Article

Journal of the Brazilian Chemical Society

https://dx.doi.org/10.21577/0103-5053.20230147

J. Braz. Chem. Soc. **2024**, *35*, 3, e-20230147, 1-15

Development and Validation of Analytical Method by HPLC-PDA and Seasonality from Gallic Acid, Catechin, and Epicatechin in Leaf Extracts from *Campomanesia adamantium*

Raphael O. Cabral,[a] Leonardo L. Borges,[b,c] Matheus G. de Oliveira,[a] Camila A. Romano,[a] Tatiana S. Fiuza[a,d] and José R. de Paula *,[a]

[a]*Faculdade de Farmácia, Universidade Federal de Goiás, 74605-170 Goiânia-GO, Brazil*

[b]*Câmpus Central, Universidade Estadual de Goiás, 75132-903 Anápolis-GO, Brazil*

[c]*Escola de Ciências Médicas e da Vida, Pontifícia Universidade Católica de Goiás, 74605-050 Goiânia-GO, Brazil*

[d]*Instituto de Ciências Biológicas, Universidade Federal de Goiás, 74690-900 Goiânia-GO, Brazil*

Campomanesia adamantium is native of the Brazilian Cerrado, and its leaves are popularly used as anti-inflammatory, antidiarrheal, and antiseptic in the urinary tract. This study aimed to develop and validate an analytical method by high-performance liquid chromatography for the simultaneous quantification and evaluation of the seasonal variability of gallic acid, catechin, and epicatechin, during 12 months, in *C. adamantium* leaf extracts. Chromatographic separations were performed with a mobile phase gradient of acetonitrile and 0.05% (v/v) trifluoroacetic acid solution, flow rate of 1.0 mL min^{-1}, detection at 210 nm, C18 column (150 × 4.6 mm, 5 µm) and column oven temperature of 35 °C. The analytical method developed was selective, linear, precise, accurate, robust, and without matrix effect. The parameters obtained in the present study meet the requirements established by national and international guidelines. The best time for leaves harvesting, with the highest levels reached, was in September (0.0626%, m/v) and August (0.044%, m/v) for gallic acid, in June (0.3953%, m/v) and July (0.3804%, m/v) for catechin, and from May (0.1622%, m/v) and June (0.1415%, m/v) for epicatechin, in the dry season. Therefore, this study contributes by providing parameters for quality control of the raw material *C. adamantium*.

Keywords: Cerrado, chemical markers, gabiroba, quality control, seasonal variability, ultrasound-assisted extraction

Introduction

Campomanesia adamantium (Cambess.) O. Berg, known as "*gabiroba*" or "*guavira*", belongs to the Myrtaceae family. It is a native species of the Cerrado distributed in several regions of Brazil and other adjacent countries, such as Argentina and Paraguay.[1-3] *C. adamantium* produces edible fruits, well appreciated due to the pleasant aroma and slightly sweet taste, used in various food products such as jams, jellies, juices, and liqueurs.[4] The *C. adamantium* leaves are used in traditional medicine as an anti-inflammatory, antidiarrheal, and antiseptic of the urinary tract.[2]

Previous studies have demonstrated several biological activities of the leaves, attributed to the identified bioactive compounds. Coutinho *et al.*[5] isolated five flavanones (7-hydroxy-5-methoxy-flavanone, 7-hydroxy-5-methoxy-6-methylflavanone, 5,7-dihydroxy-6-methylflavanone, 5,7-dihydroxy-8-methylflavanone and 5,7-dihydroxy-6,8-dimethylflavanone) and four chalcones (2',4'-dihydroxy-6'-methoxychalcone, 2',4'-dihydroxy-6'-methoxy-5'-methylchalcone, 2',4'-dihydroxy-6'-methoxy-3'-methylchalcone and 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone) from the methanol extract of the leaves, which showed antioxidant activities. Pascoal *et al.*[6] also reported antioxidant activities of the ethanol extract of leaves, where they identified isoquercitrin, myricetin, quercitrin, quercetin, 2',4'-dihydroxy-6'-methoxychalcone, 2',4'-dihydroxy-5'-methyl-6'-methoxychalcone and

*e-mail: jose_realino@ufg.br
Editor handled this article: Eduardo Carasek

2',4'-dihydroxy-3',5'-dimethyl-6'-methoxychalcone. Ferreira *et al.*[7] demonstrated the *in vitro* and *in vivo* anti-inflammatory and antinociceptive activities of leaves in aqueous and ethyl acetate fractions, which presented isolated flavonoids (quercetin, myricetin, and myricitrin). Pascoal *et al.*[8] identified several compounds, such as gallic acid, cardamonin, 2',4'-dihydroxy-5'-methyl-6'-methoxychalcone, quercetin, quercitrin, and isoquercitrin, which may be responsible for the antiproliferative activities of ethanol extract and fractions from *C. adamantium* leaves.

Gallic acid (GA) (Figure 1) is derived from benzoic acid of the phenolic acid class, is a precursor of hydrolysable tannins, and has a series of biological activities of therapeutic interest, such as anticancer, antiulcerogenic, antimicrobial, anti-inflammatory, and antioxidant.[9-11]

Catechins are flavan-3-ols from the flavonoid family and precursors of condensed tannins.[9] These phytocompounds have chiral centers that allow the formation of isomers such as catechin (CC) (Figure 1) and epicatechin (EC) (Figure 1), and have diverse biological activities such as antimicrobial, anticarcinogenic, anti-inflammatory, antioxidant, antimutagenic and antiproliferative.[12]

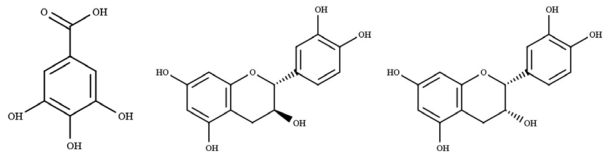

Figure 1. Molecular structures of compounds: (**1**) gallic acid ($C_7H_6O_5$), (**2**) *trans*-(+)-catechin ($C_{15}H_{14}O_6$), and (**3**) *cis*-(–)-epicatechin ($C_{15}H_{14}O_6$).

Several techniques can identify and quantify phenolic compounds in products of plant origin. High-performance liquid chromatography (HPLC) is used most in analytical methods due to its remarkable sensitivity, selectivity, and resolution.[12-14]

For implementing a non-compendial analytical method in a quality control laboratory for plant materials such as *C. adamantium*, the development and subsequent validation of the technique is necessary, according to the parameters required by the regulatory agencies, considering the operational conditions of the laboratory. Analytical validation measures the method's ability to provide reliable, precise, reproducible, and fit for purpose results.[15,16]

The chemical composition of *C. adamantium* extract may change over time due to the influence of the environment, such as seasonal and daily variations, as well as intra and inter-plant distinctions. The concentrations of secondary metabolites (GA, CC, and EC) can be influenced by biotic and abiotic factors, which involve biochemical, physiological, ecological, and evolutionary.[17]

Seeking to obtain a simple, efficient, fast, and low-cost method to be applied in the standardization of markers in the extract, this work aimed to: develop and, for the first time, validate an analytical approach by HPLC coupled to a photodiode array detector (PDA) for the simultaneous quantification of gallic acid, catechin, and epicatechin markers in *C. adamantium* leaves extracts, employing ultrasound-assisted extraction and to apply the method in the study of seasonal chemical variability for 12 months.

Experimental

Chemical reagents and solvents

Acetonitrile (J.T. Baker, Pennsylvania, USA), methanol (J.T. Baker, Pennsylvania, USA), 99.8% (v/v) ethanol (Neon, Suzano, Brazil), and the ultrapure water obtained through the Milli-Q filtration system (Merck Millipore, Molsheim, France) were used in the preparation of standard, sample, and mobile phase solutions. Acetic acid 99% (v/v) (Sigma-Aldrich, Saint Louis, USA), formic acid 98% (v/v) (Sigma-Aldrich, Saint Louis, USA), orthophosphoric acid 85% (m/v) (Sigma-Aldrich, Saint Louis, USA), and trifluoroacetic acid 99% (v/v) (Sigma-Aldrich, Saint Louis, USA) were used as acidifying agents for the mobile phase. In addition, caffeic acid (98%) (Sigma-Aldrich, Saint Louis, USA), chlorogenic acid (95%) (Sigma-Aldrich, Saint Louis, USA), ellagic acid (95%) (Sigma-Aldrich, Saint Louis, USA), gallic acid (98%) (Sigma-Aldrich, Saint Louis, USA), *p*-coumaric acid (98%) (Sigma-Aldrich, Saint Louis, USA), rosmarinic acid (98%) (Sigma-Aldrich, Saint Louis, USA), caffeine (99%) (Sigma-Aldrich, Saint Louis, USA), kaempferol (90%) (Sigma-Aldrich, Saint Louis, USA), catechin (96%) (Sigma-Aldrich, Saint Louis, USA), epicatechin (90%) (Sigma-Aldrich, Saint Louis, USA), hesperidin (80%) (Sigma-Aldrich, Saint Louis, USA), naringin (95%) (Sigma-Aldrich, Saint Louis, USA), quercetin (95%) (Sigma-Aldrich, Saint Louis, USA), rutin (94%) (Sigma-Aldrich, Saint Louis, USA), were used as analytical grade reference standards.

