catarina, Brazil) to powder form. Climatic data for the period were obtained from the National Institute of Meteorology.[19]

Measurements of phenolic compounds: total phenols, tannins, and flavonoids

The determinations of flavonoids, total phenols, and total tannins of the *E. punicifolia* leaves were carried out from September 2016 to August 2017. All experiments were performed in triplicate and the solutions of the standard curves of rutin (Sigma-Aldrich, St. Louis, USA) and tannic acid (Galena, Campinas, São Paulo, Brazil) were used for the construction of the analytical curve. Absorbance readings were taken in glass cuvettes in the spectrophotometer (METASH UV-5100, Shanghai, China) and from the equation obtained from the standard curve it was possible to calculate the concentration (mg mL^{-1}) of total phenols in the extract and the percentage present in the *E. punicifolia* leaves.

The total phenol content of the *E. punicifolia* leaves was determined by $FeCl_3$ (Vetec®, Duque de Caxias, Rio de Janeiro, Brazil) and the total tannins were quantified by the protein precipitation assay involving bovine serum albumin (Sigma®, Steinheim, Germany) using the methods of Mole and Watermand.[20] The total flavonoid content was determined by a modification of the described method by Rolim *et al*.[21]

Development of validation of the method for quantification of gallic acid and ellagic acid by HPLC

Samples consisting of sprayed and dried *E. punicifolia* leaves were weighed in triplicates, 1 g of leaves to 25 mL of MeOH (J.T. Baker®, Xalostoc, Mexico), with ultrasound-assisted maceration (Q5.9/40A, 40 kHz, Ultronique®, São Paulo, Brazil) for 15 min. Afterward, they were filtered on filter paper and a 0.45 µm polyvinylidene fluoride (PVDF) membrane (Millex®, Massachusetts, USA). The methanol extract was diluted in MeOH (J.T. Baker®, Xalostoc, Mexico) (1:1) to decrease the concentration and then samples were injected into the chromatograph. The HPLC-DAD validation method described by Assunção *et al*.[17] was employed using ellagic acid as a marker for the methanol extract of *E. punicifolia* leaves (Figure S1, Supplementary Information (SI) section). Acetonitrile (J.T. Baker®, Xalostoc, Mexico) and water acidified with 0.2% formic acid (Organics, New Jersey, USA) were used. Then, methanol was added, tested in different proportions, with a flow of 0.8 to 1.0 mL min^{-1} and different temperatures, in order to obtain a more economical method, with less execution time, better separation of the peaks, and framing in the RDC parameters.[22]

Analyzes were performed using a Waters® Chromatographic System model HPLC Alliance® (Massachusetts, USA) with e2695 separation module, 2998 diode array detector (DAD), and Empower 2.0 data processing system. Chromatographic separations were conducted on Zorbax Eclipse XDB-C18 (California, USA) reverse-phase column (250 mm × 4.6 mm, 5 µm) LN-B14036. The mobile phase employed was a mixture of HPLC grade acetonitrile (J.T. Baker®, Xalostoc, Mexico) (pump A), HPLC grade methanol (J.T. Baker®, Xalostoc, Mexico) (pump B), and ultrapure water (Milli-Q®, Molsheim, France) acidified with 0.2% formic acid (Organics, New Jersey, USA) (pump D). The mobile phase started with 8% (A) and 92% (D); 7 min 20% (A), 5% (B) and 75% (D); 10 min 25% (A), 5% (B) and 70% (D); 16 min 35% (A), 5% (B) and 60% (D), and 20 min 8% (A) and 92% (D),

with gradient elution mode and flow rate of 0.8 mL min^{-1} for 20 min, and detection at 254 nm. The injection volume was 10 µL. Analyzes were performed at a temperature of 22 °C. The mobile phase was previously filtered through a 0.45 µm polyvinylidene fluoride (PVDF) membrane (Millex®, Massachusetts, USA) and degassed using an ultrasound bath (Q5.9/40A, 40 kHz, Ultronique®, São Paulo, Brazil).

System suitability

Before performing the validation, the chromatographic system used for the analysis was evaluated to verify its ability to provide reproducible results. This assessment was achieved with system suitability compliance experiments, which can be defined as a set of tests to ensure that the equipment used can generate acceptable accuracy and precision results. The parameters according to Food and Drug Administration (FDA),[23] and Ribani *et al*.[24] are: (*i*) retention factor (k)-the peak must be well separated from other peaks and from the peak corresponding to the retention time (t_R) of an unretained compound (tm), $k > 2$; (*ii*) repeatability-relative standard deviation (RSD) < 1% for $n > 5$; (*iii*) resolution (Rs) > 2 between the peak of interest and the closest potential interferent (impurity, degradation product, or other compounds); (*iv*) tail factor (TF) ≤ 2; (*v*) the number of theoretical plates in the column (N) should generally be > 2000 for HPLC.

