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Biological studies and chromatograms aided by chemometric analysis in evaluation of seasonality and extraction method of *Croton grewioides* extracts

Vilma Menezes de Jesus Prado[1] · Raphael Amancio de Jesus[1] · Julio Manoel Andrade Oliveira[1] · Arie Fitzgerald Blank[2] · Daniel Pereira Bezerra[3] · Milena Botelho Pereira Soares[3] · Valdenizia Rodrigues Silva[3] · Luciano de Souza Santos[3] · Carmen Lúcia Cardoso[4] · Adriana Ferreira Lopes Vilela[4] · Paulo Cesar de Lima Nogueira[1] · Valéria Regina de Souza Moraes[1]

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Abstract

The genus *Croton* is the second largest genus of Euphorbiaceae family, comprising more than 1300 registered species. The species *Croton grewioides* is endemic in the northeast of Brazil and distributed mainly throughout the caatinga biome presenting, in its essential oils, various compounds with biological activities such as pupicidal, larvicidal, insecticidal, and bactericidal. The secondary metabolites commonly found in their extracts include diterpenes, phenols, steroids, and aglycone and glycoside flavonoids derivatives, which can undergo variations based on climatic changes that modify processes such as photosynthesis, transpiration and nutrient cycle. To understand the effects of seasonal variability and extraction method on the biological activities of polar extracts from *C. grewioides*, fingerprint chromatograms were analyzed aided by chemometric analysis. In vitro assays of cytotoxicity against HL-60 and HepG2 cancer cells and anti-acetylcholinesterase activity were performed. Seasonal effects could only be observed in the chemical profiles of the methanolic and ethanolic extracts from accessions 113 and 126, respectively. Considering the extraction method, the chromatograms of aqueous and hydroalcoholic extracts of all the accessions were similar, but differed from those for the corresponding methanolic and ethanolic extracts. Five hexane partitions were active against HL-60 cell line, displaying inhibition values between 81 and 93%. Only the ethanolic extract of accession 101 (winter), the methanolic partition from the methanolic extract, and the decoction of accession 113 (winter) presented high inhibition values against the acetylcholinesterase enzyme. This study enabled elucidation of the effects of seasonality and extraction method on the biological activities presented by the *C. grewioides* extracts.

Keywords Acetylcholinesterase inhibition · Chromatographic fingerprint · Cytotoxicity · Hierarchical Cluster Analysis · Principal Component Analysis

1 Introduction

Plants are able to withstand temperature and light variations that cause changes in the production of secondary metabolites (Alencar Filho et al. 2017). Beyond these, many others factors can have significant qualitative and quantitative effects on the production of secondary metabolites that represent a chemical interface between plants and the environment. Consequently, the metabolic pathways can be altered, leading to the biosynthesis of different compounds, or the same compounds in different proportions, according to the stimulus provided by the environment in which the plant is located (Morais 2009; Alvarenga-Venutolo et al. 2018).

✉ Valéria Regina de Souza Moraes
valrsmoraes@academico.ufs.br

[1] Departamento de Química, Universidade Federal de Sergipe, São Cristovão, SE 49100-000, Brazil

[2] Departamento de Engenharia Agronômica, Universidade Federal de Sergipe, São Cristovão, SE 49100-000, Brazil

[3] Instituto Gonçalo Moniz, Fundação Oswaldo Cruz, Salvador, BA 40296-710, Brazil

[4] Departamento de Química, Faculdade de Filosofia Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP 14040-901, Brazil

Seasonality can influence the chemical composition of plants, which directly influences their pharmacological properties, since climate changes modify processes such as photosynthesis, transpiration, nutrient cycle, and production of primary and secondary metabolic (Hrichi et al. 2020; Ribeiro et al. 2020).

To the best of our knowledge, studies of the effect of seasonality on *Croton* species have only been based on the chemical compositions of essential oils. In the work of Almeida et al. (2014), studying the oil composition of the leaves of *Croton conduplicatus* Kunth in two different seasons (dry and rainy seasons), it was demonstrated that there was a seasonal difference between the main constituents present, such as 1,8-cineole, *p*-cymene, spathulenol and caryophyllene oxide (dry season) and 1,8-cineole, α-phellandrene, bicyclogermacrene, (*E*)-caryophyllene and spathulenol (rainy season).

In addition to knowing the time when a plant was collected, it is important to select a suitable solvent for obtaining the extract, especially when a specific biological activity is desired. Studies have shown strong relationships between the biological activities exhibited by *Ecballium elaterium* (Cucurbitaceae) extracts and the solvents (diethyl ether, acetone and methanol) used for their preparation (Felhi et al. 2017). Based on that, Sepahpour et al. (2018) and Venkatesan et al. (2019) suggested that the nature of the solvent affected both the chemical profile and the antioxidant activity of extracts from several herbs and spices and *Pinus densiflora*.

The species *C. grewioides* Baill., belonging to the genus *Croton*, the second largest genus of the Euphorbiaceae family, with more than 1300 registered species, is endemic in the northeast of Brazil, being found in several Brazilian states, and is distributed throughout the caatinga biome. Known locally as "canelinha-de-cheiro," the tea made from the leaves and branches of this species has been popularly used for the treatment of influenza, coughs, fevers, and headache (Silva et al. 2016a; Prado et al. 2021). Most of the reported studies concerning this species have investigated the essential oil, which has shown characteristic activities including pupicidal, larvicidal, insecticidal, and bactericidal (Silva et al. 2016a; Medeiros et al. 2017; Castro el al. 2019). So far, the literature only reports two studies concerning phytochemical investigation of extracts of *C. grewioides*, one describing the isolation of derivatives of diterpenes, phenols, and steroids, and the other the isolation of aglycone and glycoside flavonoids (Medeiros 2012; Prado et al. 2021).

Many species of *Croton* display anti-inflammatory, cytotoxic, antirheumatic, antiulcerogenic, antispasmodic, antidiabetic, anticholinesterasic, and analgesic activities, among others (Silva et al. 2016a; Palmeira Júnior et al. 2006). Antitumor activity has been found for essential oils from *C. regelianus* and *C. flavens*, as well as for some compounds isolated from *C. flavens* leaf oils (Sylvestre et al. 2006; Bezerra et al. 2009).

Two pyran-2-one derivatives obtained from *C. crassifolius* Geisel roots showed good cytotoxicity against two human cancer cell lines of HeLa and NCI-446 (Li et al. 2014). Phorbol esters isolated from *C. tiglium* showed potent cytotoxicity against several cell lines including HL60, among others (Wang et al. 2015).