Botanical material

The leaves of ten individuals of *Campomanesia adamantium* were collected monthly (February 2015 to January 2016), in the morning period, in the city of Bela Vista, Goiás, Brazil (847 m, 17º02'01.1"S and 48º49'00.3"W). Dr José Realino de Paula identified the plant material, and a voucher specimen was deposited in the Herbarium of the Federal

University of Goiás (UFG), Conservation Unit/Pro-Rectory of Research and Innovation, under the code number UFG-243832. The leaves were desiccated at 40 °C in an air circulation oven, model 171 (Fabbe-Primar, São Paulo, Brazil), and crushed in a Willye-type knife mill (Tecnal, Piracicaba, Brazil). The powder was stored in containers able to provide protection from moisture and light, under refrigeration to –18 °C, in DA550 freezer (Metalfrio, Três Lagoas, Brazil), for further analysis.

In developing and validating the analytical method and evaluating seasonal variations, the extract of *C. adamantium* was prepared by the solid-liquid extraction process. 170 mg of the dried leaves powder were extracted in 10 mL of methanol, obtaining a concentration of 17 mg mL^{-1} (100%). All extractions were performed in an Ultronique Q5.9L ultrasonic washer (Indaiatuba, Brazil) for 30 min at 25 °C, with a frequency of 40 kHz. Before injection into the chromatograph, all prepared solutions were filtered through a 0.45 μm pore polytetrafluoroethylene syringe filter (Allcrom, Itajaí, Brazil).

Identification of chemical constituents present in *C. adamantium* extract

The phytochemical investigation of possible constituents to be evaluated in the chromatographic profile of the extract was carried out from literature data on the species,[7,18,19] in a Waters Alliance e2695 High-Performance Liquid Chromatography system (Milford, USA), equipped with a quaternary pump, 2998 diode array detector, and Empower™ data processing software (version 2.0).[20]

The chromatographic conditions for qualitative evaluation of the profile used acetonitrile (A) and ultrapure water (B) as mobile phases, both acidified to 0.05% (v/v) with formic acid, following the elution gradient: 0 min 100% B phase, 5 min 95% B phase, 15 min 90% B phase, 25 min 85% phase B, 35 min 80% phase B, and 50 min 80% phase B. The column used was a Zorbax Eclipse C18 (Agilent, Santa Clara, USA) reversed-phase column (250 × 4.6 mm, 5 μm) at an oven temperature of 35 °C, a flow rate of 1.0 mL min^{-1}, a wavelength (λ) of 210, 254, 327, and 366 nm, and injection volume of 10 μL.[21]

The identification of compounds was performed by comparing the peaks of the extract constituents with the peaks of the analyzed standards (caffeic acid, chlorogenic acid, ellagic acid, gallic acid, *p*-coumaric acid, rosmarinic acid, caffeine, kaempferol, catechin, epicatechin, hesperidin, naringin, quercetin, rutin) at a concentration of 50 μg mL^{-1} in methanol, concerning retention times and similarity of ultraviolet (UV) absorption spectra. Among the analyzed compounds, GA, CC, and EC were identified. They are chemical markers monitored throughout the study of this species.

HPLC-PDA method development

The chromatographic conditions to qualitatively and quantitatively evaluate GA, CC, and EC in the extracts were obtained after testing different methods reported in the literature and optimizing several of these conditions.[13,19,22,23] Chromatographic separations were tested using a Zorbax Eclipse C18 (Agilent, Santa Clara, USA) reversed-phase column (250 × 4.6 mm, 5 μm) and the XTerra (Waters™, Milford, USA) RP18 (150 × 4.6 mm, 5 μm), exploring an optimal separation between the constituents of the sample. The mobile phases tested were constituted by water, acetonitrile, or methanol, and with the addition of different acidifying agents (acetic acid, formic acid, orthophosphoric acid, and trifluoroacetic acid), through the isocratic elution mode and exploratory gradient, being previously filtered through a membrane 0.45 μm nylon (Allcrom, Itajaí, Brazil) and degassed in an ultrasonic washer. Solvent flow rates range from 0.5 to 1.0 mL min^{-1}, column oven temperature of 25, 30, and 35 °C, and λ of 210, 254, 265, 270, 271, and 280 nm. The injection volume of the chromatographic system was kept constant at 10 μL.

Suitability of the chromatographic system

Before carrying out the validation, we checked whether the chromatographic system is suitable for the analyses to be carried out and that it provided reproducible results. This test was performed using the average of three consecutive injections of GA, CC, and EC standards, and methanolic extracts of *C. adamantium* leaves, which were evaluated through the parameters of capacity factor (k'), resolution (Rs), tail factor (TF), number of plates (N) of the peaks of GA, CC, and EC, through the relative standard deviation (RSD), following the rules of the Food and Drug Administration.[24]

Method validation by HPLC-PDA

The analytical method validation followed the Agência Nacional de Vigilância Sanitária (Anvisa) rules[16,25,26] and international guidelines from the Association of Official Analytical Collaboration International and the International Conference on Harmonization.[27,28] The parameters recommended by the guidelines for the dosing assay were: selectivity, linearity, precision (repeatability and intermediate precision), accuracy, robustness, and matrix effect.

The selectivity was evaluated by comparing the chromatograms of the standard solutions, sample solutions, mobile phases, and blank (methanol) to verify the presence of possible interfering peaks by coelution. The UV spectral similarities of the GA, CC, and EC peaks in the standard solutions and the sample solution were also compared at a λ of 210 nm, and the purity of the peaks of interest was verified.

Linearity was determined by the analytical curves of the standards at seven concentration levels for GA (2.7; 7.7; 12.7; 17.7; 22.7; 27.7, and 32.7 μg mL^{-1}), CC (4.3; 14.3; 24.3; 34.3; 44.3; 54.3 and 64.3 μg mL^{-1}) and EC (7; 12; 17; 22; 27; 32 and 37 μg mL^{-1}) in methanol. Analysis was performed in triplicate for each concentration level, and the calibration curve was fitted by linear regression from the correlation between peak areas and standard concentration. Pearson correlation coefficients (R) and determination (R^2) of linear regression, analysis of variance (ANOVA), and residuals were calculated, taking into account a significance level of 5%.

Repeatability was performed through nine determinations of the samples, considering the linear range of the method, at three levels of concentration: low (80%: 4.68; 35.62; 8.83 μg mL^{-1}), medium (100%: 5.87; 45.49; 11.26 μg mL^{-1}) and high (120%: 7.13; 54.93; 13.76 μg mL^{-1}) for GA, CC, and EC in the extract, respectively, with triplicate at each level, evaluating the RSD of the results. The intermediate precision was verified through the proximity of the results through the RSD, in two days of analysis, under the same sample preparation conditions and by different analysts. For accuracy, the sample solutions were prepared at three concentration levels 80, 100, and 120%, in triplicate, covering the linear range, with and without the addition of a known concentration of the GA standard (2.53 μg mL^{-1}), of CC (14.05 μg mL^{-1}) and EC (6.8 μg mL^{-1}). This parameter was determined by the recovery capacity of the analyte, according to equation 1:

$$\text{Recovery}(\%) = \frac{(\text{Experimental mean concentration})}{(\text{Theoretical concentration})} \times 100 \quad (1)$$

The robustness was verified by injecting sample solutions at the level of 100%, in triplicate, under different chromatographic conditions: column oven temperature (34 and 36 °C), hydrogenic potential (pH) of the mobile phase (2.5 and 2.7), and mobile phase flow rate (0.9 and 1.1 mL min^{-1}). In addition, the results of the peak areas and the contents of GA, CC, and EC in each factor of method alteration were compared to the results of the original condition, evaluated by the RSD.

The effect of matrix components on the analytical response was determined by comparing the slopes of the GA calibration curves (4.1; 5; 5.9; 6.8; 7.7 μg mL^{-1}), CC (32; 38, 8; 45.7; 52.6; 59.4 μg mL^{-1}) and EC (6.2; 7.6; 8.9; 10.2; 11.6 μg mL^{-1}) in methanol, with the solutions of extract sample by 80%, added with each concentration of the analytical curve of each analyzed marker, being performed in triplicate. Proof of the absence of a matrix effect was demonstrated by the parallelism of the curve lines and confirmed using the *F* ANOVA test and the application of the *t*-test, considering the significance level of 5%. The calculations of the validation parameters were performed using the statistical software Action Stat® (version 3.7)[29] and Microsoft® Excel (version 2016).[30] The graphs of the tests were treated with the aid of the software Origin® (version 6.0).[31]

Application of the analytical method in the evaluation of the seasonal variations of chemical markers

The evaluation of qualitative and quantitative variations of chemical markers (GA, CC, and EC) in the composition of the methanolic extract of *C. adamantium* leaves during 12 months of the collection was performed in analytical triplicate, based on the chromatographic profile of the matrix, through the method validated through the HPLC system. In addition, the meteorological data (maximum and minimum temperature, pluviometric index, and relative humidity of the air) of the collection period were obtained in the climatic database of the National Institute of Meteorology,[32] from the Goiânia station, Goiás, Brazil, under the code 83423 of the World Meteorological Organization.