Validation of the analytical method

The validation was carried out by what is recommended by resolution of the collegiate board (RDC) No. 166/2017[22] for category I regarding quantitative tests for the determination of the active ingredient in pharmaceutical products or raw materials.[23]

Selectivity

The selectivity of the method was evaluated by identifying gallic acid and ellagic acid in the sample by comparing the retention times and ultraviolet absorption spectrum (190 to 400 nm) of the peaks obtained in the sample and the gallic acid (GA; VETEC®, Duque de Caxias, Rio de Janeiro, Brazil) and ellagic acid (EA; Sigma-Aldrich, Saint Louis, USA) reference standards. The chromatograms and absorption spectra of the HPLC grade methanol (J.T. Baker®, Mexico) diluent were evaluated to verify possible interfering peaks in the analysis.

Linearity and interval

To construct the standard curve, seven GA and EA concentration solutions were prepared: 5, 25, 50, 100, 150, and 200 µg mL^{-1} in HPLC grade methanol (J.T. Baker®, Mexico). Standard solutions were filtered on a 0.45 and 0.22 µm Millex® membrane (Massachusetts, USA) and injected, in triplicate, into the chromatograph. Area means of each marker concentration were plotted on the ordinate axis and the corresponding concentrations on the abscissa. The straight-line equation was obtained by the method of least squares, according to the equation 1.

$$y = a + bx \quad (1)$$

where a: inclination of the line to the axis; b: intersection of the line with the *y* axis.

The curve was plotted in Microsoft Excel 2013.[25] The test results were treated with the aid of the Statistica 7 software,[26] performing the regression significance tests by analysis of variance (ANOVA) and the normality of the residuals by the method of Anderson-Darling. All of these were calculated with a 95% confidence interval.

Limits of detection and quantification

The limits of detection and quantification were calculated with equations 2 and 3, respectively:

$$LOD = DPa \times 3IC \quad (2)$$
$$LOQ = DPa \times 10IC \quad (3)$$

where LOD: limit of detection; LOQ: limit of quantitation; DPa: standard deviation of the intercept with the Y axis of the calibration curve; IC: slope of the analytical curve.

Precision (repeatability and intermediate accuracy)

For precision evaluation, repeatability (intra-day precision) and intermediate precision (inter-day precision) were determined. Precision was assessed by determining the concentration of three points on the analytical curve: low level (32 µg mL^{-1}), medium level (40 µg mL^{-1}), and high level (48 µg mL^{-1}) at the repeatability level. Low, medium and high levels correspond to 80, 100 and 120%, respectively.

The solutions were filtered through a 0.22 µm Millex® membrane (Massachusetts, USA), and injected (in triplicate) into the chromatograph. Intermediate precision was performed by a different analyst on another day, with sample preparation respecting the above conditions. The coefficient of variation (CV), was calculated using the Microsoft Excel 2013[25] program to establish the RDC parameters.[22]

Accuracy

Accuracy was verified by adding known amounts (concentration equivalent to 5 µg mL^{-1}) of the standard gallic

acid and ellagic acid to the sample solutions at three different concentration levels. The accuracy value, in percentage, was obtained by the relationship between the concentration of the standard added in the sample and the concentration of the standard before addition, according to the equation 4.

$$\text{Accuracy} = \frac{100 \times [(\text{sample} + \text{standard}) - (\text{standard})]}{[\text{standard}]} \quad (4)$$

Robustness

Robustness was evaluated by varying the temperature from 22 °C to 25 and 20 °C, the flow rate from 0.8 mL min^{-1} to 0.7, 0.9 and 1.0 mL min^{-1}, the mobile phase pH from 3.3 to 3.1, and 3.5, and finally another Zorbax Eclipse XDB-C18 LN column B12003 (4.6 mm × 250 mm, 5 μm) (USA). CV was calculated between the peak areas of gallic acid and ellagic acid at each change concerning the area of the original method.

Matrix effect

The matrix effect is a selectivity study that investigates possible interference caused by compounds that make up the sample matrix, basically generating phenomena of decrease or increase in the signal or instrumental response.[27] Matrix effects were evaluated using the standard additions method. The calibration curve was used as described for the evaluation of the linearity of the GA and EA standards (5, 25, 50, 100, 150, 200 μg mL^{-1}) in solvent (MeOH, J.T. Baker®, Mexico) and the calibration curve of the extract in five levels (32, 36, 40, 44, 48 μg mL^{-1}) with the addition of the standard (5 μg mL^{-1}) (1:1). Each level was prepared in three independent repetitions, which were analyzed in random order. The parallelism of the straight lines is another indication of the absence of interference from the constituents of the matrix, and its demonstration must be carried out through adequate statistical evaluation. Thus, the slopes of both curves were compared by the *t*-test,[28] according to RDC 166/17.[22]

Linearity of the extract

The profile of markers in the complex matrix was checked to analyze whether their behavior is linear or not. Therefore, linearity analysis of the methanol extract was performed at concentrations of 32, 36, 40, 44, and 48 μg mL^{-1} in triplicate in the chromatograph and the analytical curve was constructed. The area means of each concentration of GA and EA were plotted to obtain the equation of the straight line by the method of least squares.