Previous studies showed that extracts from *C. zehntneri* (Lopes et al. 2022), *C. sylvaticus* (Aderogba et al. 2013a), *C. gratissimus*, and *C. zambesicus* (Ndhlala et al. 2013) and some compounds such as quercetin 3-*O*-rhamnoside, kaempferol-3-*O*-rhamnoside, protocatechualdehyde, and *p*-hydroxybenzoic acid, isolated from *C. penduliflorus* (Aderogba et al. 2013b), have already presented anticholinesterasic activities. Thereupon, in this study anti-acetylcholinesterase activity assays, which indicate the capacity of the extracts interfere in the enzymatic catalysis, based on monitoring the effect of the samples in the formation of reaction´s product (*m/z* 104) were performed (Vanzolini et al. 2013).

In addition, in this work, chemometric tools (Principal Component Analysis-PCA and Hierarchical Cluster Analysis-HCA) were used to evaluate the similarities and differences among the chromatographic profiles of aqueous (infusion and decoction), hydroethanolic, and alcoholic (methanol and ethanol) extracts of the leaves of various accessions of *C. grewioides*, collected in two seasons (winter and summer). Studies of the cytotoxic and acetylcholinesterase inhibition activities of these extracts and their partitions were performed as part of an initial screening to determine the antitumor and anti-acetylcholinesterase potentials of this species, since the literature mentions these activities for other species of this genus (Aderogba et al. 2013a, b; Queiroz et al. 2014; Wang et al. 2015; Abreu et al. 2020).

2 Material and methods

Plant material – Leaves of four accessions of *C. grewioides* belong to the Active Germplasm Bank of the Federal University of Sergipe. Voucher herbarium specimens of each accession were deposited under numbers 28256, 25138, 28251, 23259, named as 101, 107, 113 and 126, respectively, at the Federal University of Sergipe Herbarium, Department of Biology (SISGEN register number A8CCB3B), as described by Prado et al. (2021) (Supplementary Material).

Chemicals, reagents and liquid chromatography (LC) system – LC-grade acetonitrile (Tedia, Fairfield, OH, USA) and analytical-grade formic acid (88% v/v, JT Baker, Philipsburg, PA, USA) were used in the LC analysis. Methanol (JT Baker, Philipsburg, PA, USA) and ethanol (PanReac,

Barcelona, Spain) were used for sample preparation. Analytical-grade hexane (Sigma-Aldrich, Steinheim, Germany) was used for liquid–liquid extraction. Deionized water was obtained from a Milli-Q system (Millipore, São Paulo, Brazil).

The analyses employed a liquid chromatography system (Shimadzu, Kyoto, Japan) equipped with a binary solvent pump (LC-20AT), column oven (CTO-20A), degasser (DGU-20A3), autosampler (SIL-20AHT), diode array detector (DAD) (SPD-M20A), and interface (CBM-20A). Data acquisition and processing were performed using LC Solution v. 2.0 Workstation software.

Sample preparation – Aqueous extracts were prepared by the decoction as described by Prado et al. (2021) (Supplementary Material), and infusion methods. For infusion, 2 g of leaves was poured into 200 mL of ultrapure water at 92 °C (heated in a microwave oven) and allowed to rest for 10 min. Both aqueous extracts were filtered through analytical filter paper, frozen, and lyophilized (at − 54 °C and pressure below 90 µ Hg).

Hydroethanolic, ethanolic, and methanolic extracts were prepared using the maceration method described by Prado et al. (2021) (Supplementary Material).

Solid phase extraction procedures – Prior to the LC analyses, the aqueous (decoction, infusion), hydroethanolic, ethanolic, and methanolic extracts were submitted to a solid phase extraction clean-up procedure as described by Prado et al. (2021) (Supplementary Material).

Chromatographic conditions – Chromatographic analysis was performed using a Kinetex C18 analytical column (250 × 4.6 mm i.d., 5 µm particle diameter; Phenomenex, Torrance, CA, USA), maintained at 25 °C. The conditions of analysis were followed as described by Prado et al. (2021) (Supplementary Material).

In vitro cytotoxicity – Human hepatocellular carcinoma (HepG2) and Human promyelocytic leukemia (HL-60) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were cultured as described by Silva et al. (2016b). The statistical analysis was performed following the same method reported by Silva et al. (2016b).

On-flow immobilized acetylcholinesterase inhibition studies – Activity and inhibition studies using immobilized acetylcholinesterase from *Electrophorus electricus* (electric eel) were performed as described by Jesus et al. (2019). Stock solutions (5 mg mL^{-1}) of each sample were prepared in the appropriate solvents, depending on the method of extraction, as follows: ultrapure water (decoction and infusion), 50% ethanol/water (hydroethanolic solution), methanol (methanolic extracts and methanol partitions), methanol (ethanolic extracts), and hexane (hexane partitions). All the solubilizations were assisted by ultrasonication for 5 min at room temperature. Subsequently, each solution was centrifuged for 5 min at 10,000 rpm. Working solutions (2 mg mL^{-1}) were then prepared in ultrapure water.

Liquid–liquid extraction – Aliquots (40 mg) of methanolic extracts (from winter and summer collections) and ethanolic extracts (summer collection) were separately dissolved in 4 mL of methanol and transferred to separation funnels, followed by addition of 4 mL of hexane. The funnels were shaken and vented, after which the two layers were separately drained into clean round bottom flasks. This extraction with hexane was performed three times for each extract. The flowchart in Fig. 1 shows the liquid–liquid extraction process and the yields obtained for the hexane and methanol partitions.

Chemometric analysis – Chemometric analyses were carried out using Pirouette 4.0 software (Infometrix Inc., USA). The data were mean centered (preprocessing), and correlation optimized warping (COW) (Skov et al. 2006) was used for