Pearson's linear correlation analysis was performed to verify the association level between chemical markers and environmental variables to investigate the seasonal behavior of the constituents identified.

Principal component analysis (PCA) was applied to evaluate the possible interrelationships between the compounds found in the methanolic extract of the leaves collected in different months over 1 year. Hierarchical cluster analysis (HCA) was used to study the similarity of samples based on the distribution of constituents, using Ward's method of minimum variance.[33] To validate the classification proposed by the HCA, canonical discriminant analysis (CDA) and a partial least squares (PLS) analysis were used to identify the components that differ between the groups and detect the standard distribution of the samples. Results were considered significant for $p < 0.05$, and in some instances, $p < 0.1$, and all statistical analyzes were performed using the Statistica® software (version 7.0) and program Past (version 4.13).[34-36]

Results and Discussion

HPLC-PDA method development

Preliminary tests were performed to select the most suitable chromatographic conditions for the analytical method to quantify GA, CC, and EC identified in the *C. adamantium* extracts. The similarity of the chemical structure of CC and EC, as they are isomers, reflects the difficulty in separating the peaks in the extract. Therefore, it was necessary to reduce the interaction between the free hydroxyl groups and the stationary phase through acidification of the mobile phase because catechins are more stable in an acidic medium, as reported by Albuquerque.[37] In addition to acidity, it prevents the ionization of hydroxyl groups in phenolic compounds, allowing for better separation.[38]

The mobile phase of the study by Bezerra,[22] specifics for GA and CC, was composed of a solution of 0.05% trifluoroacetic acid and methanol in an isocratic system in the proportions 88:12 (v/v). This study was the base for the development of the method because, among the tested mobile phases, it was the one that presented a satisfactory separation and a suitable symmetry between the analyzed peaks.

Trifluoroacetic acid is a stronger organic acid compared to acetic acid.[22] Considering this, the choice of mobile phase pH was based on the acid dissociation constant (pK_a) of the analyzed markers, such as the GA pK_a of 4.40, the CC of 8.68, and the EC of 8.91.[11,39] The pH of the mobile phase must be below the ionization constant, as the increase in the ionized form of the analyte increases its dissolution in the aqueous phase and reduces its retention time (t_R) since the ionic form can pass through the column without retention.[40]

Phenols have chromophore groups that absorb in the UV region; catechins and gallic acid absorption were considered maximum at λ of 210-280 nm.[23] The wavelengths evaluated were 210, 254, 265, 270, 271, and 280 nm. However, it showed better absorbance of chemical markers at 210 nm, with increased sensitivity of detection of chromatographic bands and improvement in baseline and signal-to-noise ratio.

The selection of the chromatographic column occurred through the evaluation under the same conditions for two columns, the Zorbax Eclipse C18 (250 × 4.6 mm, 5 µm) and the XTerra RP18 (150 × 4.6 mm, 5 µm). Among the various methods tested, Zorbax Eclipse C18 presented a chromatographic elution analysis time of approximately 30 min. On the other hand, XTerra RP18 obtained a time of roughly 26 min. That is, there was a little faster separation of the peaks in this column. In addition, it can be seen that the decrease in column length reduced the consumption of solvents and provided a satisfactory separation of the analyzed compounds. Hence, the column chosen for the method was XTerra RP18.

However, the simple decrease in column length causes a loss in efficiency, expressed by the number of plates in each sample peak. Strategies to reduce analysis time while maintaining separation efficiency occur by increasing the average linear velocity of the mobile phase, which was tested at various flow rates from 0.5 to 1.0 mL min^{-1}. Still, due to this factor, the adequate flow rate was 1 mL min^{-1}. Another alternative was to increase the temperature of the column oven in which they were evaluated at 25, 30, and 35 °C. The selected temperature was 35 °C, which increases the solubility of the analyte in the mobile phase, reducing the total elution time and consequently improving the column performance.[41]

Therefore, after testing different methods reported in the literature and optimizing several of these conditions,[13,19,22,23] an efficient approach can be achieved with adequate separation capacity and chromatographic bands in gaussian format. The conditions obtained were acetonitrile mobile phase (A) and 0.05% (v/v) trifluoroacetic acid solution (B), with the following elution gradient: 0 min 100% B phase, 10 min 88% phase B, 20 min 88% B phase, 22 min 90% phase B and 26 min 100% phase B. The column selected was the XTerra RP18 (150 × 4.6 mm, 5 µm), with an oven temperature of 35 °C, a flow rate of 1.0 mL min^{-1}, a λ of 210 nm, and an injection volume of 10 µL.

Suitability of the chromatographic system

The system suitability parameters of GA, CC, and EC peaks in the standards and methanolic extracts of *C. adamantium* leaves follow the rules Food and Drug Administration,[24] expressed as mean values of three determinations (± RSD) (Table 1). The results ensure that the chromatographic method was adequate to separate and quantify the markers.

Method validation by HPLC-PDA

In the selectivity, the chromatographic profiles of the reference standards (GA, CC, and EC), the *C. adamantium* extract, the diluent (methanol), and the mobile phase solvents (acetonitrile and 0.05% trifluoroacetic acid solution) were obtained by HPLC-PDA. With no evidence of substances interfering in the retention time of GA, CC, and EC, even using a λ of 210 nm, an absorption region encompassing many compounds. Furthermore, the purity of the peaks of interest was evaluated by the Empower™ 20

Table 1. Mean data of triplicates of the suitability parameters of the chromatographic system, obtained from the gallic acid, catechin, and epicatechin peaks in the standards and methanolic extracts of *C. adamantium* leaves

Sample	System suitability parameters			
	k'	TF	Rs	N
GA standard (0.0327 mg mL^{-1})	2.9 (0.34)	1.5 (0.95)	–[a]	4,219.96 (1.39)
GA peak in the extract (17 mg mL^{-1})	2.88 (0.21)	1.11 (1.41)	–[a]	4,531.32 (0.63)
CC standard (0.0443 mg mL^{-1})	7.29 (0.17)	1.13 (0.43)	–[a]	16,437.27 (1.78)
CC peak in the extract (17 mg mL^{-1})	7.25 (0.07)	1.06 (0.77)	17.82 (1.14)	18,887.7 (1.97)
EC standard (0.012 mg mL^{-1})	8.94 (0.04)	1.2 (2.31)	–[a]	14,917.74 (1.08)
EC peak in extract (17 mg mL^{-1})	8.74 (0.10)	0.99 (1.77)	5.64 (0.92)	19,665.56 (1.77)
Literature specifications (US-FDA)[24]	> 2	≤ 2	> 2	> 2000

[a]Resolution evaluates the ability to separate two consecutive peaks, but some samples that were analyzed did not present conditions to obtain this parameter. Values in parenthesis are the relative standard deviation percentage (RSD / %); k': capacity factor; TF: tail factor; Rs: resolution; N: number of dishes; GA: gallic acid; CC: catechin; EC: epicatechin; US-FDA: United States Food and Drug Administration.

chromatography software, in which it was found that the purity angles of the GA, CC, and EC peaks were smaller than the purity thresholds, so there was no coelution in the threshold range that determines the noise effect. In addition, another factor that confirmed the purity was the UV spectra of the samples' markers, which were similar to those of the standards, demonstrating the selectivity of the method (Figure 2).

Linearity was verified by linear regression in the GA calibration curves, in the range of 2.7-32.7 μg mL^{-1}, presenting an equation of the straight line of $y = 82{,}237x - 13{,}007$ ($R^2 = 0.9988$; R = 0.9994), CC in the range of 4.3-64.3 μg mL^{-1}, obtaining an equation of $y = 92{,}871x - 78{,}827$ ($R^2 = 0.999$; R = 0.9995), and EC in the range of 7-37 μg mL^{-1}, demonstrating an equation of $y = 101{,}595x - 9{,}762.2$ ($R^2 = 0.9988$; R = 0.9994). The correlation coefficient must be above 0.99. The higher the value, the stronger the correlation between the two variables (R^2 and R), and the more realistic the proposed model, estimated by the ordinary least squares method. The coefficients were found to follow the specifications.[25]

The investigation of homoscedasticity by the Cochran test of the GA presented a calculated *C* of 0.361. At the same time, the CC obtained a calculated *C* of 0.3459, and the EC was 0.5386. All values were below the critical *C* of 0.561, so the data presented are homoscedastic, with variances in the levels of constant concentration.[25]

The *F* ANOVA test analyzed the significance of the angular coefficient; for the GA, a calculated *F* of 11,551.61 was obtained, while for the CC, a calculated *F* of 16,835.86 was evidenced, and for the EC of 12,911.47. All values found were greater than the tabulated *F* of 4.38, indicating that the slope is significantly different from zero, demonstrating that the model was adequate to predict the data and the method can be considered linear.[25]