Seasonality of gallic and ellagic acids in *E. punicifolia* leaves

For the evaluation of the seasonal profile, the leaves of *E. punicifolia* collected from September 2016 to August 2017 were individually extracted (in triplicate) 1 g 25 mL^{-1} in MeOH (J.T. Baker®, Mexico) in an ultrasound device at room temperature for 15 min and analyzed by HPLC-DAD. The respective areas for gallic and ellagic acid were collected and quantified.

Obtaining the crude ethanol extract (CEE)

The material previously sprayed was subjected to a cold maceration process for three days, with occasional agitation using ethanol 96° GL PA as an extracting liquid. The proportion used was one part of the sprayed material to five amounts of ethanol (Neon®, Suzano, São Paulo, Brazil) (100 g 500 mL^{-1}). After maceration, filtration was carried out with the aid of a funnel and filter paper, and the obtained extract was concentrated in a rotary evaporator at a temperature of 40 °C. The vegetal residue was extracted three more times in an analogous way to the first, thus obtaining the CEE from the leaves of *E. punicifolia*.[29]

Fractionation of the crude ethanol extract (CEE)

For the fractionation of the crude ethanol extract was dispersed in the methanol (MeOH)/water mixture at a ratio of 7:3, and submitted to successive liquid-liquid partitions with hexane (Neon, Suzano, São Paulo, Brazil), dichloromethane (Neon, Suzano, São Paulo, Brazil), and ethyl acetate (Neon, Suzano, São Paulo, Brazil). Thus, four fractions will be obtained: hexane fraction (FrH), dichloromethane fraction (FrD), ethyl acetate fraction (FrAc), and aqueous fraction (FrAq).

Obtaining tannin-rich fractions

The dried and powdered *E. punicifolia* leaves (200 g) were mechanically macerated with acetone (Synth, Diadema, São Paulo, Brazil)/water (1:1) for 3 h. Afterward, filtration was carried out and the obtained extract was concentrated in a rotary evaporator at a temperature of 40 °C. As a result, the extract was obtained, which was fractionated 3 times with 100 mL of ethyl acetate (Neon, Suzano, São Paulo, Brazil), getting the ethyl acetate fraction rich in tannin (FrAcRT) and the concentrated aqueous fraction of tannins (FrAqRT).

Partial co-validation of the HPLC method for CEE and FrAqRT

The method was co-validated for the analytical parameters of selectivity, linearity, and precision (repeatability) in sextuplicate at a concentration of 3 mg mL^{-1} and accuracy

at 1, 3, and 5 mg mL^{-1}, according to RDC No. 166/2017[22] for CEE and FrAqRT. As for the fractions: hexane (FrH), dichloromethane (FrD), ethyl acetate (FrAc), an aqueous fraction of the crude extract (FrAq), ethyl acetate fraction rich in tannin (FrAcRT), and aqueous fraction rich in tannin (FrART) where the two markers were quantified.

Statistical analysis

Pearson's correlation analyzed the relationship between phenolic compounds, GA and EA found in *E. punicifolia* leaves and environmental variables. Linearity and matrix effect analysis by the *t*-test in the statistical program PAST 4.06.[30]

Results and Discussion

Development of a method for quantification of GA and EA by HPLC-DAD

The methods with the mobile phase starting at 8% (A) and 92% (D); 7 min 20% (A), 5% (B), and 75% (D); 10 min 25% (A), 5% (B) and 70% (D); 16 min 35% (A), 5% (B) and 60% (D), 20 min 8% (A), and 92% (D), with gradient elution mode and flow rate of 0.8 mL min^{-1} for 20 min, and detection at 254 nm showed the best adequacy parameters, according to the United States Pharmacopoeia and Food and Drug Administration (FDA)[23] and Ribani *et al*.[24] The choice of method had as its main objective to guarantee an accurate and fast analysis. Thus, the standard for gallic acid came out at 4.7 min and for ellagic acid at 13.6 min (Table 1).

As for the temperature variation, it was observed that 22 ºC improved the resolution of the peaks. The balance reached between retention time and peak resolution was found with a flow of 0.8 mL min^{-1}. Then, the mobile gradient phase of acetonitrile/methanol/acidified water at a flow rate of 0.8 mL min^{-1} provided the best separation of the ellagic acid peak in the complex matrix, with a retention time of 13.639 min. These chromatographic conditions were found to be within the system adequacy parameters for the peak of pure gallic and ellagic acid and in complex matrices (tail factor (TF), resolution (Rs), retention factor (k), and a number of theoretical plates (N)) according to FDA,[23] and Ribani *et al*.[24] (Table 1).

The results showed that the method conditions are suitable for the quantification of GA and EA markers in the complex matrix, for the methanol extract from *E. punicifolia* leaves.