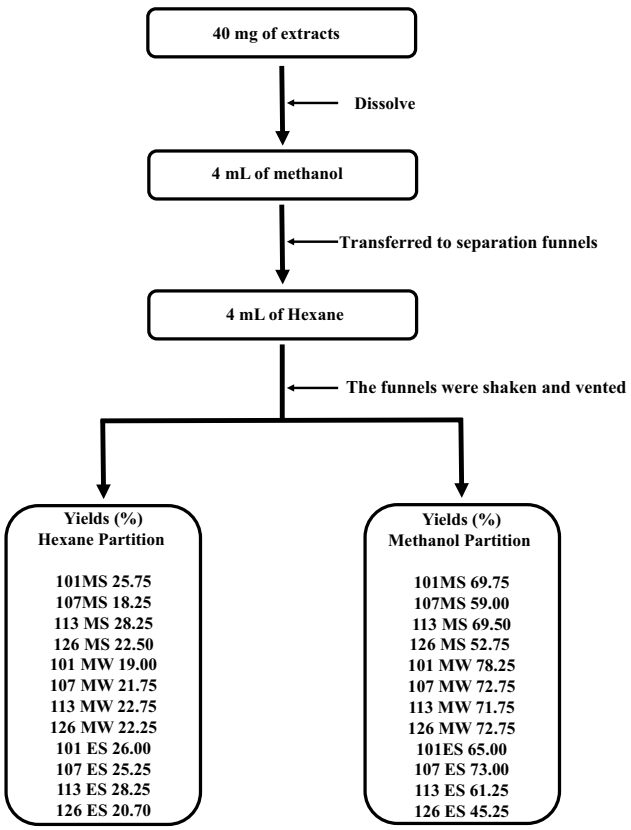

Fig. 1 Schematic flowchart for the solvent extraction process

peak alignment (pretreatment), following the methodology as described by Gomes et al. (2010).

3 Results

Fingerprint chromatograms – The mean fingerprint chromatograms obtained for each sample revealed some of the differences and similarities among them (Fig. 2). There was considerable similarity in terms of the compounds detected in the different samples, although some qualitative and quantitative differences were found, which could be observed by comparing the chromatograms for each variable separately (accession, preparation method, and collection season).

PCA and HCA analyses – A matrix containing 160 lines (samples) and 543 columns (variables, according to retention time) was submitted to PCA after data preprocessing (mean centering) and pretreatment (peak alignment). The resulting biplot of PC1 *versus* PC2 (Fig. 3) explained 49.7% of the data variance (PC1: 34.9%; PC2: 14.8%), with five groups formed from the 160 *C. grewioides* samples, considering the four accessions, five preparation methods, and two collection times (with analysis in quadruplicate in each case).

As shown in Fig. 3, methanolic and ethanolic samples from accession 101 (summer and winter) were assembled into group G1, presenting negative PC1 and positive PC2. All the methanolic and ethanolic samples from accessions 113 and 126 were assembled into group G2, characterized by negative PC1 and PC2 values. The methanolic and ethanolic samples from accession 107 were assembled into group G3, with values close to zero for PC1 and PC2. In addition, group G4 included all the infusion, decoction, and hydroalcoholic extracts from accession 101 with positive values for PC1 and PC2, while group G5 contained all the infusion, decoction, and hydroalcoholic extracts from accessions 107, 113 and 126 with positive values for PC1 and negative values for PC2.

Among these samples, groups G1, G2, and G3 presented similarities and could be combined in group G6 (negative PC1), including methanolic and ethanolic extracts. The samples from groups G4 and G5 could be combined in group G7 (positive PC1), including infusion, decoction, and hydroalcoholic extracts. These results indicated that the chemical profiles for the methanolic and ethanolic extracts were significantly different from the other three, which are in agreement with visual analysis of the differences between these two groups shown in Fig. 2.

As shown in Fig. 3, all the samples from accession 101 were well separated from the other samples obtained for the same extraction method and collection season. It can also be seen that the chemical profiles of the methanolic and ethanolic extracts of accession 107 were different to others from the same type of extraction, forming a separate group (G3). However, the other six accession 107 samples obtained by infusion/decoction/hydroalcoholic extraction (summer and winter) were grouped more closely with others from the same preparation method and collection season (group G5).

The seasonality effect was most evident in G2, highlighting the methanolic extract from accession 113 and the ethanolic extract from accession 126.

The HCA dendrogram (Fig. 4) shows two large initial groups consisting of the 40 samples of *C. grewioides*, with one group formed by the samples of the ethanolic and methanolic extracts and the other formed by the samples of the infusion, decoction, and hydroalcoholic extracts. From these two groups, there was the formation of five groups: (i) group G2, with similarity 0.643; (ii) group G3 and samples 101ES and 101EW, with similarity 0.583; (iii) samples 101MS and 101 MW, with similarity 0.679; (iv) group G5, with similarity 0.658; (v) group G4, with similarity 0.796.

In the HCA dendrogram (Fig. 4), it should be noted that samples 101ES and 101EW were separated from the methanolic samples. However, this variation was not considered significant, since there was proximity among the samples in the scores graph.

The ethanolic and methanolic extracts from accession 107 were separated from the others, showing different characteristic bands for the summer and winter extracts. The winter samples (positive PC2) hence resembling the extracts from accession 101, while the summer extracts (PC2 close to zero) had characteristic bands eluting at 40.4, 42.2, and 44.7 min.

Based on the extraction methods, the characteristic bands for the ethanolic and methanolic extracts were all those between 40.4 and 52.9 min, while the bands at 8.3, 10.4, 14.2, 15.1, 17.7, and 19.3 min were characteristic of the infusion, decoction, and hydroalcoholic extracts.

The loadings graph (Fig. 5) was used to identify the chromatographic bands that differentiated among the samples. Based on the work of Prado et al. (2021), the flavonoids quercetin 3-*O*–*β* –D– galactopyranosyl—(1 → 2)–*α*-L- rhamnopyranoside—(1 → 6)–*α*—L- rhamnopyranoside, quercetin 3-*O*-*β*-D-galactopyranosyl-(1 → 2)-*α*-apiopyranoside-(1 → 6)-*α*-L-rhamnopyranoside, and quercetin 3-*O*-glucopyranoside, which eluted at 14.2, 15.1, and 19.3 min, respectively, were responsible for differentiation of the infusion, decoction, and hydroalcoholic extracts of accessions 107, 113, and 126, relative to accession 101. The latter presented positive PC2 and differed from the other extracts by the presence of a compound that eluted at 17.7 min. Similarly, the methanolic and ethanolic extracts of accession 101 differed from the other accessions with the same preparation method, showing characteristic bands at 48.8, 51.2, 51.6, 52.1, 52.5, and