The residual analysis quantifies the distance between the actual and estimated values. When the error of the linear regression model is due only to the common variations of the study, it is expected that the residuals are independent and with a normal distribution.[25] Residual normality was analyzed using the Anderson-Darling test, with a *p*-value of 0.0842 for GA, a *p*-value of 0.1737 for CC, and a *p*-value of 0.0739 for EC. All these values were greater than the 0.05 significance level, demonstrating normal distributions. Independence was investigated using the Durbin-Watson test, with a *p*-value of 0.094 for GA, a *p*-value of 0.517 for CC, and a *p*-value of 0.4464 for EC. Furthermore, these values were higher than the 0.05 significance level, with no dependence on the observations. The results confirmed that it is a linear method that meets the guidelines of Brazilian legislation.[16,25]

Precision data (Table 2) were expressed by determining the RSD between the plant extract's triplicates of the low, medium, and high concentrations. For the repeatability parameter, 1.15% was obtained for GA, 1.33% for CC, and 1.39% for EC. In the intermediate precision, the RSD value was 0.89% for GA, 1.56% for CC, and 1.88% for EC. According to the Association of Official Analytical Collaboration International,[27] the acceptance criterion for precision must be defined through the concentration of the analyte in the matrix, with the RSD being appropriate for the concentration of GA and EC of up to 3.7% and the CC of up to 2.7%. However, higher RSD values (≤ 15%) can also be considered acceptable.[26] The results follow current specifications, being a precise method that presents proximity between a series of measurements obtained from multiple sampling of the same sample.[26]

The accuracy (Table 3) was evaluated by the recovery of the fortifications of the chemical markers in the extract, with the mean of the triplicates of each concentration

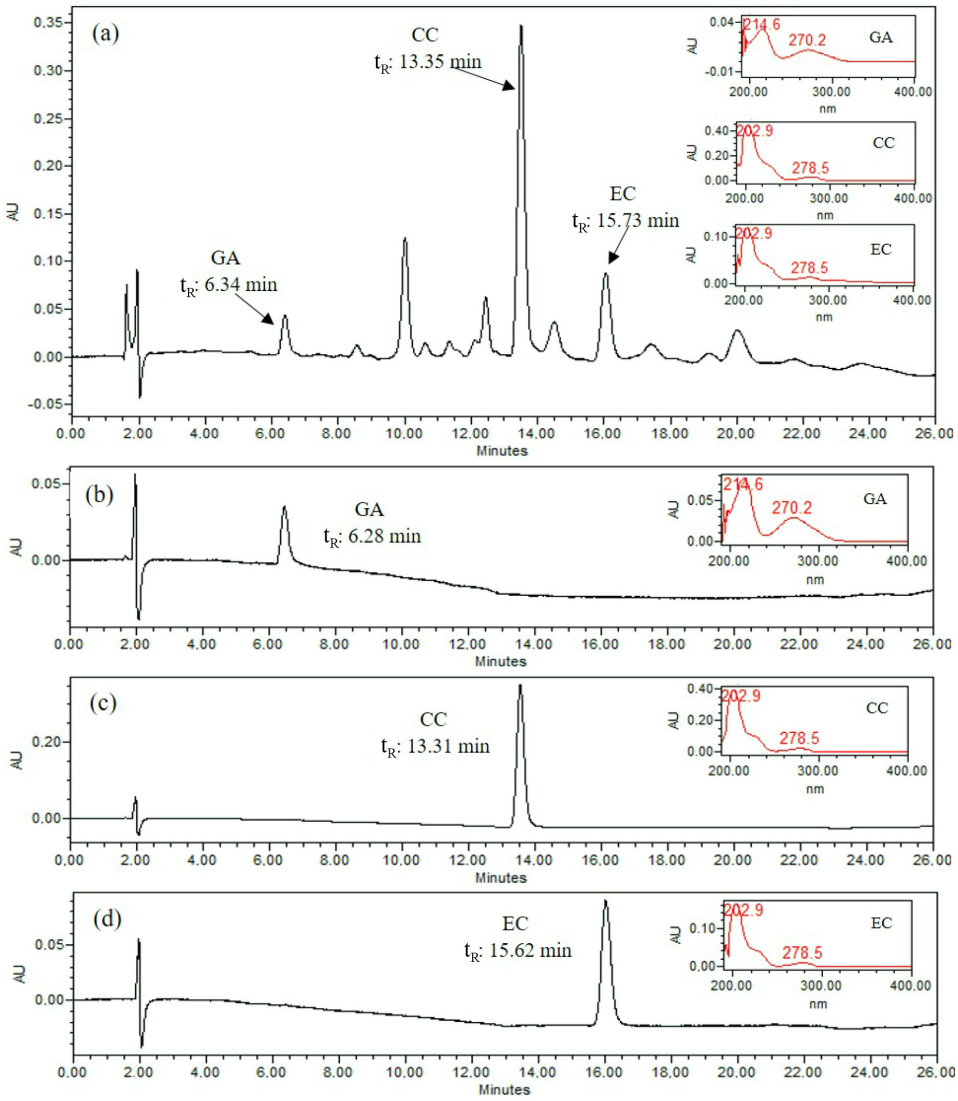

Figure 2. Chromatographic profiles and the respective ultraviolet spectra of gallic acid (GA), catechin (CC), and epicatechin (EC) at 210 nm were obtained by high-performance liquid chromatography in the validation of the analytical method by the Empower™ program.[20] (a) Sample of the methanolic extract of *C. adamantium* leaves (17 mg mL^{-1}); (b) GA standard (7.7 µg mL^{-1}); (c) CC standard (64.27 µg mL^{-1}); (d) EC standard (22.03 µg mL^{-1}); t_R: retention time. Chromatographic conditions: acetonitrile mobile phase (A) and 0.05% (v/v) trifluoroacetic acid solution (B), following elution gradient: 0 min 100% phase B, 10 min 88% phase B, 20 min 88% phase B, 22 min 90% phase B and 26 min 100% phase B. The column oven temperature was 35 °C, the flow rate of 1.0 mL min^{-1}, the wavelength of 210 nm, the injection volume of 10 µL, and the C18 column (150 × 4.6 mm, 5 µm).

analyzed ranging from 100.59 to 101.79%, with a mean of 101.31% and an RSD of 1.14% for GA, from 99.24 to 101.33%, with an average of 99.98% and RSD of 1.23% for CC and from 99.08 to 99.75%, with an average of 99.32% and RSD of 0.99% for the EC. According to the Association of Official Analytical Collaboration International,[27] the acceptance criterion for accuracy must be defined through the concentration of the analyte in the matrix, with the appropriate recovery interval for the concentration of GA and EC from 95 to 105% and CC from 97 to 103%. The results showed that the method was considered accurate due to the degree of agreement between the individual results of the technique under study concerning the value accepted as true.[16]

Robustness was analyzed using the RSD between the observed results of peak area and marker content obtained from the proposed original method and variations in the conditions of this method. The RSD values found in all markers (Table 4) were in agreement with the RSD recommended for the precision of this method.[27] The system suitability parameters of the chromatograms of the variations were within the recommended by the Food and Drug Administration.[24] The method was robust because it was able to withstand minor and deliberate variations in the analytical conditions.[16]

The absence of the matrix effect was observed by the parallelism between the concentration lines *versus* the analytical response of the GA, CC, and EC standards and

Table 2. Data from the mean of triplicates of the results obtained in the precision of the validation of the analytical method by high-performance liquid chromatography for quantification of gallic acid, catechin, and epicatechin in methanolic extracts from *C. adamantium* leaves

Precision	Sample concentration / (mg mL^{-1})	Area / ($\mu V\ s^{-1}$)	Marker concentration / (mg mL^{-1})	Content / %	RSD / %
Gallic acid					
Precision 1 day (analyst 1)-repeatability	13.6	369,864	0.0047	0.0342	1.15
	17	470,209	0.0059	0.0346	
	20.4	575,439	0.0072	0.0351	
Precision 2 day (analyst 2)	13.6	373,855	0.0047	0.0346	0.58
	17	469,261	0.0059	0.0345	
	20.4	571,891	0.0071	0.0349	
Intermediate precision				0.0346	0.89
Catechin					
Precision 1 day (analyst 1)-repeatability	13.6	3,241,675	0.0358	0.2629	1.33
	17	4,158,007	0.0456	0.2684	
	20.4	4,998,292	0.0547	0.2680	
Precision 2 day (analyst 2)	13.6	3,217,704	0.0355	0.2610	1.85
	17	4,134,409	0.0454	0.2669	
	20.4	5,046,474	0.0552	0.2705	
Intermediate precision				0.2663	1.56
Epicatechin					
Precision 1 day (analyst 1)-repeatability	13.6	887,435	0.0088	0.0649	1.39
	17	1,141,234	0.0113	0.0666	
	20.4	1,370,794	0.0136	0.0666	
Precision 2 day (analyst 2)	13.6	887,647	0.0088	0.0650	2.34
	17	1,128,032	0.0112	0.0659	
	20.4	1,405,561	0.0139	0.0683	
Intermediate precision				0.0662	1.88

RSD: relative standard deviation.