Validation of an analytical method for quantification of GA and EA by HPLC-DAD

Selectivity, linearity, and interval

The chromatographic profile and UV spectrum (Figures S2a and S2b, SI section) of gallic acid (200 µg mL^{-1}) and ellagic acid (200 µg mL^{-1}) in MeOH were obtained from HPLC-DAD analysis. The GA had a retention time of 4.658 min with maximum absorption of 220.5 and 271.4 nm, while the EA had a retention time of 13.639 min with maximum absorption of 253.6 and 364.3 nm. The sample extract in MeOH (1 g 25 mL^{-1}) (Figure S2c) showed a retention time for GA of 4.658 min (λmax 217.0 and 271.4 nm) and EA of 13.639 min (λmax 253.6 and 364.3 nm). These chromatographic profiles did not reveal compounds interfering with the retention time of GA and EA. Furthermore, the UV spectrum of the samples was considered identical to the standard, demonstrating the method selectivity.

The calibration curve for GA and EA showed a linear response within the range of 5-200 µg mL^{-1} and the linear equation for GA was $y = 22{,}997x + 40{,}537$ and for EA $y = 130{,}295x - 205{,}247$. The mean RSD% for the slope of the gallic acid calibration curve was 2.85% and ellagic acid 4.11%, which is following the limits established by the specifications (RSD < 5%).[23] The analytical curve showed a linear correlation greater than 0.99, which offers an adjustment of the data to the regression line, and demonstrates that the results obtained are directly proportional to the analyte concentration in the sample.

Table 1. Parameters of compliance with the system suitability according to FDA,[23] and Ribani *et al*.[24] of standards (GA and EA) and methanol extract of *E. punicifolia* leaves

Parameter	Gallic acid (GA)		Ellagic acid (EA)		Literature (FDA[23] and Ribani *et al.*[24])
	Standard	Extract	Standard	Extract	
Retention time (t_R) / min	4.7	4.6	13.6	13.6	
Retention factor (k)	7.9	9.9	4.2	4.3	≥ 2
Resolution (Rs)	4.6	5.3	2.6	2.7	≥ 2
Tail factor (TF)	1.0	1.3	1.3	1.4	≤ 2
Number of theoretical plates	4.62×10^3	3.30×10^3	6.44×10^4	8.41×10^3	> 2

FDA: Food and Drug Administration.

Linearity data were also evaluated by the ANOVA test, which showed that the *F* value calculated for the model was higher than the *F* value tabulated for a 95% confidence level, demonstrating that the model was adequate to predict the data.

The homoscedasticity of the data was investigated for the two markers using the Cochran test. It showed that for GA the C calculated 0.5914 < critical 0.616 and EA calculated 0.5687 < critical 0.616, therefore, the hypothesis null was accepted, and the data were homoscedastic. The significance of the angular coefficient by the *F* ANOVA test was evaluated and indicated that the calculated 2,424.178 > critical (4.49) for GA and the EA the calculated 3,661.766 > critical (4.49). Then the hypothesis null was rejected, and the peak area (*y*) varies as a function of the concentration of analytes (*x*), demonstrating that the method is linear. The angular coefficient was also evaluated using the Student's *t* test, and it was found that *T* calculated for GA (3.04×10^{-7}) and EA (1.04×10^{-7}) was greater than *T* critical (2.5706), so it rejected the null hypothesis and, therefore, there is evidence that the angular coefficient is statistically different from zero.[31]

Limits of detection and quantification

The limit of detection (LOD) value was 1.6 μg mL^{-1} for GA and 1.9 μg mL^{-1} for EA, representing the smallest amount of detectable analyte in the sample not necessarily quantified. Regarding the limit of quantification (LOQ) value, it was determined to be 5.1 μg mL^{-1} for GA and 5.8 μg mL^{-1} for EA, which represents the lowest measurable concentration of analyte in the sample by the proposed method.[24] The experiments were carried out in a range above the limits and, therefore, the concentration values obtained for GA and EA were adequate.

Precision

As for method precision (Table 2), the RSD values were less than 5% between the low, medium, and high concentration triplicates, as recommended by the specifications.[32] The precision in the repeatability level indicates the correlation between the results of the method executed under the same conditions within a period. The intermediate precision means that although with different analysts on different days, the technique can provide the same results.[32]

Accuracy

In accuracy (Table 3), the method provided recovery ranging from 92.48 to 111.05% with an average of 99.83% and an average RSD of 1.88 for GA, and for EA, the recovery ranged from 81.28 to 105.22% with an average of 95.03% and an average of RSD of 1.90. These data followed with the acceptable recovery ranges for tests on complex matrices (80-120%), such as natural products.[33] The recovery test quantifies the amount of analyte added to the test material that is extracted and amenable to quantification.[34]

Robustness

Regarding robustness, variations in column temperature, pH, flow, and column resulted in RSD values below 5% for peak area and GA and EA content (Table 4), demonstrating the method's ability to remain unchanged with these tested variations, in addition to contributing to the transfer of the analytical process to other laboratories.[35]

Matrix effect

The matrix effect was evaluated according to RDC 166/17,[22] and the proof of the parallelism of the lines must be carried out through adequate statistical evaluation, and the confirmation that the lines are parallel is indicative of the absence of interference from the matrix constituents, so it was used the *t* test by the statistical program PAST 4.06.[30] All regression assumptions were confirmed for the calibration curves with a combination of solvent and matrix. The matrix effect was not significant regarding the solvent slopes and matrix curves, for GA and EA in the studied ranges ($p > 0.05$). The *p*-values 0.1 and 0.2 for GA and EA, respectively, of the parallelism test (Table S1, SI section), are greater than 0.05, so the hypothesis that the slopes are equal to the significance level is not rejected. In this case, the lines are parallel.