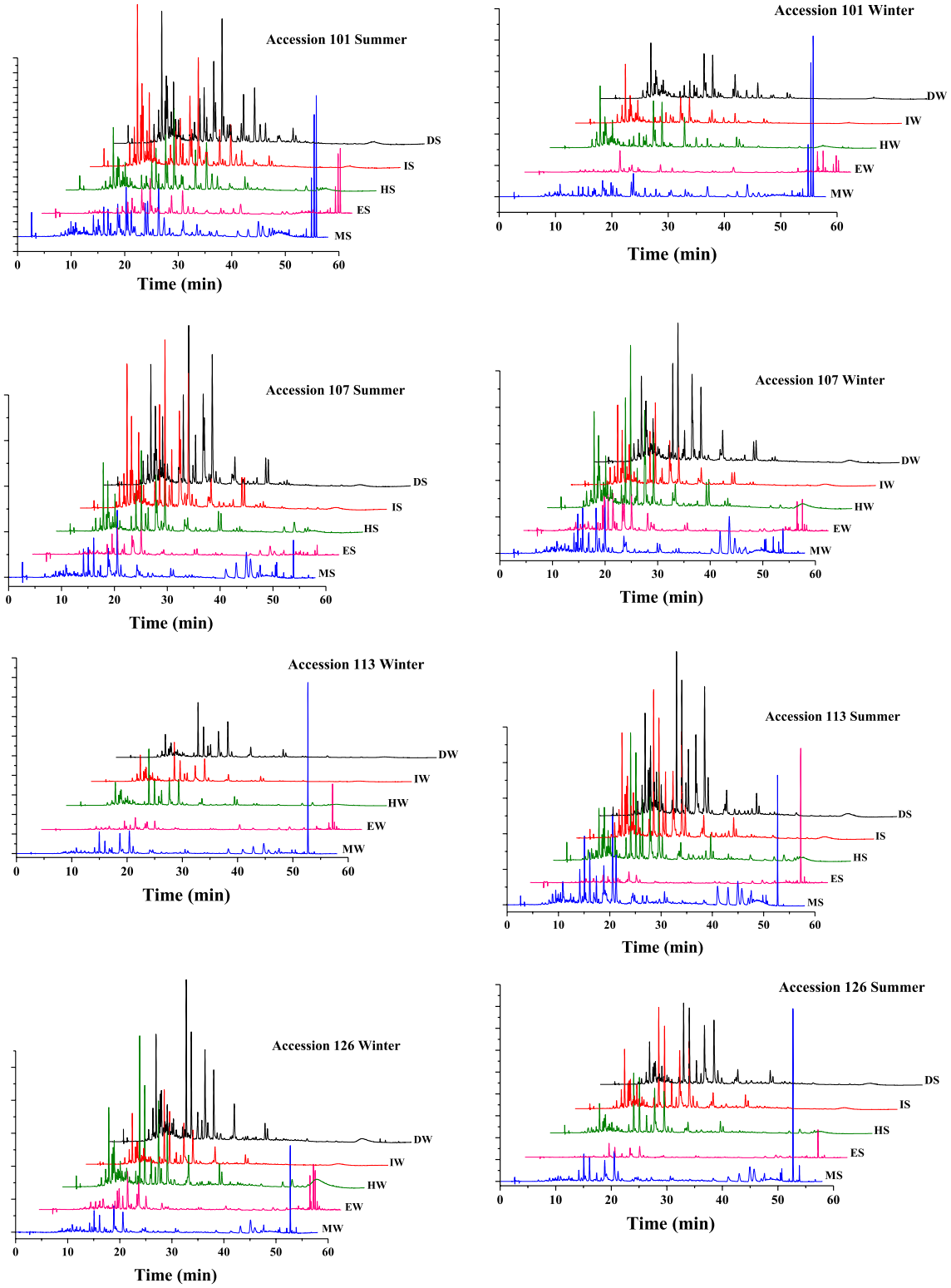

Fig. 2 Fingerprint chromatograms, with detection at 320 nm, for the four accessions of *C. grewioides*

52.9 min. Samples 113 and 126 of these extracts differed from the others, due to the presence of a band at 49.6 min.

In vitro assays of cytotoxicity and inhibition of acetylcholinesterase activity – In vitro cytotoxicity and anti-acetylcholinesterase activity assays were used in initial screening to evaluate the antitumor and anti-acetylcholinesterase

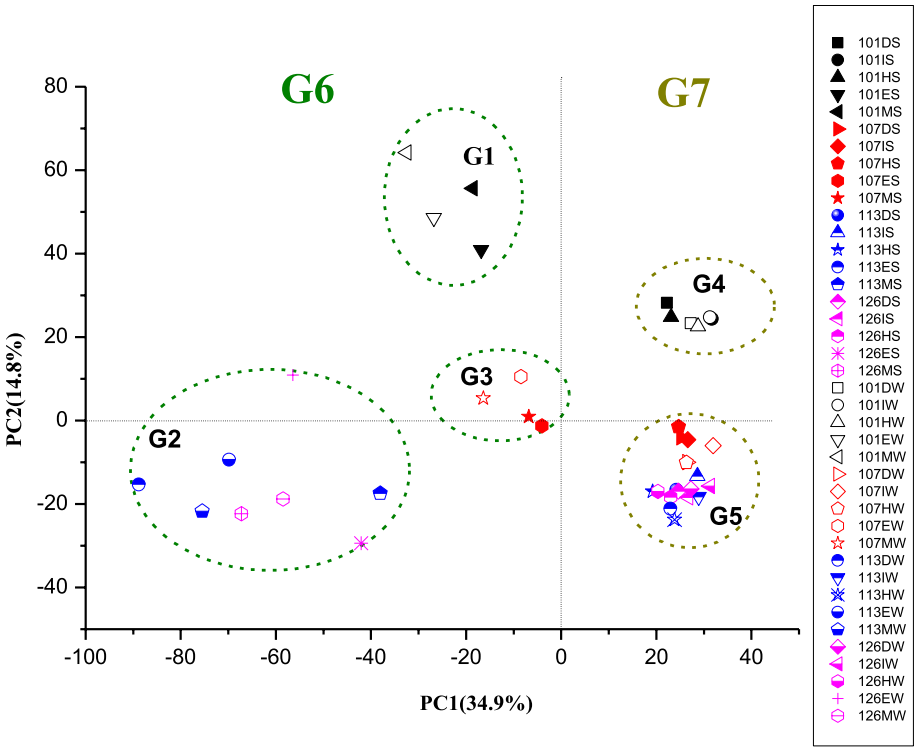

Fig. 3 Scores plot (PC1 vs. PC2) for the samples from *C. grewioides* (accessions 101 in black, 107 in red, 113 in blue, and 126 in pink). G1: 101MS, 101 MW, 101ES, and 101EW. G2: 113MS, 113 MW, 113ES, 113EW, 126MS, 126 MW, 126ES, and 126EW. G3: 107MS, 107 MW, 107ES, and 107EW. G4: 101DS, 101DW, 101HS, 101HW, 101IS, and 101IW. G5: 107DS, 107DW, 107HS, 107HW, 107IS, 107IW, 113DS, 113DW, 113HS, 113HW, 113IS, 113IW, 126DS, 126DW, 126HS, 126HW, 126IS, and 126IW