Table 3. Data from the mean of triplicates of the results obtained in the accuracy of the validation of the analytical method by high-performance liquid chromatography for the quantification of gallic acid, catechin, and epicatechin in methanolic extracts from *C. adamantium* leaves

Sample concentration / (mg mL^{-1})	Marker area in the sample / ($\mu V\ s^{-1}$)	Marker area in swatch + marker pattern / ($\mu V\ s^{-1}$)	Recovered marker pattern concentration / (mg mL^{-1})	Recovery / %	RSD / %
Gallic acid (theoretical concentration of standard GA added: 0.0025 mg mL^{-1})					
13.6	367,722	566,520	0.0026	101.79	1.14
17	477,382	675,633	0.0026	101.53	
20.4	573,835	770,133	0.0026	100.59	
Catechin (theoretical concentration of CC standard added: 0.0141 mg mL^{-1})					
13.6	3,232,637	4,448,419	0.0139	99.24	1.23
17	4,192,389	5,435,546	0.0142	101.33	
20.4	5,012,151	6,229,671	0.0140	99.37	
Epicatechin (theoretical concentration of EC pattern added: 0.0068 mg mL^{-1})					
13.6	882,714	1,558,156	0.0067	99.14	0.99
17	1,112,191	1,791,895	0.0068	99.75	
20.4	1,329,356	2,004,407	0.0067	99.08	

RSD: relative standard deviation; GA: gallic acid; CC: catechin; EC: epicatechin.

Table 4. Mean data (RSD, between the original method and variations in conditions) of the triplicates of the chromatographic parameters evaluated in the robustness of the validation of the analytical method by high-performance liquid chromatography for quantification of gallic acid, catechin, and epicatechin in methanolic extracts from *C. adamantium* leaves

Parameter	Area of GA / (μV s^{-1})	Area of CC / (μV s^{-1})	Area of EC / (μV s^{-1})	Content of GA / %	Content of CC / %	Content of EC / %
Original method	469,749	4,186,501	1,160,040	0.0345	0.2702	0.0677
Column oven temperature / °C						
34	468,180 (1.04)	4,115,517 (1.16)	1,167,272 (0.93)	0.0344 (1.01)	0.2657 (1.14)	0.0682 (0.92)
36	469,464 (0.72)	4,078,673 (1.57)	1,138,796 (1.17)	0.0345 (0.70)	0.2633 (1.54)	0.0665 (1.16)
Mobile phase pH						
2.5	485,307 (1.90)	4,137,986 (0.98)	1,232,848 (3.41)	0.0356 (1.85)	0.2671 (0.96)	0.0719 (3.38)
2.7	487,159 (2.11)	4,154,149 (1.01)	1,235,828 (3.49)	0.0358 (2.06)	0.2661 (0.99)	0.0721 (3.46)
Flow rate / (mL min^{-1})						
0.9	481,252 (1.52)	4,187,375 (0.65)	1,224,298 (3.01)	0.0354 (1.48)	0.2702 (0.64)	0.0715 (2.99)
1.1	479,405 (1.36)	4,101,216 (1.28)	1,223,608 (3.12)	0.0352 (1.32)	0.2648 (1.26)	0.0715 (3.09)

GA: gallic acid; CC: catechin; EC: epicatechin; RSD: relative standard deviation; pH: potential of hydrogen. Values in parenthesis are the relative standard deviation percentage (RSD / %).

the sample solution fortified with the standards (Figure 3). The *F* ANOVA test analyzed the significance of the angular coefficients of the estimated lines. GA had a calculated *F* of 5.1 for the standard and 8.28 for the fortified extract. CC showed a calculated *F* of 5.86 for the standard and 6.09 for the fortified extract. EC obtained a calculated *F* of 5.96 for the standard and 4.85 for the fortified extract. All values found were greater than the critical *F* of 4.67, which indicates that the slope is significantly different from zero.[16,25] The *t*-test was applied to the GA data, which presented a calculated *T* of 2, the CC test showed a calculated *T* of 1.01, and the EC test gave a calculated *T* of 1.98. All being lower than the critical *T* of 2.06, demonstrating that the angular coefficients are statistically similar and confirming that there is no significant matrix effect, indicating that the unidentified components of the matrix in which the marker is inserted do not interfere with the quantification of GA, CC, and EC.[16,25]

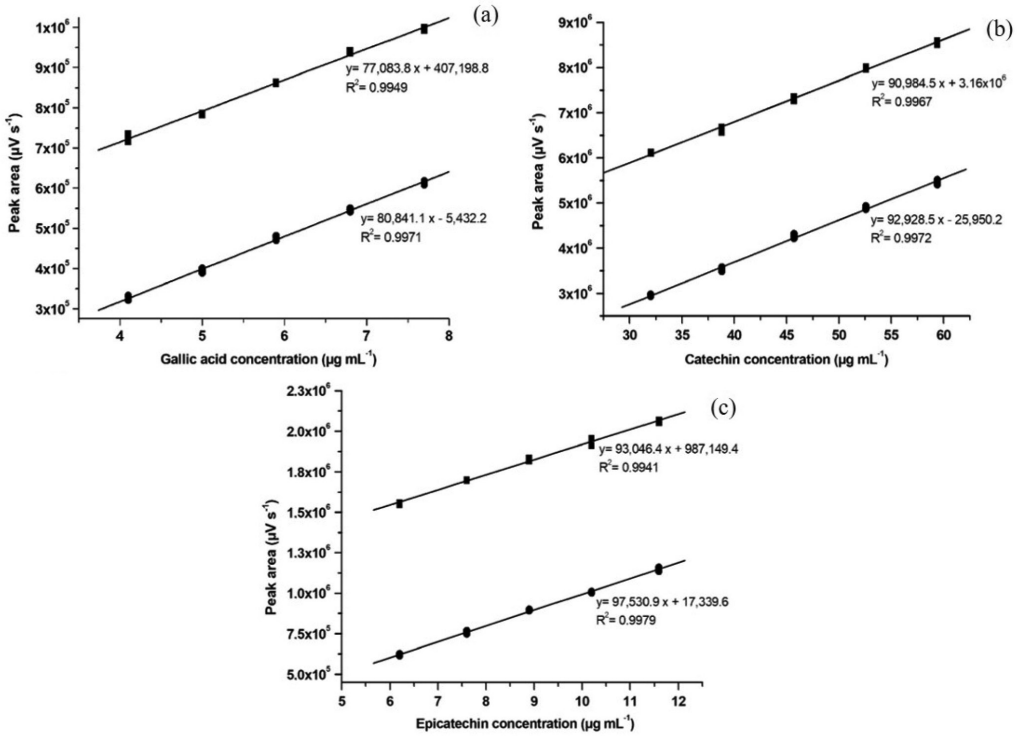

Figure 3. The parallelism between the lines, constructed with the pattern of gallic acid (a), catechin (b), epicatechin (c), represented by the line with ●, and the methanolic extract of *C. adamantium* leaves, fortified with the pattern of gallic acid (a), catechin (b), epicatechin (c), represented by the line with ■, concerning concentration × area, obtained by the Origin program.[31]

Application of the analytical method in the evaluation of seasonal variations of chemical markers

C. adamantium is native of the Cerrado, which has a predominantly tropical climate, with two well-defined seasons throughout the year, with rainfall rates that reflect the rainy and dry seasons.[42-44] Climatic data from Bela Vista of Goiás of the period in which *C. adamantium* leaves were collected are described in Table 5. The rainy season was from February to March 2015 and from November 2015 to January 2016, with values ranging from 155.1 to 484.8 mm, with the lowest rainfall in February and the highest in January, with average temperatures ranging from 21 to 33.9 °C. The dry period occurred from April to October 2015, with values ranging from 0 to 70.7 mm, reaching the lowest index in June and the highest in May, with temperatures ranging from 16.7 to 36.7 °C. The same behavior was observed for relative humidity, with higher humidity in the rainy season (January) and lower in the dry season (August), with values of 80.2 and 38.4%, respectively.

The biosynthesis of secondary metabolites in plant species can be influenced by endogenous factors such as genotype and physiological conditions and exogenous factors that reflect the biotic and abiotic environmental stimuli that occur during the growth period of the plant, including feeding on phytophagous insects or animals herbivores, other factors such as light and water availability, soil composition, temperature and interaction with pathogens and parasites.[45] It was observed in the present study that most of the phytochemical profiles obtained by HPLC were similar in terms of chemical composition throughout the seasons, with the constituents being compromised only in October, in which it was impossible to detect EC. However, the profiles were significantly different in quantity (Figure 4).