Linearity of GA and EA in the complex matrix

The pure compound within a pre-established interval must present a linear behavior. Therefore, the linearity of the GA and EA marker (Figure S3, SI section) was verified, showing that even in the complex matrix (the extract), the compounds has a linear character with a correlation coefficient equal to 0.9969 for GA and 0.9958 for EA in the conditions under which the analytical curve of the standard was tested.

Seasonality of phenolic compounds, GA, and EA in the *E. punicifolia* leaves

Through liquid chromatography, it was possible to confirm that the chromatographic profile is very similar in terms of retention time (t_R) for all seasonal methanol extracts, with differences only in peak height and area (quantitative).

As for the climatic factors (Table 5), the maximum temperature was between 28.9 to 35.6 ºC, and the minimum of 14.6 to 20.7 ºC. The precipitation was 0 mm from June

Table 2. Precision data from the HPLC analytical method at repeatability and intermediate precision levels to quantify the content GA and EA in the methanol extract of *E. punicifolia* leaves (λ = 254 nm)

Gallic acid (GA)				
Method linear range concentration level	Theoretical concentration of the sample / (μg mL^{-1})	Area / (μV S^{-1})	GA content / (μg mL^{-1})	GA / %
Low 80%	32.000	1,610,922	66.523	0.207
		1,635,256	67.581	0.211
		1,584,558	65.377	0.204
Medium 100%	40.000	2,049,840	85.609	0.214
		2,074,488	86.681	0.216
		2,015,100	87.6209	0.219
High 120%	48.000	2,473,556	104.0345	0.216
		2,540,380	106.9403	0.222
		2,416,312	101.5453	0.211
RSD / %				2.679
Intermediate precision				
Low 80%	32.000	1,714,486	71.0272	0.221
		1,729,820	71.6940	0.224
		1,709,370	70.8047	0.221
Medium 100%	40.000	2,101,752	87.8670	0.219
		2,067,448	86.3754	0.215
		2,050,148	85.6231	0.214
High 120%	48.000	2,416,312	101.5453	0.211
		2,417,618	101.6021	0.211
		2,434,292	102.3272	0.213
RSD / %				2.197
Ellagic acid (EA)				
Method linear range concentration level	Theoretical concentration of the sample / (μg mL^{-1})	Area / (μV S^{-1})	EA content / (μg mL^{-1})	EA / %
Low 80%	32.000	5,037,166	41.8102	0.130
		5,151,326	42.6864	0.133
		5,089,396	42.2111	0.131
Medium 100%	40.000	6,570,094	53.5753	0.133
		6,423,172	52.4476	0.131
		6,218,600	50.8776	0.127
High 120%	48.000	8,094,724	65.2766	0.135
		8,065,908	65.0555	0.135
		8,042,244	64.8738	0.135
RSD / %				2.138
Intermediate precision				
Low 80%	32.000	5,158,280	42.7397	0.133
		5,113,450	42.3957	0.132
		5,040,016	41.8321	0.130
Medium 100%	40.000	6,458,606	52.7198	0.131
		6,384,262	52.1490	0.130
		6,566,638	53.5487	0.133
High 120%	48.000	7,845,524	63.3640	0.132
		7,972,942	64.3420	0.134
		7,979,232	64.3902	0.134
RSD / %				1.083

RSD: relative standard deviation.

Table 3. Accuracy data of the HPLC analytical method for the quantification of GA and EA in methanol extract (λ = 254 nm)

Gallic acid (GA)					
Method linear range concentration level	GA area in the sample	GA area in the sample + GA pattern / (μV S^{-1})	GA recovery area	GA concentration / (mg mL^{-1})	GA recovery / %
Low (32 μg mL^{-1})	1,648,106	1,864,599	216,493	7.651	92.48
	1,610,922	1,836,441	225,519	8.043	97.22
	1,535,950	1,760,372	224,422	7.996	96.65
Medium (40 μg mL^{-1})	2,015,100	2,232,443	217,343	7.688	92.93
	2,026,194	2,245,150	218,956	7.758	93.77
	2,074,488	2,298,205	223,717	7.965	96.28
High (48 μg mL^{-1})	2,542,240	2,794,068	251,828	9.187	111.05
	2,542,240	2,789,588	247,348	8.992	108.70
	2,545,404	2,794,068	248,664	9.050	109.39
Theoretical concentration of the GA standard					4.74
Recovery average (RSD) / %					99.83
Ellagic acid (EA)					
Method linear range concentration level	EA area in the sample	EA area in the sample + EA pattern / (μV S^{-1})	EA recovery area	EA content / (mg mL^{-1})	EA recovery / %
Low (32 μg mL^{-1})	5,159,042	5,918,623	759,581	4.254	82.12
	4,678,554	5,447,210	768,656	4.324	83.47
	4,800,070	5,553,965	753,895	4.210	81.28
Medium (40 μg mL^{-1})	6,570,094	7,425,141	855,047	4.987	96.27
	6,423,172	7,305,869	882,697	5.199	100.36
	6,218,600	7,109,955	891,355	5.265	101.64
High (48 μg mL^{-1})	8,094,724	9,010,202	915,478	5.450	105.22
	8,065,908	8,961,039	895,131	5.294	102.20
	8,042,244	8,940,997	898,753	5.322	102.74
Theoretical concentration of the EA pattern					5.18
Recovery average (RSD) / %					95.03

RSD: relative standard deviation.