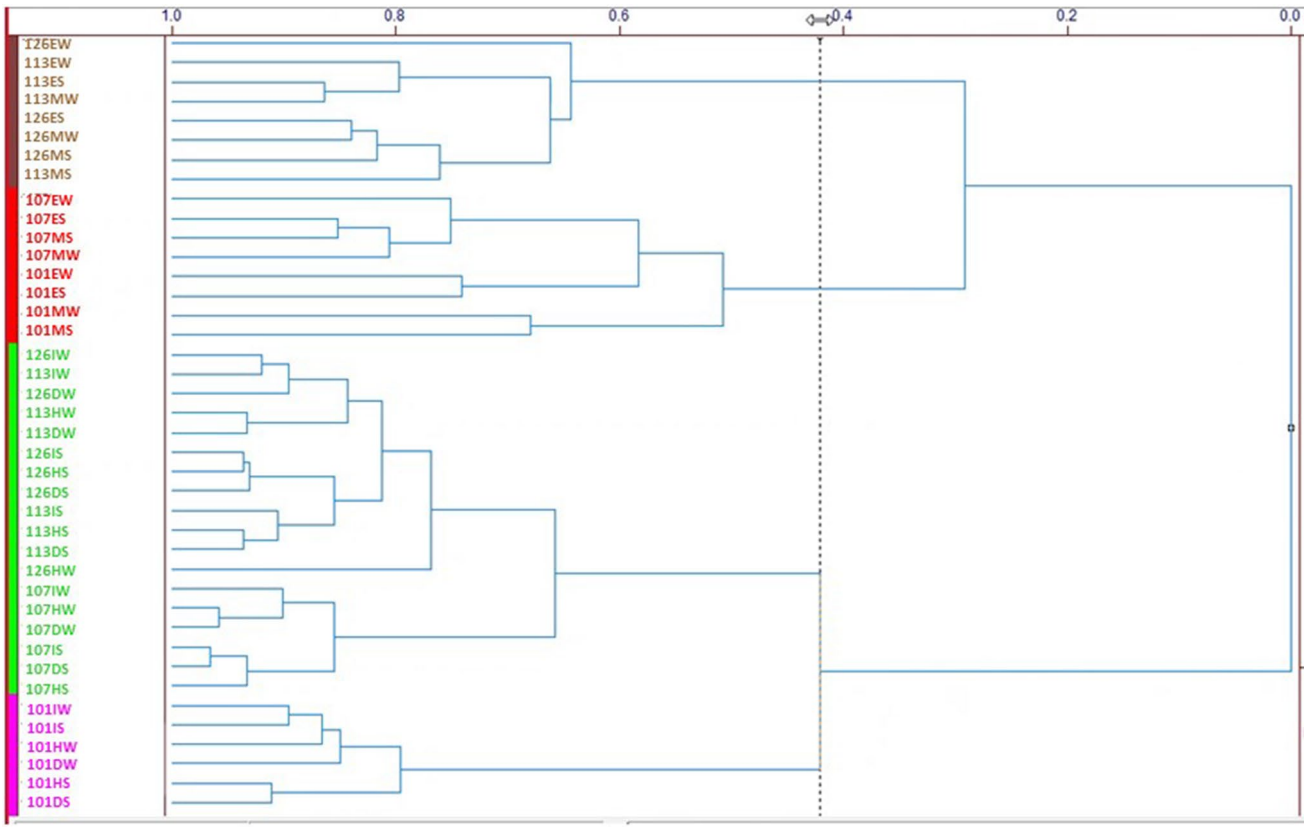

Fig. 4 Dendrogram for the samples obtained from *C. grewioides*

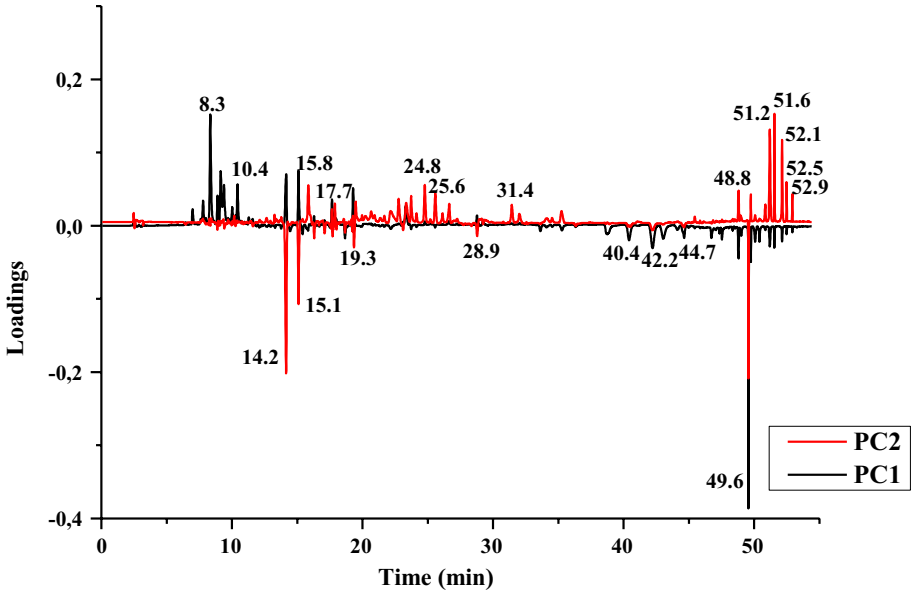

Fig. 5 Loadings plot of the samples obtained from *C. grewioides*

potentials of the *C. grewioides* extracts. Cytotoxicity assays were performed against two human cancer cell lines, HepG2 and HL-60. The extracts were tested at a final concentration of 50 µg mL^{-1}, using the Blue Alamar assay after incubation for 72 h, with doxorubicin as a positive control. The results are shown in Table 1.

The 107MS, 107EW, 101EW, 107ES, and 126EW samples showed the highest percentage inhibitions of HL-60 cell proliferation, with values of 49.26, 47.38, 45.16, 44.19, and 41.07%, respectively. These samples were considered the most promising, suggesting that the chromatographic bands with retention times longer than 37.5 min should be important for these activities, which present negative values for PC1 (Fig. 5). Samples 113IS and 126IS, characterized by the presence of compounds with retention times of 14.2, 15.1, and 19.3 min, presented the next highest inhibition percentages of 38.30 and 33.33%, respectively, against this cell line. These compounds were previously isolated from the decoction extract of sample 107DS and were identified as quercetin 3-*O*-*β*-D-galactopyranosyl-(1 → 2)-*α*-L-rhamnopyranoside-(1 → 6)-*α*-L-rhamnopyranoside, quercetin 3-*O*-*β*-d-galactopyranosyl-(1 → 2)-*α*-apiopyranoside-(1 → 6)-*α*-L-rhamnopyranoside, and quercetin 3-*O*-glucopyranoside, respectively (Prado et al. 2021).