Figure 5 shows the seasonal variations of the averages of GA, CC, and EC contents in the methanolic extract of *C. adamantium* leaves from February 2015 to January 2016, ranging from 0.0313 to 0.0626% (m/v), from 0.1599 to 0.3953% (m/v) and from 0 to 0.1622% (m/v), respectively. The lowest levels of GA, CC, and EC were obtained at the end of the drought in October with the lowest rates and at

Table 5. Climatic data during the period of collection of *C. adamantium* leaves in Bela Vista of Goiás

Date	Total rainfall / mm	Relative humidity / %	Average temperature / °C	
			Maximum	Minimum
02/20/2015	155.1	68.9	33.2	21
03/31/2015	156.2	67.8	32.3	21.2
04/30/2015	1.3	50.3	33.4	20.2
05/31/2015	70.7	66	29.7	18.4
06/30/2015	0	56.3	30.2	17
07/31/2015	2.7	50.9	31.4	16.7
08/31/2015	3.6	38.4	33.3	17.5
09/30/2015	30.4	42.5	30.4	20.3
10/31/2015	18.2	43.5	36.7	22.1
11/30/2015	354.8	63.9	33.9	21
12/31/2015	207.7	66	33	21.2
01/31/2016	484.8	80.2	29.8	21

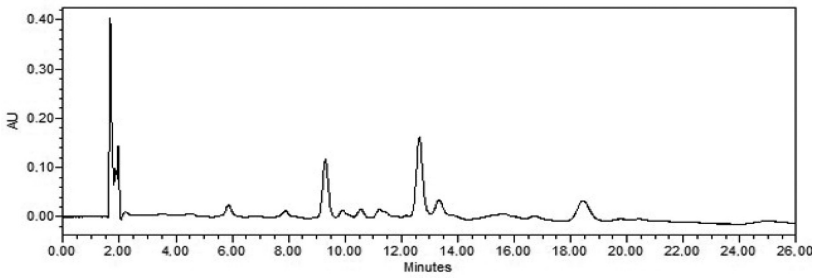

Figure 4. Chromatographic profile of the methanolic extract of *C. adamantium* leaves (17 mg mL^{-1}), collected in October 2015, showing the absence of the epicatechin compound, obtained by high-performance liquid chromatography in the Empower™ program.[20] Chromatographic conditions: acetonitrile mobile phase (A) and 0.05% (v/v) trifluoroacetic acid solution (B), following elution gradient: 0 min 100% phase B, 10 min 88% phase B, 20 min 88% phase B, 22 min 90% phase B and 26 min 100% phase B. The column oven temperature was 35 °C, the flow rate 1.0 mL min^{-1}, the wavelength 210 nm, the injection volume 10 µL, and C18 column (150 × 4.6 mm, 5 µm).

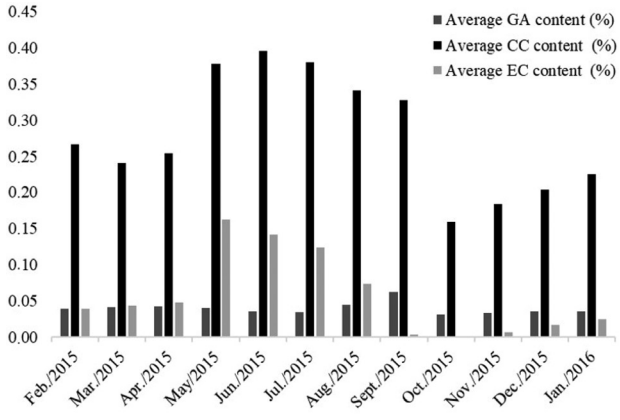

Figure 5. Seasonal variations in the mean contents of triplicates of gallic acid, catechin, and epicatechin in methanolic extracts from *C. adamantium* leaves collected from February 2015 to January 2016; the graph was obtained by the Microsoft Excel program.[30]

the beginning of the rainfall in November with 0.0336 and 0.1839% (m/v) for GA and CC; however, EC also showed a lower content in September of 0.0035% (m/v) near the end of the drought (Table 6).

Primary and secondary metabolites can share the same precursors and intermediates, which results in competition for common substrates in phenolic biosynthesis and growth processes.[45] In the present study, there was a decrease in the production of markers in the flowering and early fruiting stages of the plant species (September to November), suggesting that leaf photosynthates were allocated with greater contribution to the developing reproductive organs, which seek a rapid fruit growth. This is due to a more intense selective pressure in this region, which required subsidies of nutrients such as carbon and nitrogen, with the production of secondary metabolites being inversely related to plant growth.[45,46] Cao *et al.*[45] reported that the leaves of *Cyclocarya paliurus* showed similar behavior, with a reduced content of phenolic compounds in the vegetative and reproductive growth period of the species.

The maximum levels reached (Table 6) for GA were 0.044% (m/v) in August and 0.0626% (m/v) in September; for CC, it was 0.3804% (m/v) in July and 0.3953% (m/v) in June and for EC it was 0.1415% (m/v) in June and 0.1622% (m/v) in May. The catechins showed similarity in the months of higher levels, but each marker of the species in question showed peculiarities. The average content of GA, CC, and EC in the months of the best collection showed an increase in yield of 80.93, 48.44 and 145.02%, respectively, concerning the average content obtained in the validation of the analytical method, demonstrating the impact that seasonality provides on marker concentrations.

The highest levels of marker levels were obtained in the dry period. Therefore, there may be a need for adaptation of the species to the environment through strategies that minimize dehydration during this period, such as the readjustment of its physiology through the reduction in stomatal conductance associated with the drop in leaf water potential and induction of abscission of leaves at the end of this season. These factors contributed to maintaining the photosynthetic performance and the continuous synthesis of markers in this species during this period.[44]

Pearson's linear correlation was used to establish the association level between GA, CC, and EC compounds and environmental variables. Significant correlations were achieved by CC in combination with rainfall, obtaining an R = |–0.545|. However, this correlation was moderate, suggesting that the increase in precipitation leads to a lower

Table 6. Mean contents of triplicates of gallic acid, catechin, and epicatechin in methanolic extracts from *C. adamantium* leaves collected from February 2015 to January 2016

Date	Average GA content / %	RSD / %	Average CC content / %	RSD / %	Average EC content / %	RSD / %
02/20/2015	0.0393	1.95	0.2663	0.62	0.0390	0.65
03/31/2015	0.0414	1.47	0.2410	0.45	0.0430	0.5
04/30/2015	0.0423	2.29	0.2540	0.67	0.0482	0.5
05/31/2015	0.0401	0.57	0.3783	0.98	0.1622	0.95
06/30/2015	0.0357	2.73	0.3953	1.09	0.1415	1
07/31/2015	0.0344	1.53	0.3804	0.21	0.1231	0.44
08/31/2015	0.0440	0.79	0.3416	1.18	0.0734	1.32
09/30/2015	0.0626	0.19	0.3275	0.33	0.0035	2.68
10/31/2015	0.0313	0.34	0.1599	0.9	–	–
11/30/2015	0.0336	1.28	0.1839	0.31	0.0067	0.63
12/31/2015	0.0350	1.77	0.2041	0.87	0.0164	0.57
01/31/2016	0.0355	2.45	0.2252	0.65	0.0244	1.87

GA: gallic acid; RSD: relative standard deviation; CC: catechin; EC: epicatechin.

marker content. CC and EC were associated with maximum temperature, with R = |–0.6721| and R = |–0.5432|, respectively. This demonstrates that higher temperatures can reduce the concentrations of these constituents. The EC also showed an expressive interaction with the minimum temperature, showing an R = |–0.8498|, indicating that the lower temperatures lead to greater extraction of the EC. GA did not present a statistical correlation with any analyzed environmental factor.

The results obtained from the PCA and HCA showed chemical variability among samples of extracts obtained from *C. adamantium* leaves. Figure 6 indicates the relative position of three clusters by a two-dimensional axis system, with components PCA that describe 78.22% of the total data variation (first principal component (PC-1): 50.72% and the second (PC-2): 27.5%) and provide discriminatory information about the samples.

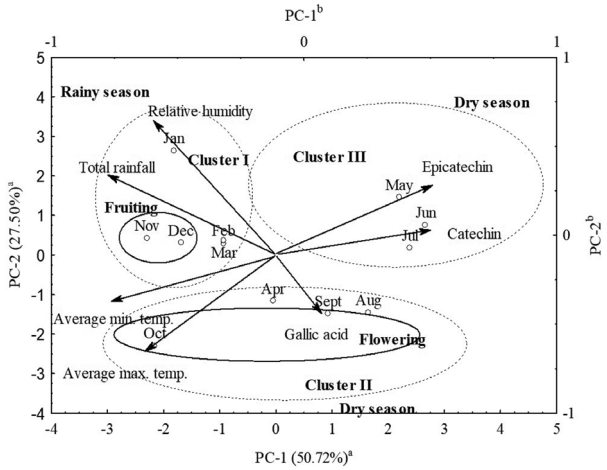

Figure 6. Scatterplot of principal component analysis of the methanolic extracts from the leaves of *C. adamantium* collected from Bela Vista/Goiás belonging to clusters I, II, and III. PC-1: first principal component. [a]Axes refer to the scores of samples; PC-2: second principal component; [b]axes refer to the scores of discriminant constituents of the extracts represented as vectors from the origin, obtained by the Statistica program.[34]

This analysis suggests that cluster I is constituted by the extracts from leaves collected in January, February, March, November, and December, with no discriminant, characterized by months of higher rainfall and fruiting (November and December). Cluster II represents the extracts from leaves collected in April, August, September, and October, being discriminated by the gallic acid compound, characterized by the dry and flowering (August, September, and October) period, with the highest average temperatures. Finally, cluster III contains the extracts from leaves collected in May, June, and July, being discriminated by the compounds epicatechin and catechin in the dry period.

The trends observed through the PCA were confirmed through the dendrogram obtained by the HCA (Figure 7), forming three groups with similar relations between the same constituents of Figure 6. Furthermore, groups are associated with rainy and dry periods, characteristic of the Cerrado in the Brazilian Midwest.

