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Article

Characterization of Flavonoids Profiles in Polar Extracts from *Croton grewioides* Baill. Using Ultra-High Resolution Mass Spectrometry

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Research to identify plant bioactive compounds led to the evolution of extraction methods. This study optimized ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE) to extract the flavonoids of *Croton grewioides* Baill. species contributing to characterize the polar extract profile and understand the antioxidant potential of these plant constituents. The use of experimental design and statistical treatments enabled the determination of the ideal conditions for each technique. The extracts of five accessions of *C. grewioides* were obtained under the optimized extraction conditions and were analyzed using ultra high-resolution mass spectrometry (FT-Orbitrap MS) operating at negative ionization mode for flavonoids detection. The accurate experimental mass obtained to the main compounds was used to attribute the molecular formula. Chemical structures of the main compounds detected were proposed using structure data bases. Chemometric analysis were performed with two FT-Orbitrap MS spectra samples using the identified metabolites and, the antioxidant activity data, showing that for this species the MAE was most effective in extracting the antioxidant compounds. It was possible to propose the structures for forty compounds in the *C. grewioides* extracts, demonstrating the excellent performance of the FT-Orbitrap MS in providing information on the chemical profile of polar compounds in plant extracts.

Keywords: quercetin, ultrasound-assisted extraction, microwave-assisted extraction, metabolites, antioxidant activity, flavonoids

Introduction

Ultra-high resolution mass spectrometry (UHRMS) is one of the most powerful techniques for analysis of the chemical constituents of complex matrices, offering high sensitivity and resolution, together with a wide detection range. The use of this mass spectrometry technique can assist in the characterization of the constituents in plant extracts, enabling in-depth studies of secondary metabolites, without any requirement for their isolation.[1]

Flavonoids, a class of secondary metabolites widely found in plants, have antioxidant and anti-inflammatory characteristics, and in many cases can be used as nutraceutical and pharmaceutical components.[2] Several studies[2-5] have shown the protective effects of flavonoids against infections caused by bacteria and viruses, as well as other important properties including antitumor, anti-acetylcholinesterase, antinociceptive, antidepressant, and antidiabetic activities.

A recent review by Russo *et al.*[6] suggested that flavonoids such as quercetin, baicalin, luteolin, hesperetin, gallocatechin gallate, epigallocatechin gallate, scutellarin, amentoflavone, and papyriflavonol-A could potentiate the action of drugs against coronavirus infection, given their capacities to inhibit key proteins involved in the infective cycle. Among these flavonoids, the compounds baicalin, quercetin and its derivatives, hesperidin, and catechins

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have been most studied in terms of their antiviral activities, including inhibition of viral protease, ribonucleic acid (RNA) polymerase, messenger ribonucleic acid (mRNA), virus replication, and infectivity.[7]

In previous work, Prado *et al.*[8] studied the species *Croton grewioides* Baill. as a candidate for obtaining derivatives of this class of compounds. Four flavonoids derived from quercetin were isolated and identified, namely 3-*O*-methylquercetin, isoquercetin, quercetin-3-*O*-β-*D*-galactopyranosyl-(1→2)-α-apiopyranoside-(1→6)-α-L-rhamnopyranoside, and quercetin-3-*O*-β-*D*-galactopyranosyl-(1→2)-α-L-rhamnopyranoside-(1→6)-α-L-rhamnopyranoside, which indicated that this species could be an important source of a wide range of flavonoids.[8]

One of the most important biological activities presented by flavonoids is their antioxidant capacity, enabling their use in maintaining oxidative stress at safe levels, preventing cellular damage associated with various health conditions such as diabetes, cancer, cardiovascular diseases, neurodegenerative diseases, and age-related diseases, among others. Therefore, it is important to study extraction methods that allow obtaining higher yield of these metabolites from plants.[2-5,9-14]

The increasing use of ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE) is related to the possibility of improving the extraction efficiency, reducing the amount of solvent, and shortening the extraction time, while simultaneously increasing the yield of extracted compounds and improving the selectivity of the process, compared to conventional extraction methods. Furthermore, these techniques meet the requirements of "green extraction", due to the possibility of using environmentally friendly solvents.[13,15-17]

The aim of this work was to extend the study of this plant species by performing a chemical mapping and identification of the flavonoids present in the polar extracts of its leaves, using the UHRMS technique. UAE and MAE were employed, with data analysis using response surface methodology and principal component analysis (PCA), in order to identify the most effective extraction technique for the production of an extract with high antioxidant activity.

Experimental

Botanical material

The leaves of five accessions (104, 106, 107, 112, and 120) of *C. grewioides* (SisGen No. A8CCB3B) were collected on the Sertão campus of the Federal University of Sergipe, in the municipality of Nossa Senhora da Glória, on August 8th, 2019. The accessions had origins different from their collection site: accession 104 was from the municipality of Poço Verde in Sergipe state (10º55'17.8"S, 37º06'04.1"W), while accessions 106 (09º58'06.5"S, 37º51'48.4"W), 107 (09º58'06.5"S, 37º51'48.4"W), 112 (09º58'06.9"S, 37º51'49.1"W), and 120 (09º58'06.9"S, 37º51'49.1"W) were from Poço Redondo in Sergipe state.

The plant materials were dried for 89 h, at 50 ºC, in an oven with forced air circulation (model MA 035, Marconi, Piracicaba, São Paulo, Brazil), followed by trituration using a domestic blender (Eletronic Pro 2 in 1, Britânia, Joinville, Brazil). The material obtained was separated using a sieve, obtaining two fractions with different granulometries (30 and 60 mesh).

Experimental design

The extraction processes were optimized using full experimental designs (2^3), with the phenolic content as the response variable and the matrix/solvent ratio fixed at a concentration of 0.067 g mL^{-1}. The different parameters of each technique were varied in order to evaluate their effects on the extracts.

Microwave-assisted extraction (MAE) and ultrasound-assisted extraction (UAE)

For each optimization processes to maximize the total phenolic content (TPC) of the extract, using MAE and UAE, 1 g of the leaves from accession 106 was extracted with 15 mL of hydroethanolic solution, following the experimental conditions of a full factorial design (2^3) with three independent variables: ethanol concentration (20 and 50%) and time (5 and 10 min) for MAE and UAE; microwave power (300 and 600 W) for MAE; and ultrasound power (30 and 90%) for UAE. The experimental design assays for MAE were performed in duplicate, totaling 16 assays. For UAE, three replicates of the central point were used to calculate the experimental error, totaling 11 assays. The experiments were conducted in random order to ensure the validity of the optimization process (Table 1).