In the inhibition assays against the HepG2 tumor cell line, only sample 101EW displayed a moderate inhibition value of 34.01%. This sample was characterized by the presence of the compounds that eluted after 45.0 min, with the exception of the compound that eluted at 49.6 min.

Based on these results and considering that all the methanolic and ethanolic extracts presented compounds with variable polarities, a liquid–liquid partitioning with hexane was applied to separate the less polar compounds from the more polar ones present in these extracts, enabling determination of the potential cytotoxicity and anti-acetylcholinesterase activity of the resulting partitions. This partitioning was not performed with the ethanolic extracts (winter), due to a lack of sufficient material (Table 1).

As shown in Table 1, with the exception of the methanolic partitions of 113 MW and 126 MW used against the HepG2 tumor cell line, all the partitions displayed higher inhibition percentages against the two cell lines tested, compared to the corresponding original extracts. Notably, the hexane partitions 107MS-H, 126MS-H, 13MS-H, 107 MW-H, and 113ES-H presented HL-60 inhibition percentages of 92.93, 84.66, 84.43, 83.75, and 81.17%, respectively.

Anti-acetylcholinesterase assays are based on obtaining AChE inhibitors that alter central cholinergic function by inhibiting the enzymes that degrade acetylcholine. Table 2 shows the AChE activity inhibition percentages for all the extracts at a concentration of 200 µg mL^{-1}, with galanthamine as the positive control.

Although no extract showed significant activity against AChE, tests were also performed using the partitions from the methanolic (summer and winter) and ethanolic extracts (summer), in order to evaluate the inhibition potentials of the constituents present when the compounds were separated according to their polarity. The only sample that showed increased activity was the methanol partition from 113 MW, with 39.7% inhibition.

None of the extracts evaluated showed strong inhibition of the acetylcholinesterase enzyme, with the highest values being presented by the 101EW (40.8%), 113DW (34.5%), and 101 MW (32.0%) samples. Considering the loadings graph (Fig. 5), the most important

Table 1 Growth inhibition effects of the methanolic and ethanolic extracts and their partitions used against the HepG2 and HL-60 tumor cell lines

Sample[a]	Cell[b] growth inhibition percentage (GI%)[c]		Sample[a]	Cell[b] growth inhibition percentage (GI%)[c]	
	HepG2	HL-60		HepG2	HL-60
101DW	6.28 ± 2.08	15.22 ± 7.75	101DS	3.57 ± 5.96	16.47 ± 4.37
107DW	1.36 ± 0.42	18.73 ± 7.19	107DS	7.16 ± 6.42	23.69 ± 5.77
113DW	1.73 ± 0.98	3.02 ± 1.40	113DS	6.70 ± 2.40	4.04 ± 1.16
126DW	6.98 ± 1.11	4.98 ± 1.19	126DS	1.42 ± 0.83	6.46 ± 1.79
101IW	1.89 ± 4.77	8.04 ± 2.12	101IS	0.79 ± 0.50	15.79 ± 5.82
107IW	10.85 ± 5.41	1.74 ± 9.74	107IS	6.21 ± 1.26	15.29 ± 7.41
113IW	10.62 ± 5.46	11.33 ± 3.96	113IS	1.40 ± 0.60	38.30 ± 5.71
126IW	6.67 ± 1.88	12.45 ± 3.73	126IS	14.92 ± 1.74	33.33 ± 4.84
101HW	17.13 ± 4.43	5.53 ± 1.19	101HS	16.90 ± 5.94	15.81 ± 7.03
107HW	14.93 ± 5.26	22.22 ± 2.79	107HS	23.39 ± 9.35	8.76 ± 1.34
113HW	4.76 ± 1.94	14.41 ± 5.38	113HS	18.03 ± 6.44	16.23 ± 5.46
126HW	9.33 ± 7.91	14.99 ± 3.56	126HS	8.70 ± 1.04	5.04 ± 1.05
101EW	34.01 ± 2.58	45.16 ± 9.67	101ES	4.90 ± 1.23	21.01 ± 8.19
107EW	9.17 ± 3.01	47.38 ± 5.93	107ES	4.25 ± 1.64	44.19 ± 3.74
113EW	2.10 ± 4.10	19.39 ± 7.03	113ES	1.64 ± 0.65	15.13 ± 7.78
126EW	4.43 ± 1.85	41.07 ± 4.65	126ES	1.77 ± 0.69	16.68 ± 7.84
101 MW	2.70 ± 1.24	26.12 ± 5.78	101MS	3.59 ± 1.53	22.69 ± 7.20
107 MW	2.83 ± 1.86	15.44 ± 4.47	107MS	11.29 ± 6.85	49.26 ± 3.16
113 MW	6.42 ± 2.67	2.94 ± 1.59	113MS	13.18 ± 9.21	29.64 ± 7.48
126 MW	10.23 ± 1.89	28.70 ± 6.73	126MS	1.78 ± 0.90	19.65 ± 5.82
Doxorubicin[d]	87.91 ± 5.56	91.28 ± 4.02			
Partitions					
101 MW-H	36.56 ± 8.42	67.07 ± 1.22	101MS-H	28.92 ± 1.92	68.71 ± 6.98
101 MW-M	27.92 ± 7.45	55.78 ± 6.63	101MS-M	18.00 ± 8.48	37.68 ± 5.14
107 MW-H	48.43 ± 9.22	83.75 ± 1.20	107MS-H	38.18 ± 7.82	92.93 ± 0.47
107 MW-M	15.43 ± 1.22	36.03 ± 2.94	107MS-M	19.87 ± 1.89	49.99 ± 3.76
113 MW-H	42.54 ± 7.68	62.78 ± 1.18	113MS-H	63.71 ± 7.12	84.43 ± 0.58
113 MW-M	5.19 ± 2.04	37.25 ± 4.35	113MS-M	38.21 ± 2.06	39.83 ± 0.40
126 MW-H	15.76 ± 4.03	68.13 ± 1.90	126MS-H	15.73 ± 8.14	84.66 ± 0.79
126 MW-M	4.57 ± 3.98	35.18 ± 2.99	126MS-M	15.50 ± 4.37	36.08 ± 1.18
101ES-H	13.92 ± 1.89	53.68 ± 8.29	113ES-H	50.16 ± 1.76	81.17 ± 10.60
101ES-M	21.79 ± 1.36	47.33 ± 5.17	113ES-M	42.24 ± 8.55	43.84 ± 8.12
107ES-H	30.3 ± 2.71	73.38 ± 10.18	126ES-H	26.91 ± 1.13	62.59 ± 9.48
107ES-M	9.39 ± 5.65	28.61 ± 1.01	126ES-M	23.2 ± 3.47	28.58 ± 8.71
Doxorubicin[d]	100.2 ± 1.80	98.27 ± 8.75			