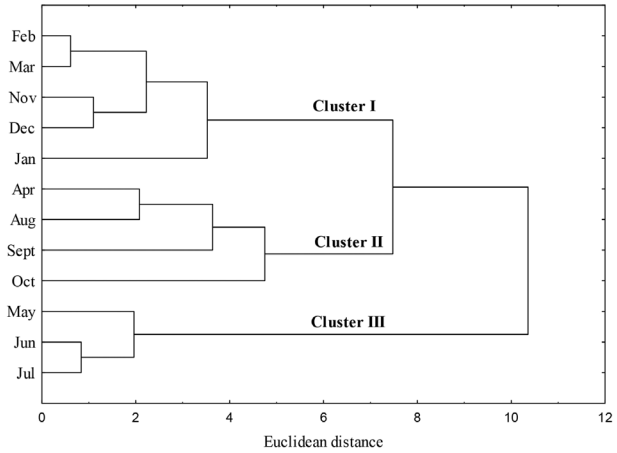

Figure 7. Dendrogram representing the similarity relations of the chemical composition of the methanolic extracts of *C. adamantium* leaves according to Ward's variance minimization method, obtained by the Statistica program.[34]

CDA (Table 7) was performed to help predict the grouping of the cluster analysis. The predictive variables employed were gallic acid and catechin; the discriminant functions retained 83.3% of well-classification in the original clusters by cross-validation. Thus, the canonical discriminant analysis evidenced the proposed category, and their employed variables are suitable to show the findings of the HCA and the PCA are consistent. To corroborate the CDA, the PLS was performed to verify the classification proposed by the PCA and HCA analyses (Figure 8). Based on these two analyses, it can be suggested that the classification proposed by the PCA and HCA analyses seems adequate. For the PLS analysis, the following variables were used gallic acid, catechin, epicatechin, and total rainfall.

Comparing this study with several data from the literature on the chemical profile of *C. adamantium* leaves,[5-8,19,43] we can observe the incidence of chemical variability, which may be related to several conditions already mentioned, that the species are exposed in the environment, but according to the statistical analyzes, the climatic elements have been highlighted.

Sá *et al.*[42] showed an increase in the content of phenols and total tannins in *C. adamantium* leaves from Bela Vista of Goiás in the period of low precipitation, presenting a behavior similar to that of the analyzed markers, with August and September being the ones with the highest levels, the same months obtained by the GA as satisfactory in the study. Frequent rain can lead to a loss of water-soluble

Table 7. Canonical discriminant analysis summary of the methanolic extracts of *C. adamantium* leaves, obtained by the Statistica program[34]

	Eingenvalues functions	Canonical R	Wilk's Lambda	*p*-level[a]
F1[b]	5.17	0.91	0.13	0.0015
	Standardized coefficients for Canonical variables			
Gallic acid	–1.22			
Catechin	1.49			
Eigenvalues	5.17			
Cumulative proportion	0.951			
	Total well-classification / %	Cluster I	Cluster II	Cluster III
		$p = 0.5$	$p = 0.5$	
Cluster I	100	5	0	0
Cluster II	50	2	2	0
Cluster III	100	0	0	3
Total	83.3	7	2	3

[a]$p < 0.05$; [b]F1: discriminant function.

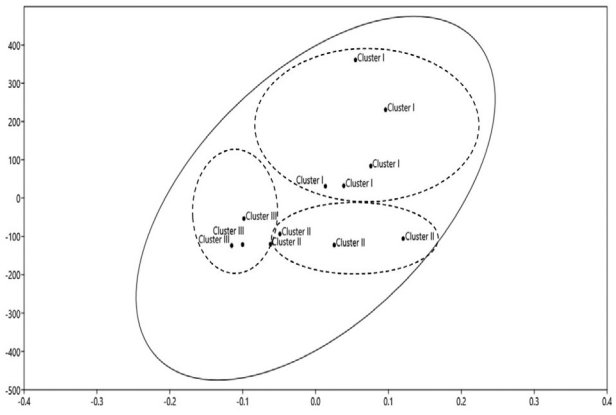

Figure 8. 2D representation obtained by partial least squares analysis of the distribution of samples according to the classification proposed by the principal component analysis and hierarchical cluster analysis, through the program Past.[35]

substances from the leaves by leaching, which is a factor that may have influenced the results obtained since the analyzed markers are soluble in water.[12,46,47]

According to Coutinho *et al.*,[43] in early spring (September), it was possible to verify, through HPLC analysis, an increase in the content of flavanones and chalcones in *C. adamantium* leaves collected in the same geographic region as the study carried out, indicating that it may be related to the accumulation of flavonoids on the leaf surface. This can provide absorption and dissipation of solar energy, thus making it difficult for the inner tissues to be damaged by UV radiation, aiming at protection against photodestruction. Another factor that may be related is the defense against herbivore attacks, which may favor the synthesis and accumulation of metabolites and the inhibition of enzymes in the digestive tract of the herbivores, reducing the palatability of the plant, which would be an effective mechanism against the herbivory.[46,48] The same period reached by Coutinho *et al.*[43] with the highest concentrations of flavonoids was observed by GA.

Drought is capable of causing climatic stress in plant species, inducing cellular oxidation, causing lipid peroxidation in cell membranes, and, consequently, producing reactive oxygen species (ROS) such as hydroxyl radicals. The metabolites GA, CC, and EC of *C. adamantium*, have antioxidant effects due to the presence of hydroxyl groups in the aromatic rings of the molecular structures, promoting the stabilization and neutralization of the effects of ROS, with the inhibition of the formation of free radicals in this species, these reactions potentiate the concentrations of the markers.[49]

Tannins can complex with carbohydrates and cell wall proteins, persisting after leaf senescence, leading to delayed decomposition.[48] Top *et al.*[48] reported that in the wet treatment *Quercus rubra* L. leaves showed a high proportion of tannins not extractable by solvent, assuming that tannins, in this condition, a greater capacity for complexing due to their longer chain length. This would consequently reduce the extraction in leaf tissues, indicating that the dry treatment would be the most suitable for extraction.

Therefore, the dry season (May to September), with temperatures from 16.7 to 33.3 °C, presented the highest levels of GA, CC, and EC in *C. adamantium* leaves, but with different groups because they present different biosynthetic pathways. These climatic conditions can induce the expression of genes that encode the enzymes of the metabolic pathways, interfering in the production of

these markers. The influence of rainfall and temperature on variations in the content of the markers shows some similarity correlations and interrelationships, demonstrating that the compounds do not act in isolation.

Conclusions

The analytical method was developed and first validated by HPLC-PDA for the simultaneous quantification of GA, CC, and EC in methanolic extracts from *C. adamantium* leaves. This method proved selective, linear, precise, accurate, robust, and with no matrix effect, being reliable and reproducible for analysis. The best time for leaf collection, with the highest levels reached, was in September and August for GA, in June and July for CC, and from May and June for EC, dry season, with temperatures from 16.7 to 33.3 °C.

Therefore, the present study contributes by providing parameters for quality control of the raw material *C. adamantium*, and the chemical variability profile related to seasonality, being a species that has therapeutic potential for future application in various technological products of the plant protection industry.

Acknowledgments

The authors are grateful for the financial support of the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), the Fundação de Amparo à Pesquisa do Estado de Goiás (FAPEG) and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), which also granted a scholarship of studies.

Author Contributions

Raphael O. Cabral was responsible for the conceptualization, methodology, investigation, formal analysis, data curation, writing of original draft, writing review, and editing; Leonardo L. Borges for the statistical analysis, writing review, suggestion, and editing; Matheus G. de Oliveira for the planning of the experiment and analysis of data obtained; Camila A. Romano for the writing-review and editing; Tatiana S. Fiuza for the writing-review, suggestion, and editing; José R. de Paula for the conceptualization, funding acquisition, resources, project supervision, investigation, writing-review, and editing.