Preparation of the extracts

The *C. grewioides* extracts obtained using MAE and UAE were filtered using a vacuum system, followed by rotary evaporation (Buchi model R-3) for removal of the ethanol (NEON, Suzano, São Paulo, Brazil).

The material was then frozen in an ultrafreezer (Liotop UFR30, Liobras, São Carlos, São Paulo, Brazil), at

Table 1. Experimental designs for MAE and UAE processes, and response values. Conditional variables are at low (–1), center point (0) and high (+1) levels

Microwave-assisted extraction (MAE)				
Trial	Ethanol concentration / %	time / min	Power / W	TPC[a] / (mg GAE kg^{-1} extract)
1	20 (–1)	5 (–1)	300 (–1)	175.88 ± 4.02
2	50 (1)	5 (–1)	300 (–1)	210.52 ± 2.56
3	20 (–1)	10 (1)	300 (–1)	149.80 ± 5.11
4	50 (1)	10 (1)	300 (–1)	166.47 ± 18.81
5	20 (–1)	5 (–1)	600 (1)	148.97 ± 20.15
6	50 (1)	5 (–1)	600 (1)	184.80 ± 7.07
7	20 (–1)	10 (1)	600 (1)	203.61 ± 10.74
8	50 (1)	10 (1)	600 (1)	237.07 ± 24.02
Trial	Ethanol concentration / %	time / min	Power / %	TPC[b] / (mg GAE kg^{-1} extract)
Ultrasound-assisted extraction (UAE)				
1	20 (–1)	5 (–1)	30 (–1)	226.05 ± 19.14
2	50 (1)	5 (–1)	30 (–1)	145.34 ± 12.59
3	20 (–1)	10 (1)	30 (–1)	233.67 ± 7.97
4	50 (1)	10 (1)	30 (–1)	221.29 ± 5.54
5	20 (–1)	5 (–1)	90 (1)	214.27 ± 2.88
6	50 (1)	5 (–1)	90 (1)	216.17 ± 6.43
7	20 (–1)	10 (1)	90 (1)	237.36 ± 7.51
8	50 (1)	10 (1)	90 (1)	200.58 ± 2.58
9[c]	35 (0)	7.5 (0)	60 (0)	236.53 ± 7.89
10[c]	35 (0)	7.5 (0)	60 (0)	241.05 ± 8.09
11[c]	35 (0)	7.5 (0)	60 (0)	227.72 ± 8.89

[a]TPC: total phenolic content, values are expressed as mean ± standard deviation (n = 2); [b]TPC: values are expressed as mean ± standard deviation (n = 3); [c]central point of the experimental design. GAE: gallic acid equivalent.

−80 ºC for 12 h, followed by lyophilization (Liotop L101, Liobras, São Carlos, São Paulo, Brazil) for 72 h at −50 ºC and pressure below 500 μHg. The extracts obtained were subsequently stored in a freezer until analysis.

Solid phase extraction (SPE)

Portions (10 mg) of the extracts were dissolved in 1 mL of 1:1 ethanol:water solution, obtaining a solution with concentration of 10 mg mL^{-1}. The solution was stirred for several seconds and centrifuged (Eppendorf MiniSpin, BioResearch, São Paulo, Brazil) at 13,300 rpm (11,866 g force) for 5 min.

For the clean-up procedure, the cartridge (C_{18}, 100 mg, 40 μm APD, 60 Å, JT Baker, Philipsburg, PA, USA) was first conditioned with 1 mL of methanol (JT Baker, Philipsburg, PA, USA), followed by 1 mL of purified water (Millipore, São Paulo, Brazil). A 1 mL aliquot of the extract solution (10 mg mL^{-1}) was then added to the cartridge, followed by sequential elution with 1 mL volumes of 50% MeOH:H_2O and 100% MeOH, producing two fractions.

Total phenolic content

The total phenolic content (TPC) was determined for all extracts obtained with the two extraction methods (MAE and UAE), using an adaptation of the methodology described by Woisky and Salatino.[18] Aliquots (12.5 μL) of the extracts were pipetted onto a microplate, in triplicate, followed by the addition of 12.5 μL volumes of Folin-Ciocalteu reagent (Sigma-Aldrich, São Paulo, Brazil) and 200 μL volumes of distilled water. After 3 min of reaction, addition was made of 25 μL volumes of saturated sodium carbonate (Na_2CO_3) solution. The plate was kept at room temperature for 1 h, protected from light, and the absorbance at 720 nm was measured using a spectrophotometer. The results were expressed as mg of gallic acid equivalent (GAE) kg^{-1} of extract,[19] determined from a standard curve, using equations 1 (equation of the straight line) and 2.

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

The value of *x* obtained according to equation 1 was

used to determine the relative phenolic content *per* g of extract:

$$\text{mg GAE kg}^{-1}\text{ extract} = \frac{\text{concentration GAE (mg L}^{-1})}{\text{concentration of extract (kg L}^{-1})} \quad (2)$$

Total flavonoids content

Flavonoids were determined using an adaptation of the methodology of Woisky and Salatino.[18] Aliquots of 25 µL of the extracts (in triplicate) were transferred to a microplate, followed by the addition of aliquots of 100 µL of distilled water and 7.5 µL of 5% NaOH. After allowing to rest for a few minutes, 7.5 µL aliquots of 10% $AlCl_3$ were added. After 6 min, 100 µL volumes of 4% NaOH and 10 µL volumes of distilled water were added, the mixtures were allowed to rest for 15 min, and measurements of the absorbance at 510 nm were made using a spectrophotometer (BioTek -Model Synergy H1, Santa Clara, United States). Blank samples (without addition of the extract) were included and the results were expressed in mg of catechin equivalent *per* 100 g of extract, obtained using a catechin (Sigma-Aldrich, São Paulo, São Paulo, Brazil) calibration curve.