[a]*DW* decoction winter, *DS* decoction summer, *IW* infusion winter, *IS* infusion summer, *HW* hydroalcoholic winter, *HS* hydroalcoholic summer, *EW* ethanolic winter, *ES* ethanolic summer, *MW* methanolic winter, *MS* methanolic summer, *MW-H* methanolic winter-hexane, *MW-M* methanolic winter-methanolic, *MS-H* methanolic summer-hexane, *MS-M* methanolic summer-methanolic, *ES-H* ethanolic summer-hexane, *ES-M* ethanolic summer-methanolic

[b]Cell lines: HepG2 (human hepatocellular carcinoma) and HL-60 (human promyelocytic leukemia)

[c]GI% values are presented as the mean ± SD from three replicates measured by the MTT assay after 72 h of incubation. All extracts were tested at a concentration of 50 μg mL^{-1}

[d]Doxorubicin was used as the positive control

contributors to these activities could have been the compounds quercetin 3-*O*-*β*-D-galactopyranosyl-(1 → 2)-*α*-L-rhamnopyranoside-(1 → 6)-*α*-L-rhamnopyranoside (Rt: 14.2 min), quercetin 3-*O*-*β*-D-galactopyranosyl-(1 → 2)-*α*-apiopyranoside-(1 → 6)-*α*-L-rhamnopyranoside (Rt: 15.1 min), and those that eluted after 37.5 min (except the compound that eluted at 49.6 min).

Table 2 Acetylcholinesterase inhibition activities of the extracts

Sample[a]	% inhibition ± SD[c] ICER-AChE$_{ee}$	Sample[a]	% inhibition ± SD[c] ICER-AChE$_{ee}$
101DW	9.0 ± 1.4	101DS	19.3 ± 5.5
107DW	11.9 ± 0.5	107DS	18.5 ± 4.0
113DW	34.5 ± 1.2	113DS	24.3 ± 2.8
126DW	18.3 ± 5.0	126DS	21.9 ± 0.4
101IW	14.1 ± 1.5	101IS	12.1 ± 3.0
107IW	11.9 ± 0.0	107IS	16.2 ± 0.6
113IW	12.7 ± 0.3	113IS	14.7 ± 2.1
126IW	9.4 ± 1.2	126IS	15.8 ± 0.3
101HW	29.3 ± 2.1	101HS	22.5 ± 0.6
107HW	10.4 ± 2.0	107HS	14.0 ± 2.8
113HW	19.1 ± 1.5	113HS	24.3 ± 4.3
126HW	24.4 ± 0.3	126HS	16.8 ± 0.5
101EW	40.8 ± 0.4	101ES	25.3 ± 1.3
107EW	10.2 ± 0.9	107ES	19.2 ± 2.7
113EW	23.1 ± 0.3	113ES	18.9 ± 0.0
126EW	22.6 ± 1.8	126ES	17.3 ± 2.0
101 MW	32.0 ± 4.9	101MS	23.9 ± 3.7
107 MW	23.4 ± 2.7	107MS	13.1 ± 2.9
113 MW	17.6 ± 0.7	113MS	27.4 ± 3.8
126 MW	20.9 ± 0.7	126MS	15.5 ± 3.4
Galanthamine[b]	87.4 ± 2.2		

[a]*DW* decoction winter, *DS* decoction summer, *IW* infusion winter, *IS* infusion summer, *HW* hydroalcoholic winter, *HS* hydroalcoholic summer, *EW* ethanolic winter, *ES* ethanolic summer, *MW* methanolic winter, *MS* methanolic summer

[b]Positive control of AChE (100 μM)

[c]Mean ± standard deviation

4 Discussion

In this study, to evaluate the individuality of each accession, the effects of seasonality and extraction method on the chemical profiles of the extracts from *C. grewioides*, a method based on LC-DAD analysis was developed to obtain the fingerprint chromatograms. The accessions of *C. grewioides* belong to the Active Germplasm Bank (AGB) of the Federal University of Sergipe, which aims to create and maintain genetic diversity (Prado et al. 2021).

Thus, the influence of seasonality was explored from leaves of four accessions of this species collected during winter (June 2016) and summer (January 2017), while the effect of the extraction method was assessed from the preparation of five polar extracts: decoction, infusion, hydroethanolic, ethanolic, and methanolic. The Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA) pattern recognition methods were applied for evaluation of data in the chromatographic fingerprinting, allowing the classification of the analyzed samples.

Chemometrics is an interfacial discipline that enables the extraction of useful information from large sets of chemical and biochemical data, employing different mathematical and statistical procedures (Kucharska-Ambrożej and Karpinska 2020).

There are several chromatographic techniques including liquid chromatography (LC) that have been used to construct fingerprint chromatograms as an analytical method that can evaluate the relationship between the chemical information and the characteristics of each plant sample, such as differentiation between botanically similar species, the variability between plants collected in different geographical locations, under different climatic and cultivation conditions. This method is recognized as an important quality control tool of herbal samples in view of constantly growing search for natural origin medicines.

In a previous study, the combination of chemical fingerprints and biothermal activities of *Salvia miltiorrhizae*, from different sources, helped to establish fingerprint-activity relationships aided by important chemometrics tools. These findings provided an understanding between these relationships allowing additional exploration of the active components of this species that contribute to the anti-*Pseudomonas aeruginosa* activity (Kong et al. 2017).

In view of our results, it is possible to reinforce the suggestion that accession 101 is a specific genetic variety, since all its extracts were well separated from its other extracts obtained for the same extraction method and collection season, through its projection in the scores plot (Fig. 3). These findings will support our research partners of the "Research Team of Medicinal, Aromatic, Spice and Vegetable Crops," which works with the ex situ harvesting and conservation of native and introduced medicinal and aromatic plants with pharmacological potential. Thus, the combination of chemical and genetic information will help to understand which accession is most promising from a pharmacological point of view, considering also the best extraction method and the time of collection.