References

1. Vallilo, M. I.; Lamardo, L. C. A.; Gaberlotti, M. L.; de Oliveira, E.; Moreno, P. R. H.; *Food Sci. Technol.* **2006**, *26*, 805. [Crossref]
2. Lorenzi, H.; de Lacerda, M. T. C.; Bacher, L. B.; *Frutas no Brasil: Nativas e Exóticas (de Consumo in natura)*, 1st ed.; Instituto Plantarum de Estudos da Flora: São Paulo, Brazil, 2015.
3. Instituto de Pesquisas Jardim Botânico do Rio de Janeiro, *Reflora - Lista de Espécies da Flora do Brasil*, [Link], accessed in March 2023.
4. Dresch, D. M.; Masetto, T. E.; Scalon, S. P. Q.; *An. Acad. Bras. Ciênc.* **2015**, *87*, 2217. [Crossref]
5. Coutinho, I. D.; Coelho, R. G.; Kataoka, V. M. F.; Honda, N. K.; Silva, J. R. M.; Vilegas, W.; Cardoso, C. A. L.; *Ecletica Quim.* **2008**, *33*, 53. [Crossref]
6. Pascoal, A. C. R. F.; Ehrenfried, C. A.; Eberlin, M. N.; Stefanello, M. E. A.; Salvador, M. J.; *Nat. Prod. Commun.* **2011**, *6*, 969. [Crossref]
7. Ferreira, L. C.; Grabe-Guimarães, A.; de Paula, C. A.; Michel, M. C. P.; Guimarães, R. G.; Rezende, S. A.; de Souza Filho, J. D.; Saúde-Guimarães, D. A.; *J. Ethnopharmacol.* **2012**, *145*, 100. [Crossref]
8. Pascoal, A. C. R. F.; Ruiz, A. L. T. G.; de Carvalho, J. E.; Stefanello, M. E. A.; Sawaya, A. C. H. F.; Salvador, M. J.; *Planta Med.* **2012**, *78*, 182. [Crossref]
9. Angelo, P. M.; Jorge, N.; *Rev. Inst. Adolfo Lutz.* **2007**, *66*, 1. [Link] accessed in August 2023
10. Badhani, B.; Sharma, N.; Kakkar, R.; *RSC Adv*. **2015**, *5*, 27540. [Crossref]
11. Fernandes, F. H. A.; Salgado, H. R. N.; *Crit. Rev. Anal. Chem.* **2016**, *46*, 257. [Crossref]
12. Gadkari, P. V.; Balaraman, M.; *Food Bioprod. Process.* **2014**, *93*, 122. [Crossref]
13. Stalikas, C. D.; *J. Sep. Sci.* **2007**, *30*, 3268. [Crossref]
14. Lanças, F. M.; *Cromatografia Líquida Moderna: HPLC/CLAE*, 2nd ed.; Átomo: Campinas, Brazil, 2016.
15. Marson, B. M.; Concentino, V.; Junkert, A. M.; Fachi, M. M.; Vilhena, R. O.; Pontarolo, R.; *Quim. Nova* **2020**, *43*, 1190. [Crossref]
16. Agência Nacional de Vigilância Sanitária (ANVISA); Resolução da Diretoria Colegiada (RDC) No. 166, de 24 de julho de 2017, *Dispõe sobre a Validação de Métodos Analíticos e dá outras Providências*; Diário Oficial da União (DOU), Brasília, No. 141, de 25/07/2017, p. 87. [Link] accessed in August 2023
17. Gouvea, D. R.; Gobbo-Neto, L.; Sakamoto, H. T.; Lopes, N. P.; Lopes, J. L. C.; Meloni, F.; Amaral, J. G.; *Quim. Nova* **2012**, *35*, 2139. [Crossref]
18. Pascoal, A. C. R. F.; Ehrenfried, C. A.; Lopez, B. G.-C.; de Araújo, T. M.; Pascoal, V. D. B.; Gilioli, R.; Anhê, G. F.; Ruiz, A. L. T. G.; de Carvalho, J. E.; Stefanello, M. E. A.; Salvador, M. J.; *Molecules* **2014**, *19*, 1843. [Crossref]
19. Sá, S.; Chaul, L. T.; Alves, V. F.; Fiuza, T. S.; Tresvenzol, L. M. F.; Vaz, B. G.; Ferri, P. H.; Borges, L. L.; de Paula, J. R.; *Rev. Bras. Farmacogn*. **2018**, *28*, 303. [Crossref]

20. *Empower™ 2*, version 2.0; Waters Corporation, Milford, Massachusetts, United States of America, 2007.
21. Marinho, T. A.; Oliveira, M. G.; Menezes-Filho, A. C. P.; Castro, C. F. S.; Oliveira, I. M. M.; Borges, L. L.; Melo-Reis P. R.; Silva-Jr., N. J.; *Braz. J. Biol.* **2022**, *82*, e234476. [Crossref]
22. Bezerra, B. P.: *Desenvolvimento e Validação de Método Analítico para Determinação de Teor de* Ácido *Gálico e Catequina no Fitoterápico Sanativo® por Cromatografia Líquida de Alta Eficiência*; MSc Dissertation, Universidade Federal do Rio Grande do Norte, Natal, Brazil, 2012. [Link] accessed in August 2023
23. Wang, H.; Helliwell, K.; You, X.; *Food Chem.* **2000**, *68*, 115. [Crossref]
24. United States Food and Drug Administration (US-FDA); *Reviewer Guidance, Validation of Chromatographic Methods*; Center for Drug Evaluation and Research (CDER), Rockville, Maryland, United States of America, 1994. [Link] accessed in August 2023
25. Agência Nacional de Vigilância Sanitária (Anvisa); Guia No. 10, versão 1, de 30 de agosto de 2017, *Guia para Tratamento Estatístico da Validação Analítica*; ANVISA: Brasília, 2017. [Link] accessed in August 2023
26. Agência Nacional de Vigilância Sanitária (Anvisa); Instrução Normativa No. 4, de 18 de junho de 2014, *Guia de Orientação para Registro de Medicamento Fitoterápico e Registro e Notificação de Produto Tradicional Fitoterápico*; Diário Oficial da União (DOU), Brasília, de 20/06/2014. [Link] accessed in August 2023
27. Association of Official Analytical Collaboration (AOAC) International; *Appendix F: Guidelines for Standard Method Performance Requirements*; AOAC: Rockville, Maryland, 2016. [Link] accessed in August 2023
28. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH); *Validation of Analytical Procedures: Text and Methodology Q2 (R1)*; ICH: London, 2005. [Link] accessed in August 2023
29. *Action Stat*, version 3.7; EstatCamp Group; São Carlos, São Paulo, Brazil, 2014.
30. *Microsoft Excel*, version 2016; Microsoft Office Professional Plus; São Paulo, Brazil, 2016.
31. *Origin*, version 6.0; Microcal™ Software Inc.; Northampton, Massachusetts, United States of America, 1999.
32. Instituto Nacional de Meteorologia (INMET); Ministério da Agricultura, Pecuária e Abastecimento, [Link], accessed in October 2017.
33. Ward Jr., J. H.; *J. Am. Stat. Assoc.* **1963**, *58*, 236. [Crossref]
34. *Statistica*, version 7.0; StatSoft Inc.; Tulsa, Oklahoma, United States of America, 2004.
35. Hammer, Ø.; Harper, D. A. T.; Ryan, P. D.; *Past: Paleontological Statistics*, version 4.13; Natural History Museum, University of Oslo, Oslo, Norway.
36. Hammer, Ø.; Harper, D. A. T.; Ryan, P. D.; *Palaeontol. Electron.* **2001**, *4*, 1. [Link] accessed in August 2023
37. Albuquerque, B. R.: *Desenvolvimento de um Aditivo Natural* à *Base de Catequina: Otimizaçãoda Extração e Estabilização a Partir de Frutos de Arbutus unedo L*; MSc Dissertation, Escola Superior Agrária de Bragança, Pará, Brazil, 2016. [Link] accessed in August 2023
38. Lopes, G. C.; Sanches, A. C. C.; de Toledo, C. E. M.; Isler, A. C.; de Mello, J. C. P.; *Braz. J. Pharm. Sci.* **2009**, *45*, 135. [Crossref]
39. Herrero-Martínez, J. M.; Sanmartin, M.; Rosés, M.; Bosch, E.; Ràfols, C.; *Electrophoresis* **2005**, *26*, 1886. [Crossref]
40. Sousa, S. A.; Pascoa, H.; da Conceição, E. C.; Alves, S. F.; Diniz, D. G. A.; de Paula, J. R.; Bara, M. T. F.; *Braz. J. Pharm. Sci.* **2011**, *47*, 269. [Crossref]
41. Lanças, F. M.; *Sci. Chromatogr.* **2009**, *1*, 39. [Crossref]
42. Sá, S.; Fiuza, T. de S.; Borges, L. L.; da Cunha, L. C.; de Oliveira Neto, J. R.; de Paula, J. R.; *Front., J. Soc., Technol. Environ. Sci.* **2022**, *11*, 206. [Crossref]
43. Coutinho, I. D.; Kataoka, V. M. F.; Honda, N. K.; Coelho, R. G.; Vieira, M. C.; Cardoso, C. A. L.; *Rev. Bras. Farmacogn.* **2010**, *20*, 322. [Crossref]
44. Kuster, V. C.; de Paula, M. S. A. M.; de Castro, S. A. B.; Vale, F. H. A.; *Rev.* Árvore **2016**, *40*, 973. [Crossref]
45. Cao, Y.; Fang, S.; Fu, X.; Shang, X.; Yang, W.; *Forests* **2019**, *10*, 624. [Crossref]
46. Gobbo-Neto, L.; Lopes, N. P.; *Quim. Nova* **2007**, *30*, 374. [Crossref]
47. Daneshfar, A.; Ghaziaskar, H. S.; Homayoun, N.; *J. Chem. Eng. Data* **2008**, *53*, 776. [Crossref]
48. Top, S. M.; Preston, C. M.; Dukes, J. S.; Tharayil, N.; *Front. Plant. Sci.* **2017**, *8*, 423. [Crossref]
49. Souza, R. T. A.; Silva, D. K. A.; dos Santos, M. V. F.; Naumann, H. D.; Magalhães, A. L. R.; de Andrade, A. P.; *Rev. Ciênc. Agron.* **2020**, *51*, 1. [Crossref]

Submitted: June 13, 2023
Published online: August 30, 2023