Antioxidant activity

DPPH• method

Analyses were performed using an adaptation of the procedure described by Brand-Williams *et al.*[20] Aliquots (50 µL) of the extracts were transferred (in triplicate) to a microplate, followed by addition of 150 µL volumes of DPPH (2,2-diphenyl-1-picrylhydrazyl, Sigma-Aldrich, Steinheim, Germany) solution at a concentration of 0.006 mM diluted in methanol. After allowing to rest for 30 min, the reduction of the radical was measured at 515 nm, using a spectrophotometer. All the determinations included a control (without the presence of the extract). The results were expressed as percentage of inhibition.

Ferric reducing antioxidant power (FRAP) assays

The ferric reducing capacity was determined using an adaptation of the method described by Oyaizu.[21] In a dark environment, 27 µL aliquots of the extracts were transferred (in triplicate) to a microplate, followed by the addition of 270 µL volumes of the FRAP reagent solution (2,4,6-tris(2-pyridyl)-*S*-triazine, ferric chloride, and phosphate buffer). The microplate was placed in an oven at 37 ºC for 30 min, followed by absorbance measurement at 595 nm, using a spectrophotometer. The results were expressed in µmol eq. Trolox kg^{-1} of extract, using a Trolox calibration curve.

FT-Orbitrap MS analysis

The samples were prepared for analysis by dilution in methanol of 10 µL of the extract solution obtained after the clean-up process, with a final volume of 1 mL, resulting in a solution with an approximate concentration of 50 ppm. The analyses were performed in HESI(−) mode, with spray voltage of 3.5 kV, vaporization region temperature of 40 ºC, capillary temperature of 300 ºC, sheath gas at 0 a.u., auxiliary gas at 10 a.u., and sweep gas at 10 a.u.

The mass spectra were acquired using an FT-Orbitrap MS Exactive HCD Plus system (Thermo Scientific, Bremen, Germany), in the *m/z* range from 100 to 1000, with accumulation of 100 microscans and resolution of 140,000 full width at half maximum (at *m/z* 200). The final mass spectrum was obtained by subtraction of the blank spectrum from the sample spectrum. The processing of the spectra and assignment of the molecular formulae of the ions employed Xcalibur v. 3.1 software (Thermo Fisher Scientific), with acceptability criterion of an error of up to 3 ppm between the experimental and assigned *m/z* values.

Chemometric and statistical analysis

Regression analysis of the experimental data was performed using Statistica v. 10.0.1011.0 software[22] (StatSoft, Inc., USA). Analysis of variance (ANOVA) was used to determine the statistical significance of the independent variables evaluated ($p < 0.05$). The statistical parameters used to assess the fits of the proposed models to the experimental data were the coefficient of determination (R^2), adjusted coefficient of determination ($R^2_{adjusted}$), and Fisher's *F*.

For each experimental design, the relation between the dependent and independent variables was obtained using a polynomial function (equation 3).

$$Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{23}X_2X_3 + \beta_{123}X_1X_2X_3 \quad (3)$$

where, Y is the measured response variable; X_1, X_2 and X_3 are the independent variables evaluated in each design; β_0 is a constant; β_1, β_2 and β_3 are the regression coefficients of the model; β_{12}, β_{13} and β_{23} are the regression coefficients for the interaction terms between two variables; and β_{123} is the regression coefficient for the interaction term among three variables.

For the calculations employed in the determination of the optimum conditions for extraction using UAE or MAE, maximizing TPC, extractions were performed in triplicate for UAE and MAE, separately, employing

the corresponding optimum conditions predicted by the experimental design, according to each extraction method.

Multivariate data analysis was performed using Pirouette v. 4.0 software (Infometrix, USA).[23] The data were preprocessed for peak alignment using the correlation optimized warping (COW) algorithm,[24] with mean-centering of the original data, using MATLAB R2009a software[25] (The MathWorks, Natick, MA, USA).

Results and Discussion

Experimental design applied to the ultrasound and microwave extractions

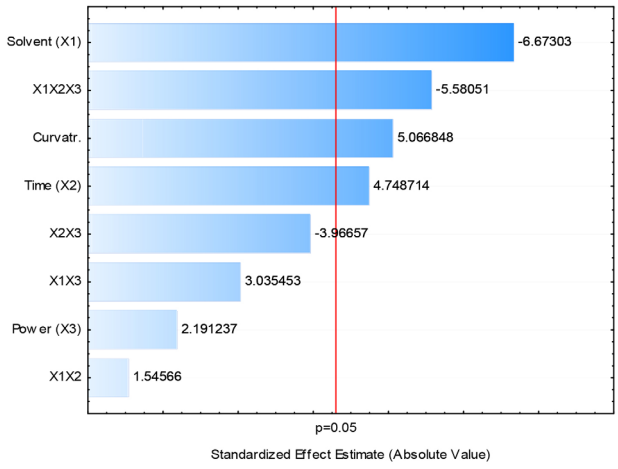
The TPC values obtained in the experimental design assays using MAE and UAE were employed as the model response variables, enabling evaluation of the parameters that influenced extraction of these compounds.[15,26] The results obtained in the assays were presented as standardized Pareto charts, supported by the corresponding response surfaces.

For the UAE method, the Pareto chart (Figure 1) showed that the most significant variables influencing the extraction of the phenolic compounds were extraction time and solvent composition, as also evidenced by the response surface graph interpolation.

The best result for UAE of the phenolic compounds was obtained with the lowest solvent composition (20%), longest extraction time (10 min), and highest power (90%), corresponding to assay 7. This clearly demonstrated progress towards optimization of the process applied to *C. grewioides*.

The statistical treatment of the results for the assays of the experimental design for the MAE method showed that the "time *versus* power" combination was most relevant for extraction of the phenolic compounds. As shown in the Pareto chart (Figure 2), all the statistically significant variables (solvent, power, and "time *versus* power") presented positive values.

The results revealed that use of the maximum values of these parameters was most effective for the extraction of phenolic compounds from *C. grewioides* employing MAE. The constructed model presented R^2 = 0.9116, similar to values reported in the literature.[27] The effects of the significant factors, especially the "solvent *versus* power" combination, could be clearly observed in the response surface plot. The statistical analysis showed that the best result for extraction of the phenolic compounds was achieved using the highest value for the solvent composition (50%), the longest extraction time (10 min), and the highest power (600 W), corresponding to assays 15 and 16.