As important as knowing the botanical identity of the plant under study for medicinal purposes is to know when it was collected and how the extracts were prepared.

A recently published study showed qualitative and quantitative variations in the composition of polyphenols, chlorophylls, and carotenoids of eight extracts of *Convolvulus althaeoides* L. leaves, collected in two different seasons (winter and spring), and extracted successively with four solvents of increasing polarity and their effects on antioxidant and antifungal activities (Hrichi et al. 2020). The results of this research showed that there are important variations in the compounds between the seasons and that ethyl acetate and ethanol extracts displayed greater antioxidant and antifungal activities against strains

of *Trichophyton rubrum*, *Trichophyton metagrophytes*, *Microsporum canis*, and *Candida* spp.

In the current study, with the aid of scores plot (Fig. 3), it was possible to highlight the variations in the chemical profiles between the methanolic and ethanolic extracts (group G6) of those extracts prepared by infusion, decoction, and hydroalcoholic (group G7). However, a more detailed analysis of this latter group, it can be seen that the extracts from accession 101 formed a separated cluster (group G4) from the others, reinforcing the idea that it is a particular accession.

All the extracts, as well as the partitions from the methanolic and ethanolic extracts, were evaluated against two different tumor cell lines and the acetylcholinesterase enzyme. None of the extracts displayed significant inhibition percentages in these assays, although 107MS and 101EW showed moderate inhibitory effects against HL-60 and HepG2 cell lines, 49.26 and 34.01%, respectively.

Apart from two methanolic partitions (113 MW-M and 126 MW-M) all the partitions displayed higher antitumor activities compared to the corresponding original extracts. The highlights among the tested samples are the hexane partitions 113ES-H, 107 MW-H, 113MS-H, 126MS-H, and 107MS-H showing inhibition percentages against HL-60 ranged from 81 to 92%.

These samples must clearly have contained higher amounts of compounds that acted to increase the inhibition values, relative to the other samples, indicating the specificity of each accession and the effect of seasonality.

These partitions could be considered active against this tumor cell line, and these data corroborate the proposal discussed in the results section that this activity was related to the compounds eluting after 37.5 min, which could have been present as major compounds in the hexane partitions.

Aglycone and glycoside flavonoids derivatives have already been isolated from *Croton* species. Quercetin and 3-*O*-methyl-quercetin, the latter already isolated from *C. grewiodes* (Prado et al. 2021), have also been reported to be responsible for the decrease proliferation of the gliomas cells. Furthermore, it was observed that 3-*O*-methyl-quercetin showed the most significant impact on cell proliferation in glioma cell lines C6 and U87 playing a crucial role for the antiproliferative effect of the extract from *Achyrocline satureioides* (Asteraceae) (Souza et al. 2018).

In this work, the moderate inhibitory effects against HL-60 and HepG2 cell lines suggest that the presence of quercetin-derived flavonoids, in the extracts, is responsible for the activity although more studies are necessary.

Two flavonoid glycosides, quercetin 3-*O*-rhamnoside and kaempferol-3-*O*-rhamnoside isolated from *C. penduliflorus*, exhibited inhibitory properties against acetylcholinesterase through microplate assay, presenting IC_{50} values of 133.9 and 87.9 µM, respectively (Aderogba et al. 2013b). Encouraged mainly by this study, the current work investigated the extracts of *C. grewioides* for their anticholinesterase potentials, considering the fact that these glycoside flavonoids derivatives are metabolites found in *C. grewioides* (Prado et al. 2021).

Unfortunately, only the ethanolic extract of accession 101 (winter collection, 101EW) and the methanol partition from the methanolic extract of accession 113 (winter collection, 113 MW-M) presented higher inhibition values. These samples are characterized, mainly, by the compounds quercetin 3-*O*-*β*-D-galactopyranosyl-(1 → 2)-*α*-L-rhamnopyranoside-(1 → 6)-*α*-L-rhamnopyranoside and quercetin 3-*O*-*β*-D-galactopyranosyl-(1 → 2)-*α*-apiopyranoside-(1 → 6)-*α*-L-rhamnopyranoside. Unlike the results obtained in the antitumor tests, most of the partitions from the methanolic and ethanolic extracts showed no increases in AChE inhibition activity.

The seasonality significantly influenced the chemical profiles of the methanolic and ethanolic extracts for accessions 113 and 126, respectively, belonging to group G2 (Fig. 3). However, among the extracts 113MS, 113 MW, 126ES and 126EW, the most significant difference in inhibition values, against the HL-60 cell line, has been observed for samples from accession 126, showing 16.68% for summer samples and 41.07% for winter samples (Table 1).

On the other hand, among the most active extracts, the winter and summer ethanolic extracts from accession 107 did not differ significantly in their inhibition values against HL-60 cell line, displaying 47.38% and 44.19%, respectively.

Although the observed activities were modest, further investigations of these extracts and their partitions are warranted, with modifications in terms of their preparation and the compounds isolated, given that studies reported in the literature have demonstrated the antitumor and anti-acetylcholinesterase activities of flavonoid derivatives, which occur naturally in this plant species.

Given these results, *C. grewioides* could be considered a potential source of compounds exhibiting dual antitumoral and anti-acetylcholinesterase activities, with the effects of seasonality and methods of extraction and cultivation, among others, influencing the pharmacological activities of the extracts. Future studies will aim to further elucidate the effects of variations of these parameters on the activities, together with isolation and identification of the constituents, based on literature reports suggesting that flavonoid derivatives may be responsible for these two activities (Khan et al. 2018; Patil and Masand 2019; Tavsan and Kayali 2019).

To the best of our knowledge, the antitumoral and anti-acetylcholinesterase activities of these extracts have not been previously reported. Thus, this study provided a preferred way to explore the bioactive secondary metabolites from *C.*

grewioides in a more rational way without the need for their prior isolation and purification.

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Author contributions VMJP (PhD student) and JMAO (undergraduate student) contributed in collecting plant sample, running the laboratory work, analysis of the data and drafted the paper. RAJ contributed to chromatographic analysis. AFB designed the Active Germplasm Bank of the Federal University of Sergipe and supervised the laboratory work on accessions. DPB and CLC designed the biological studies and supervised the laboratory work. MBPS, VRS, LSS and AFLV contributed to biological studies. PCLN supervised the laboratory work and contributed to critical reading of the manuscript. VRSM designed the study, supervised the laboratory work, analysis of the data, drafted the paper and contributed to critical reading of the manuscript. All the authors have approved the submission of the manuscript.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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