Table 4. Robustness of the method considering the variation in column temperature, flow, pH, and column compared to the original method developed (λ = 254 nm)

Gallic acid (GA)						
Condition		Area / (μV S^{-1})	Average	Average between parameters	SD	RSD / %
Original method developed		2,139,456		–	–	–
		2,245,452	2,236,577	–	–	–
		2,324,822		–	–	–
Temperature	20 °C	2,133,334				
		2,117,168	2,113,443	2,175,010	87,068	4.00
		2,089,828				
	24 °C	2,166,702				
		2,153,600	2,143,979	2,190,277	65,476	2.98
		2,111,634				
pH	3.1	2,354,206				
		2,062,596	2,257,003	2,246,789	14,443	0.64
		2,354,206				
	3.5	2,582,168				
		2,166,682	2,259,999	2,248,287	16,561	0.73
		2,031,146				

Table 4. Robustness of the method considering the variation in column temperature, flow, pH, and column compared to the original method developed (λ = 254 nm) (cont.)

Condition		Area / (μV S^{-1})	Average	Average between parameters	SD	RSD / %
Flow	0.7 mL min^{-1}	2,321,856	2,360,214	2,298,395	87,424	3.80
		2,388,388				
		2,370,398				
	0.9 mL min^{-1}	2,175,300	2,123,969	2,180,273	79,625	3.65
		2,096,056				
		2,100,552				
	1.0 mL min^{-1}	2,180,458	2,108,010	2,172,293	90,910	4.18
		2,062,576				
		2,080,996				
Column	Agilent Eclipse XDB-C18 L.N. B12003 (USA)	2,210,510	2,271,962	2,254,269	25,021	1.10
		2,183,628				
		2,421,748				
Ellagic acid (EA)						
Original method developed		6,687,960	6,881,200	–	–	–
		6,766,050		–	–	–
		7,189,590		–	–	–
Temperature	20 °C	6,743,620	6,700,969	6,791,084	127,442	1.87
		6,638,088				
		6,721,198				
	24 °C	6,584,196	6,597,167	6,726,445	200,841	2.98
		6,612,786				
		6,594,518				
pH	3.1	7,034,096	6,944,509	6,912,854	44,766	0.64
		6,802,250				
		6,997,182				
	3.5	7,281,060	6,871,042	6,876,121	7,182	0.10
		6,654,726				
		6,677,340				
Flow	0.7 mL min^{-1}	6,866,490	7,349,295	7,115,247	330,993	4.65
		7,650,134				
		7,531,262				
	0.9 mL min^{-1}	6,573,190	6,439,343	6,660,271	312,439	4.69
		6,390,446				
		6,354,394				
	1.0 mL min^{-1}	6,491,558	6,423,957	6,652,578	323,319	4.86
		6,251,578				
		6,528,734				
Column	Agilent Eclipse XDB-C18 L.N. B12003 (USA)	6,859,118	6,679,689	6,780,444	142,489	2.10
		6,606,648				
		6,573,302				

SD: standard deviation; RSD: relative standard deviation.

to August, and in the other months, it varied from 26.5 to 215.5 mm with relative humidity from 36.88 to 70.68% and insolation from 17.71 to 22.18 MJ m^{-2} day^{-1}.

The total phenols had a content between 0.374 to 0.548%, total tannins from 0.264 to 0.346%, and total flavonoids from 0.213 to 0.264% by the spectrophotometric method, whereas the GA had a content between 0.067 to 0.168% and the EA from 0.081 to 0.199%. No significant difference was found in the contents of phenols, tannins, flavonoids, GA, and EA during the months that the samples were collected. It is noteworthy that there are no studies on method development, validation, and seasonal quantification of phenolic compounds, GA, and EA for *E. punicifolia* leaves.