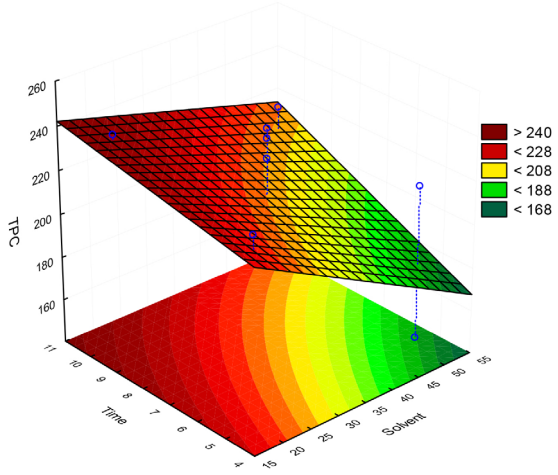


Figure 1. Determination of the main variables and their interactions for optimization of the extraction of phenolic compounds by UAE, employing response surface methodology and Pareto chart.

Application of analysis of variance (ANOVA, Tables S1 and S2, Supplementary Information (SI) section) showed that the individual models obtained to describe the TPC of the extracts obtained by UAE and MAE could be considered significant, with high *F*-values of 19.50 for UAE ($p < 0.05$) and 11.79 for MAE ($p < 0.001$). The models showed good fits to the observed responses, as evidenced by the high coefficients of determination (R^2) of 0.9873 and 0.9116 for the UAE and MAE models, respectively. These results indicated that the models had high capacity for predicting the relationship between the independent and dependent (response) variables.[10,28,29] Hence, the models were suitable for use in prediction of the TPC values of the *C. grewioides* extracts.

Based on the statistical results, the optimal TPC values predicted by the models (equations S1 and S2, SI section), under the optimized conditions for extraction by UAE (20% ethanol concentration, 10 min, and 90% ultrasound power) and MAE (50% ethanol concentration, 10 min, and 600 W microwave power) were 237.36 and 237.10 mg GAE kg^{-1}

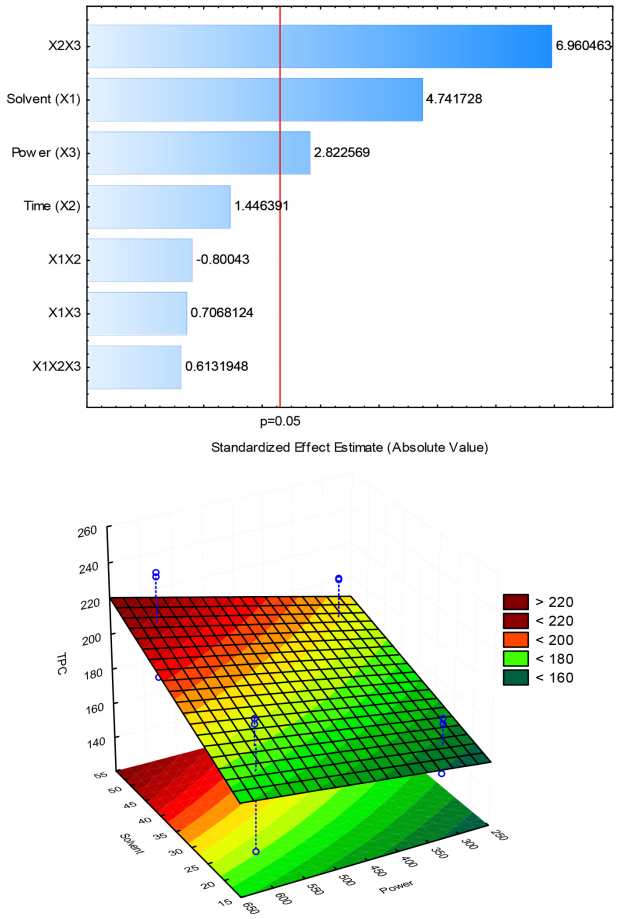


Figure 2. Determination of the main variables and their interactions for optimization of the extraction of phenolic compounds by MAE, employing response surface methodology and Pareto chart.

of extract, respectively. The statistical analysis showed that the variables ethanol concentration and time had the greatest influence on TPC for the extracts obtained using UAE, while the variables ethanol concentration and microwave power were most important for the extracts obtained using MAE.

Croton grewioides extracts obtained under the optimal UAE and MAE conditions, and quantification of total flavonoids

Having identified the optimal extraction conditions, method validation was performed applying the two extraction methodologies (UAE and MAE) to the materials from all accessions. The quantification of bioactive compounds considered the total flavonoid content, determined as the catechin equivalent *per* gram of extract. The yields obtained are shown in Table 2.

It was evident from the results (Table 2) that the two extraction methods acted differently in the recovery of the *C. grewioides* flavonoids. For accessions 104, 106, 107, and 112, the most effective method for obtaining the flavonoids

Table 2. Total flavonoid contents (TFC) and yields obtained using UAE and MAE applied to the *C. grewioides* accessions

Accession	Yield / %		TFC / (eq catechin 100 g^{-1} extract)	
	MAE	UAE	MAE	UAE
104	24.06	25.26	156.00 ± 11.60	97.17 ± 6.22
106	19.93	17.92	158.35 ± 10.61	141.88 ± 6.22
107	16.87	16.84	99.53 ± 0	57.17 ± 1.36
112	12.65	20.76	177.17 ± 32.63	165.41 ± 5.43
120	21.65	21.46	257.17 ± 11.12	278.35 ± 33.30

MAE: microwave-assisted extraction; UAE: ultrasound-assisted extraction.

was MAE, while UAE was most suitable in the case of accession 120. This difference could be explained by a combination of the effects of the extraction methodology and the characteristics of each accession, suggesting that accession 120 differed genetically from the other accessions.

Proposed structures of the compounds present in the *C. grewioides* extracts

The accurate masses of the main negative ions $[M - H]^-$ detected by FT-Orbitrap MS were converted into a molecular formula, such as $C_xH_yN_zO_wS_k$, presenting errors below 3 ppm. The chemical structures were proposed considering the flavonoid structures available in the ChemSpider database and those reported previously in scientific articles for plants of this species.[8] The mass region considered for the analysis was $m/z \geq 285$, where kaempferol flavonoids and their derivatives, commonly reported in *Croton* species, are detected. Forty chemical structures of compounds belonging to the flavonoid class (see SI section, Tables S3 and S4 have been arranged in ascending order of theoretical mass) were proposed, of which seven were present in all the accessions, irrespective of the extraction methodology applied (UAE or MAE), suggesting that these flavonoids could be used as phylogenetic markers of the genus *Croton*. The mass spectral profiles for each accession are shown in Figure 3.

Kaempferol (**1**), quercetin (**2**), 3-*O*-methylquercetin (**3**), isoquercetin (**4**), rutin (**5**), quercetin-3-*O*-β-*D*-galactopyranosyl-(1→2)-α-apiopyranosyl-(1→6)-α-L-rhamnopyranoside (**6**), and quercetin-3-*O*-β-*D*-galactopyranosyl-(1→2)-α-L-rhamnopyranosyl-(1→6)-α-L-rhamnopyranoside (**7**) (Figure 4) are compounds that can be considered representative of this plant species, so they could be used as phylogenetic markers. Table 3 shows the relative abundances of these compounds in the samples analyzed.

These seven chemical marker compounds have been reported previously for this genus and the same species studied here. The most recent work was that of

Prado *et al.*,[8] who isolated compounds **3**, **4**, **6**, and **7** from an aqueous extract prepared by decoction of an accession of *C. grewioides*, with compound **7** being considered a previously unpublished natural product.

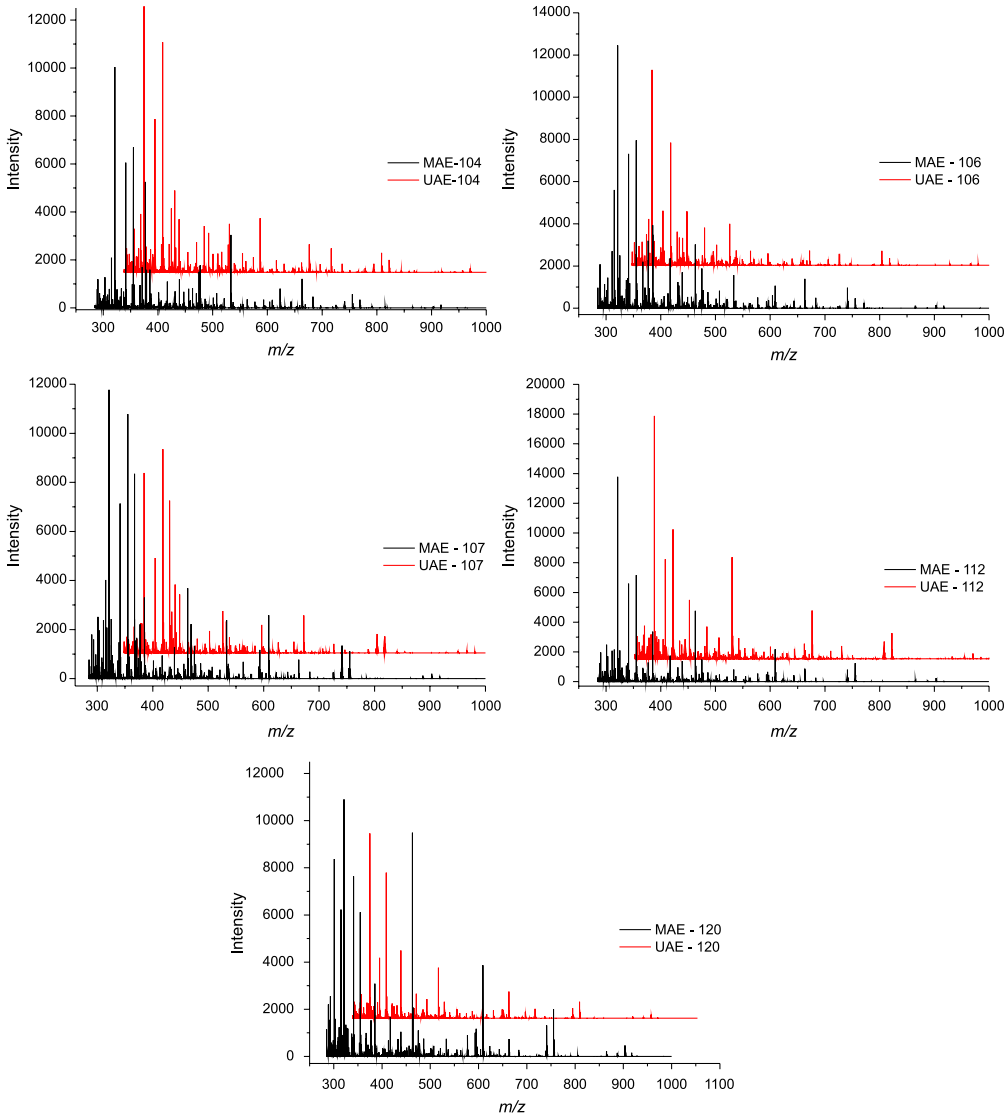


Figure 3. Mass spectrum fingerprints of *C. grewioides* extracts from five different accessions, obtained using MAE and UAE.

Table 3. Main compounds selected to represent the flavonoids profile of *C. grewioides*

Compound	T.M. $[M-H]^-$ (*m/z*)	UAE					MAE				
		Relative abundance / %					Relative abundance / %				
		104	106	107	112	120	104	106	107	112	120
Kaempferol (**1**)	285.04046	0.34	0.29	0.28	0.19	0.96	0.51	0.19	1.94	2.03	10.49
Quercetin (**2**)	301.03538	1.90	3.90	5.75	9.84	8.52	5.26	6.75	21.39	18.08	76.81
3-*O*-Methylquercetin (**3**)	315.05103	8.00	23.72	14.68	10.04	8.41	17.59	44.98	34.16	15.69	57.13
Isoquercetin (**4**)	463.08820	7.34	21.24	20.61	41.84	27.35	8.20	24.24	31.28	34.60	87.03
Rutin (**5**)	609.14611	3.48	7.44	18.43	19.88	14.40	3.38	8.54	21.96	15.80	35.49
Quercetin-3-*O*-β-*D*-galactopiranosil-(1→2)-α-apio-pyranosil-(1→6)-α-L-rhamnopiranoside (**6**)	741.18837	3.06	7.38	9.30	9.30	5.50	2.71	7.81	11.47	5.87	12.09
Quercetin-3-*O*-β-*D*-galactopiranosil-(1→2)-α-L-rhamno-piranosil-(1→6)-α-L-rhamnopiranoside (**7**)	755.20402	6.97	3.60	8.18	8.18	8.89	5.74	3.75	9.45	9.06	18.37

T.M. $[M-H]^-$: theoretical mass presented with the absence of a proton; UAE: ultrasound-assisted extraction; MAE: microwave-assisted extraction.