Co-validation for crude ethanol extract (CEE) and tannin-rich aqueous fraction (FrAqRT)

The analytical method was co-validated to measure the content of GA and EA in the CEE and FrAqRT because

Table 5. Climatic data and seasonal variation of total phenols, tannins, flavonoids, GA, and EA of *E. punicifolia* leaves collected from September 2016 to August 2017

Month	Temperature / ºC		Rainfall / mm	Relative humidity / %	Insolation / (MJ m^{-2} day^{-1})	Phenolic compounds / %				
						Spectrophotometer			HPLC-DAD	
	Max.	Min.				Phenols	Tannins	Flavonoid	GA	EA
September	35.6	20.5	26.5	39.07	22.18	0.374	0.339	0.251	0.067	0.081
October	33.3	20.6	209.2	55.58	21.97	0.548	0.341	0.262	0.069	0.096
November	30.4	20.6	110.4	71.20	18.75	0.329	0.269	0.264	0.130	0.134
December	31.7	20.7	169.6	67.35	22.11	0.458	0.303	0.254	0.157	0.146
January	31.1	20.6	176.1	66.69	20.66	0.548	0.264	0.242	0.168	0.199
February	31.3	20.4	153.2	70.68	20.96	0.472	0.310	0.219	0.152	0.118
March	32.2	20.5	215.5	68.91	20.13	0.526	0.313	0.241	0.119	0.116
April	31.9	20.4	200.1	64.48	19.20	0.469	0.306	0.222	0.108	0.090
May	31.5	18.0	48.2	59.50	18.11	0.450	0.315	0.248	0.164	0.137
June	30.4	16.9	0	53.03	17.71	0.548	0.318	0.242	0.135	0.139
July	28.9	14.6	0	46.42	19.14	0.459	0.344	0.250	0.134	0.141
August	34.1	17.0	0	36.88	20.91	0.563	0.346	0.213	0.100	0.085

Max: maximum; Min: minimum; mm: millimeter; GA: gallic acid; EA: ellagic acid; MJ m^{-2} day^{-1}: megajoule *per* meter frame *per* day; HPLC-DAD: high-performance liquid chromatography diode array detector.

it is a different matrix from the one used in the validated method (methanol extract of *E. punicifolia*). In the analysis of the method's selectivity, the peak corresponding to the GA and EA pattern in the CEE was observed with a retention time of approximately 4.223 and 13.651 min, respectively, which was similar to that observed in the methanol extract (Figure S2c). In FrAqRT the retention time was 4.622 and 13.590 min (Figure S4f, SI section). The absorbance spectrum in the ultraviolet region determined for the GA and EA standard, through the DAD detector, reveals equivalent absorbance regions of the standards with the CEE and FrAqRT (Figures S4a-S4f). The absorption spectra demonstrate that the method is capable of measuring the compounds GA and EA in the presence of other constituents, being selective as defined by the RDC No. 166/2017.[22]

The method was linear, with a linear regression coefficient (r) of 0.996 for AG and 0.9989 for AE. The technique was also accurate for CEE (Table S2, SI section) and FrAqRT (Table S3, SI section), as the RSD value of repeatability and intermediate precision obtained for GA and EA was less than 5% of RSD. This value is acceptable for a complex matrix, demonstrating the accuracy of the method for measuring the two markers.

As for the accuracy of the CEE, a recovery was obtained for GA between 83.55 to 96.28%, with an average of 88.56%, and for EA from 96.08 to 116.35% with an average of 107.50%, being an average RSD of 2.39 and 1.5%, respectively. As for FrAqRT, the recovery for GA was between 94.89 to 111.07% and for EA from 102.54 to 115.38% with an average of 108.90%, and average RSD of 1.23 and 1.04%, respectively.

Determination of GA and EA in the CEE and fractions of liquid-liquid fractionation

After co-validation, the markers were quantified in the fractions obtained from the partition of the crude ethanol extract: hexane fraction (FrH), dichloromethane fraction (FrD), ethyl acetate fraction (FrAc), and aqueous fraction (FrAq), tannin-rich ethyl acetate fraction (FrAcRT) and the tannin-rich aqueous fraction (FrAqRT). It is observed in Figure S4 of the SI section the chromatograms and the UV spectra of the markers in the extract and fractions that show the selectivity of the method according to the retention time and UV spectrum.

It is possible to observe in the chromatograms that there are significant qualitative differences between the fractions, mainly in FrH and FrD. Still, it is also possible to observe similarities in the chromatographic profile of the methanol extract (Figure S2c), ethanol extract, and the FrAc, FrAq, FrAcRT, and FrART (Figure S4). The chromatographic profile plays an important role in identifying plant species as if it were a "fingerprint" of chemical characteristics, and this constancy of secondary metabolites is related to the biological activities of the species.[18]

The developed and co-validated method was able to quantify the GA and EA in the extract and the fractions (Table 6), except for the GA in the hexane fraction, which was below the limit of quantification. There is a higher

content of GA (16.57%) and EA (6.83%) in the ethyl acetate fraction extracted from the liquid-liquid partition of the CEE, thus indicating a higher content of these tannins in this fraction.

Table 6. GA and EA content in ethanol extract and fractions of *E. punicifolia* leaves at 3 mg mL^{-1}

Extract/fractions	GA / %	EA / %
Crude ethanol extract	7.86	4.25
Hexane fraction	< LOQ	1.33
Dichloromethane fraction	0.50	2.72
Ethyl acetate fraction	16.57	6.83
Aqueous fraction	1.06	0.91
Ethyl acetate fraction rich in tannin	3.43	2.72
Aqueous fraction rich in tannin	1.47	1.05

GA: gallic acid; EA: ellagic acid; < LOQ: less than limit of quantification.