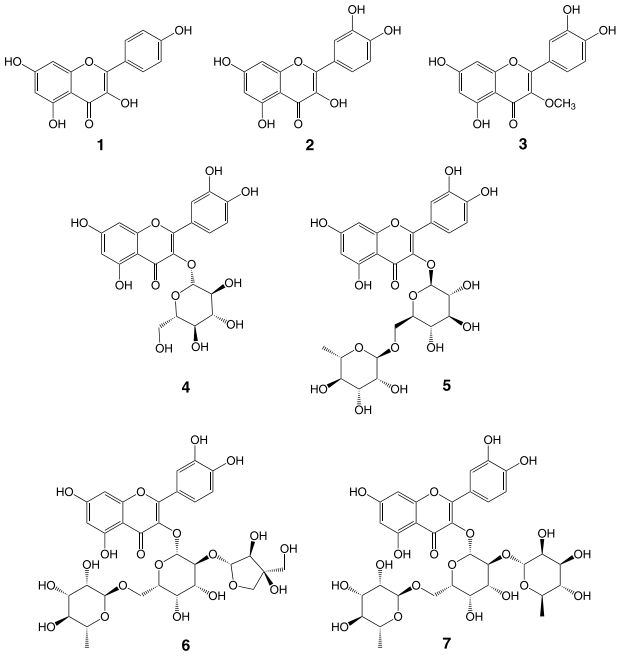


Figure 4. Structures of the main flavonoid compounds suggested as phylogenetic markers for *C. grewioides*.

Chemometric analysis for the samples using FT-Orbitrap MS spectra data

PCA of the data employed a matrix containing 10 lines (samples) and 40 variables (ions with attributed molecular formulae). This analysis was used to statistically differentiate the *C. grewioides* extracts in terms of accession and the extraction methodology applied.

The first two components, PC1 (33.8%) and PC2 (19.3%), described 53.1% of the total variance of the data and were used in the subsequent procedures. The scores plot of PC1 against PC2 (Figure 5) showed the formation of four large groups.

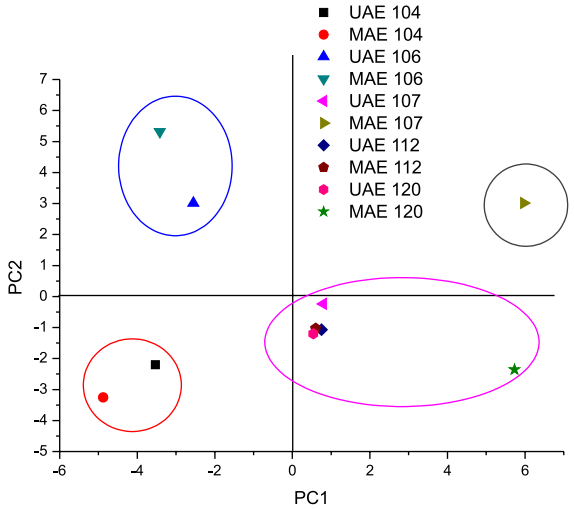


Figure 5. PC1 (33.8%) *vs.* PC2 (19.3%) biplot for the *C. grewioides* samples.

Group 1 (G1) was formed by the UAE and MAE extracts from accession 106, with negative PC1 and positive PC2. Group 2 (G2) consisted only of the MAE extract for accession 107, with positive PC1 and PC2. Group 3 (G3) was composed of the UAE and MAE extracts for accession 104, with negative PC1 and PC2. Group 4 (G4) consisted of the UAE extract for accession 107, together with the UAE and MAE extracts for accessions 112 and 120, with positive PC1 and negative PC2.

These results showed that in terms of the components identified in the FT-Orbitrap MS analyses, the UAE and MAE extracts were quite similar, except for accessions 107 and 120, for which there were differences between the chemical profiles of the UAE and MAE extracts. Although accession 120 was included in G4, there was a greater distance between the positions for the two extracts in the quadrant of positive PC1 and negative PC2. It could also be seen that the extracts with greatest similarity were those for accession 112, suggesting that they had very similar chemical profiles.

These differences and similarities could have been related to both the extraction technique, with the two methods favoring the extraction of different compounds, and the characteristics of each accession.

The loadings graph (Figure 6) was used to identify the variables (metabolites) that contributed to the differences and similarities observed among the samples, enabling identification of the ions characteristic of each group highlighted in the scores graph, showing their prevalence in the different extracts. This also allowed evaluation of the effect of geographic origin, since accession 104 was from Poço Verde, while the other accessions originated from Poço Redondo.

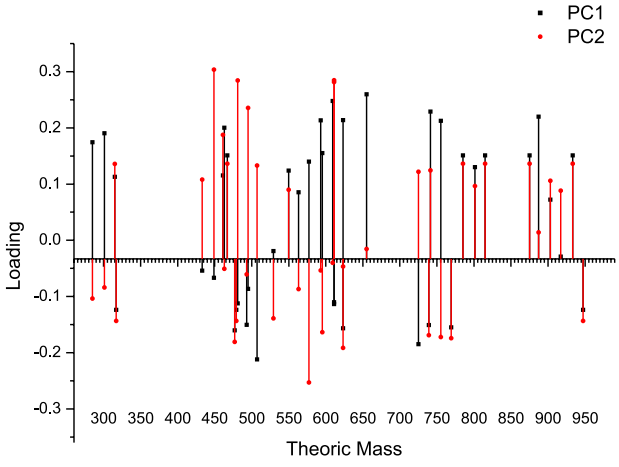


Figure 6. Loadings plot for the *C. grewioides* samples.

The compounds characterizing the extracts for the different groups are shown in Table 4. As shown in the

Table 4. Characteristic compounds of the *C. grewioides* accession extracts obtained by MAE and UAE, identified using PCA

Group 1: PC1– and PC2+			Group 2: PC1+ and PC2+			Group 3: PC1– and PC2–			Group 4: PC1+ and PC2–		
MAE[a] and UAE[b] (106)			MAE[a] (107)			MAE[a] and UAE[b] (104)			UAE[b] (107); UAE[b] and MAE[a] (112); UAE[b] and MAE[a] (120)		
m/z	Comp.	Molecular formula	*m/z*	Comp.	Molecular formula	*m/z*	Comp.	Molecular formula	*m/z*	Comp.	Molecular formula
449.07255	**9a**, **9b, 9c**	$C_{20}H_{17}O_{12}$	315.05103	**3**	$C_{16}H_{11}O_7$	317.03029	**8**	$C_{15}H_9O_8$	285.04046	**1**	$C_{15}H_9O_6$
495.11441	**16**	$C_{22}H_{23}O_{13}$	461.10893	**10**	$C_{22}H_{21}O_{11}$	477.10385	**12a**, **12b**, **12c**	$C_{22}H_{21}O_{12}$	301.03538	**2**	$C_{15}H_9O_7$
507.11441	**17**	$C_{23}H_{23}O_{13}$	467.11950	**11**	$C_{21}H_{23}O_{12}$	479.08311	**13a**, **13b**	$C_{21}H_{19}O_{13}$	463.08820	**4**	$C_{21}H_{19}O_{12}$
611.12537	**24a**, **24b**	$C_{26}H_{27}O_{17}$	549.88590	**19a**, **19b**	$C_{24}H_{21}O_{15}$	481.09876	**14**	$C_{21}H_{21}O_{13}$	563.17701	**20**	$C_{27}H_{31}O_{13}$
611.16176	**25**	$C_{27}H_{31}O_{16}$	741.18837	**6**	$C_{32}H_{37}O_{20}$	493.09876	**15**	$C_{22}H_{21}O_{13}$	577.13515	**21a**, **21b**, **21c**	$C_{30}H_{25}O_{12}$
725.19345	**29**	$C_{32}H_{37}O_{19}$	785.19345	**32**	$C_{37}H_{37}O_{19}$	529.13515	**18**	$C_{26}H_{25}O_{12}$	593.13006	**22**	$C_{30}H_{25}O_{13}$
917.23571	**38a, 38b**	$C_{42}H_{45}O_{23}$	801.20950	**33**	$C_{34}H_{41}O_{22}$	623.16176	**27**	$C_{28}H_{31}O_{16}$	595.13046	**23a**, **23b**	$C_{26}H_{27}O_{16}$
–	–	–	815.20402	**34**	$C_{38}H_{39}O_{20}$	739.20910	**30**	$C_{33}H_{39}O_{19}$	609.14611	**5**	$C_{27}H_{29}O_{16}$
–	–	–	875.22515	**35a**, **35b**	$C_{40}H_{43}O_{22}$	769.21967	**31**	$C_{34}H_{41}O_{20}$	623.14063	**26a, 26b**	$C_{31}H_{27}O_{14}$
–	–	–	887.22515	**36**	$C_{41}H_{43}O_{22}$	947.26740	**40a**, **40b**, **40c**	$C_{40}H_{51}O_{26}$	655.15159	**28a, 28b**	$C_{28}H_{31}O_{18}$
–	–	–	903.22006	**37**	$C_{41}H_{43}O_{23}$	–	–	–	755.20402	**7**	$C_{33}H_{39}O_{20}$
–	–	–	933.25175	**39a, 39b**, **39c**	$C_{39}H_{49}O_{26}$	–	–	–	–	–	–

MAE: microwave-assisted extraction; UAE: ultrasound-assisted extraction; Comp.: compounds.

loadings plot (Figure 6), the G1 samples MAE-106 and UAE-106 are related to the ions corresponding to the metabolites derived from quercetin, myricetin, epigallocatechin, syringetin, and kaempferol, since they presented negative values in PC1 and positive values in PC2 in the scores plot (Figure 5). The G2 sample MAE-107 showed positive values in PC1 and PC2, related to the characteristic ions corresponding to the metabolites derived from quercetin, diosmetin, dihydromyricetin, and kaempferol. For the G3 samples MAE-104 and UAE-104, with negative values in PC1 and PC2, the characteristic ions were slightly different, compared to the other samples, presenting metabolites derived from myricetin, isorhamnetin, mearnsitrin, quercetin, and kaempferol. Finally, for the G4 samples UAE-107, UAE-112, MAE-112, UAE-120, and MAE-120, which presented positive values in PC1 and negative values in PC2 (Figure 5), the characteristic ions corresponded to the metabolites derived from kaempferol, quercetin, pinocembrin, procyanidin, epigallocatechin, and isorhamnetin.

These results allowed to propose the presence of 10 different flavonoid skeleton structures, demonstrating the chemical diversity of *C. grewioides*. An interesting feature was the predominance of quercetin and kaempferol derivatives in all four groups differentiated by ultra-high resolution mass spectrometry, in agreement with previous work showing the presence of these derivatives in different species of the genus *Croton*.[30]

Determination of antioxidant activity

Table 5 provides the values obtained for the DPPH radical inhibition percentage and the Trolox equivalent (FRAP assay) for the different extracts. Statistical evaluation of the results was performed using the Tukey's test. The extracts presented DPPH radical inhibition values exceeding 80%, while the FRAP assay results indicated that the accessions interacted differently with the reagent, with varying Trolox equivalent values for the oxidation-reduction reaction employed in this test.