The concentrations of GA and EA did not show significant differences during the months in which the samples were collected. As for the climatic relationships and the GA and EA content, through correlation analysis, the total GA content ($r = -0.702$, $p = 0.01$) and EA ($r = -0.669$, $p = 0.01$) had a strong inverse or negative relationship with a maximum temperature. These data corroborate with Rezende *et al.*[36] in the leaves of *Syzygium jambos* (L.) Alston, collected in Rio Verde and Nova América, Goiás, in which tannins had a negative correlation with the temperature. The GA ($r = 0.563$, $p = 0.05$) showed a moderate positive correlation with moisture. That is, they are directly correlated.

The drop in the concentration of GA and EA from April and July to October may be related to the low rainfall that is related to low humidity, corroborated by data from Santos *et al.*[37] in which the months with lower rainfall had lower levels of phenols and tannins condensed in the bark of "*barbatimão*" species. In a study with *E. uniflora* leaves, Santos *et al.*[38] concluded that in the dry season (May to October) there is an increase in the amount of phenols and flavonoids. In the rainy season (November to March), the hydrolysable tannins increase. This corroborates with this work where there is a greater amount of GA and AE in the months of greater humidity. Seasonal effects have a direct influence on the production of secondary metabolites in plants, such as period, time and method of plant collection, drying and storage of the sample, soil and nutrients, water stress, climatic factors (temperature, humidity, precipitation, insolation), geographic, ecological, physiological and genetic may affect qualitatively and quantitatively the active constituents during the year.[37-41] The high-performance liquid chromatography method developed in this work presented itself as a highly accurate instrument used to identify and quantify the compounds GA, and EA, and standardize the methanol extract of *E. punicifolia* leaves. There was no significant qualitative variation in the profile of secondary metabolites during the period analyzed, which is essential for the standardization of plant extracts and quality control of herbal products.

Phenolic compounds have more affinity with organic solvents such as methanol, ethanol, and aqueous solutions with acetone. Santos *et al.*[14] found that ethanol and methanol at 100% were the best solvents for extracting phenolic compounds from *E. punicifolia* leaves. In the liquid-liquid partition of CEE from *E. punicifolia* leaves, to separate the secondary metabolites according to their polarities, FrAc was the most concentrated of AG and EA, which corroborates with Cechinel Filho and Yunes,[42] in which the phenolic compounds in general, tannins and flavonoids are more retained in the extraction with ethyl acetate. This fact is related to the greater interaction between the hydrophilic portion of ethyl acetate with the hydroxyls present in GA and EA, which promotes greater retention of these compounds in this solvent, due to its moderate solubility in water and poor solubility in non-polar solvents.[43-45] Similar results were found by Falcão *et al.*[46] from *E. uniflora* leaves, which presented higher GA and EA content in the ethyl acetate fraction (0.872 and 0.323%, respectively), than in the crude ethanol extract (0.459 and 0.200%) and aqueous fraction (0.328 and 0.035%). Bezerra *et al.*[18] found that ethyl acetate fraction had higher concentrations of GA (0.899%) and EA (0.323%), followed by hydro ketone extract (0.459 and 0.200%), aqueous fraction (0.365 and 0.035%) and hexane fraction (0.058 and 0.060%, respectively) of *E. uniflora* leaves. These results suggest that FrAc has a higher content of phenolic compounds in general, including hydrolyzable tannins, ellagitannins, and flavonoids, and may have greater biological effects related to these metabolites.

There are no studies on the quantification of these markers in extracts and fractions of *E. punicifolia*. Hydro ethanol and ethanol extracts and aqueous solutions with acetone are traditionally used to extract phenolic compounds, with ethanol being the most used due to its low toxicity.[14,47] In addition, hydro ethanol solutions are similar to Brazilian medicinal preparations.[48] The CEE was extracted from samples collected in December 2016, a time correlated with higher levels of GA and EA.

Conclusions

The HPLC-DAD analytical method developed for the quantification of GA and EA was validated and demonstrated

to be selective, linear, precise, accurate, robust, and without matrix effect, being useful for the analysis of these tannins in extracts from the leaves of *E. punicifoila*, as from the crude ethanol extract and fractions. Thus, the results may be useful for the quality assessment and standardization of the species' raw materials.

Supplementary Information

Supplementary information (Figures S1-S4, Tables S1-S3) is available free of charge at http://jbcs.sbq.org.br as PDF file.

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Author Contributions

Liliane de S. Silva was responsible for conceptualization, data curation, formal analysis, investigation, methodology, writing-review and editing, writing-original draft; Matheus G. de Oliveira for implementation of the experiment; Christiane F. Martins for implementation of the experiment, writing-review and editing; Leonardo L. Borges for statistical analysis; Tatiana de S. Fiuza for writing-review and editing, Edemilson C. da Conceição for review and editing, José Realino de Paula for professor, conceptualization, funding acquisition, investigation, project administration, resources, supervision, writing-original draft, writing- review and editing.

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