Table 5. Antioxidant activity values for the *C. grewioides* accessions, obtained using the DPPH and FRAP methods

Accession	DPPH (inhibition) / %		FRAP / (μmol eq. Trolox kg^{-1} of extract)	
	UAE	MAE	UAE	MAE
104	81.02 ± 0.87	81.20 ± 0.15	1569.78 ± 1.92	2180.89 ± 84.94
106	80.21 ± 0.15	80.93 ± 0.15	1545.33 ± 14.53	1832.00 ± 17.64
107	80.12 ± 0.56	80.58 ± 0.93	1174.22 ± 10.72	1590.89 ± 55.21
112	80.48 ± 0.31	80.93 ± 0.62	1580.89 ± 40.32	1939.78 ± 13.47
120	80.30 ± 0.27	82.01 ± 0.15	2124.22 ± 26.94	2935.33 ± 86.67

UAE: ultrasound-assisted extraction; MAE: microwave-assisted extraction; DPPH: 2,2-diphenyl-1-picrylhydrazyl; FRAP: ferric reducing antioxidant power.

The antioxidant activity results (Table 5) showed that the extracts obtained using both MAE and UAE presented DPPH radical inhibition greater than 80%, with no statistically significant difference between the two methods (Tukey's test). The antioxidant activities obtained using the FRAP methodology indicated that the most promising extracts were those obtained using the microwave method, especially the extract from accession 120, in agreement with the DPPH results.

The DPPH and FRAP assays showed that the extracts obtained using both extraction methods presented high antioxidant activities, with the best results for the MAE method. This suggested that the MAE methodology enabled the extraction of compounds with greater oxidation-reduction capacity and, consequently, higher antioxidant potential.

Among all the extracts obtained by MAE, the extract from accession 120 presented the best antioxidant activity according to both assays (DPPH and FRAP). This activity must have been related to the predominant chemical constituents, as indicated by the PCA results that identified the ions corresponding to compounds **1**, **2**, **4**, **5**, **7**, **20**, **21a**, **21b**, **21c**, **22**, **23a**, **23b**, **26a**, **26b**, and **28a**, **28b** (Table 6).

As shown in Table 6, with the exception of compound **20**, all the compounds characteristic of group G4, mentioned above, showed higher relative abundance values for the MAE extract from accession 120. This suggested that it was not only their presence, but also their greater abundance, that led to this extract having the highest antioxidant activity.

Conclusions

The findings of this work showed the value of using an experimental design applied to the process of optimizing the extraction of the target compounds. The optimized conditions for the UAE method were lower ethanol concentration (20%), longer extraction time (10 min), and higher power (90%), represented by test 7 (Table 1). In the case of MAE, the best conditions were higher ethanol concentration (50%), longer extraction time (10 min), and higher power (600 W), represented by test 8 (Table 1).

It was possible to identify 40 different flavonoids, based on the FT-Orbitrap MS analyses, together with the determination of different flavonoid structural skeletons and seven flavonoids that could act as phylogenetic markers for *C. grewioides* species. In terms of the antioxidant potential of this species, the main compounds contributing to this activity were the flavonoids kaempferol (**1**), quercetin (**2**), isoquercetin (**4**), rutin (**5**), quercetin-3-*O*-β-*D*-galacto-piranosil-(1→2)-α-L-rhamnopiranosil-(1→6)-α-L-rhamnopiranoside (**7**), pinocembrin-7-rhamnosylglucoside (**20**), (+)-procyanidin B2 (**21a**), procyanidin B1 (**21b**), procyanidin B4 (**21c**), tiliroside (**22**), peltatoside (**23a**), quercetin-3-sabubioside (**23b**), epigallocatechin-(4β-8)-4'-*O*-methyl-gallocatechin (**26a**), 5,7-dihydroxy-2-(4-hydroxy-3-methoxyphenyl)-4-oxo-4*H*-chromen-3-yl-6-*O*-[(2*E*)-3(4-hydroxyphenyl)-2-propenoyl]-β-*D*-glucopyranoide (**26b**), petuletin-3-*O*-gentiobioside (**28a**), patutetin-7-diglucoside (**28b**).

Finally, it is known that the use of ethanol, water, or a mixture of the two, for the extraction of flavonoids and their derivatives from plants, is already widely adopted in the areas of food, nutraceuticals, and cosmeceutics. The present results, showing that the polar extracts of *Croton grewioides* are rich in flavonoid derivatives, suggest that this species is a strong candidate for use by manufacturers developing products based on the antioxidant potential of plant flavonoids.

Table 6. Relative abundance values for the characteristic ions in the extracts from accessions 107, 112, and 120, obtained using ultrasound extraction (UAE), and from accessions 112 and 120, obtained using microwave extraction (MAE)

Compound	*m/z*	UAE-107	UAE-112	MAE-112	UAE-120	MAE-120
1	285.04046	0.28	0.19	2.03	0.96	10.49
2	301.03538	5.75	9.84	18.08	8.52	76.81
4	463.0882	20.61	41.84	34.60	27.35	87.03
20	563.17701	5.21	2.30	1.98	2.08	2.37
21a, **21b**, **21c**	577.13515	1.55	4.06	3.95	4.27	8.28
22	593.13006	1.65	1.34	3.51	1.41	9.20
23a, **23b**	595.13046	0.87	6.21	4.74	4.84	10.75
5	609.14611	18.43	19.88	15.8	14.4	35.49
26a, **26b**	623.14063	–	–	1.47	–	4.01
28a, **28b**	655.15159	0.87	0.73	0.59	0.64	1.14
7	755.20402	8.18	10.53	9.06	8.89	18.37

Supplementary Information

Supplementary data are available free of charge at http://jbcs.sbq.org.br as PDF file.

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Author Contributions

Pedro E. S. do Nascimento was responsible for formal analysis, investigation, methodology, project administration, supervision and writing-original draft; Vilma M. J. Prado for the data curation, formal analysis, project administration, supervision, and writing-original draft and writing- review and editing; Raphael A. de Jesus for the formal analysis, methodology, project administration, supervision, writing-original draft; Wenes Ramos da Silva for the formal analysis, investigation, methodology and supervision; Alberto Wisniewski Jr. for the conceptualization, funding acquisition, methodology, project administration, supervision and writing-review and editing; José C. F. S. Filho for the formal analysis and methodology; Arie F. Blank for the conceptualization, investigation, methodology and supervision; Daniel A. de Souza for the formal analysis, investigation and methodology; Elma R. S. A. Wartha for the formal analysis, funding acquisition, investigation, methodology, project administration, supervision and validation; Paulo C. L. Nogueira for the conceptualization, funding acquisition, methodology and project administration; Valéria R. S. Moraes for the project administration, supervision, visualization and writing original draft.

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