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Antioxidant and Cytotoxic Activities and Chemical Profile of Five Amaranthaceae Plants Collected in the South of Brazil

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Abstract

In this study, the antioxidant and cytotoxic activity, the phenolic content and the HPLC-DAD/ESI-MS profile of the ethanolic extracts of five Amaranthaceae plants collected in the south of Brazil were investigated: these were *Alternanthera philoxeroides* (Mart.) Griseb. (I), *Alternanthera hirtula* (Mart.) R.E. Fr. (II), *Alternanthera praelonga* A. St. –Hil. (III), *Froelichia tomentosa* (C. Mart.) Moq. (IV) and *Pfaffia tuberosa* (Spreng.) Hicken (V). The antioxidant potential was determined using the DPPH and ORAC-FL assays. The total phenolic content was measured using the Folin-Ciocalteu reagent. The ethanolic extracts of I, II, IV and V showed high levels of phenolic compounds (3.6-20.0 mg GAE/Kg), as well as high antioxidant activity in both methods. The cytotoxicity of the extracts was investigated *in vitro* against panel of human cancer cell lines and against VERO control. The extract of V exhibited anti-proliferative activity against all cancer cell lines studied (Total Growth Inhibition, TGI<100 μg/mL), except for VERO cell line (TGI=125.2 μg/mL). HPLC-UV/DAD and ESI-MS analyses revealed that the extracts investigated appear to contain phenolic acids and flavonoids as main constituents. Findings from this study demonstrated that the extracts of the Amaranthaceae plants from the south of Brazil may be considered as promising sources of antioxidant and anti-proliferative compounds.

Keywords: Amaranthaceae; Anti-proliferative activity; Antioxidant capacity; Phenolic

Introduction

Free radicals are responsible for lipid peroxidation occurring during production and storage of nutrients and are directly involved in some cancers, cardiovascular disorders, diabetes, Alzheimer's disease, and others human pathologies [1,2]. Antioxidant compounds can be useful to prevent several degenerative diseases or as preservative in food, toiletries and pharmaceutical products [3-7]. Species of the Amaranthaceae family are able to accumulate phenolic substances and these are closely related to the antioxidant activity due to their capacity to scavenge free radicals, protect against lipid peroxidation and quench reactive oxygen species [3-5,8,9]. Members of this plant family are used in the extraction of natural pigments for application as food colorants and antioxidants [1,4,5]. In addition, species of Amaranthaceae have been used in Brazilian folk medicine for the treatment of diseases such as infections and inflammation [10-15].

Previous chemical analyses of Amaranthaceae plants have demonstrated the occurrence of phenolic acids, aurones, betacyanins, betalains, betaxanthins, chromoalkaloids, ecdysteroids, flavonoids, protoalkaloids, saponins, steroids, and triterpenes [1,2,4,11-15].

These facts prompted us to investigate the chemical composition and antioxidant and cytotoxic activities of the ethanolic extracts of five Amaranthaceae plants collected in the south of Brazil; these were *A. philoxeroides* (Mart.) Griseb. (**I**), *A. hirtula* (Mart.) R.E. Fr. (**II**), *A. praelonga* A. St. –Hil. (**III**), *F. tomentosa* (C. Mart.) Moq. (**IV**) and *P. tuberosa* (Spreng.) Hicken (**V**).

Materials and Methods

Plant material

The whole plants of *A. philoxeroides* (Mart.) Griseb. (**I**), *A. hirtula* (Mart.) R.E. Fr. (**II**), *A. praelonga* A. St. –Hil. (**III**), *F. tomentosa* (Mart.) Moq. (**IV**) and *P. tuberosa* (Spreng.) Hicken (**V**) were collected in Rio Grande do Sul State, Brazil. Voucher specimens were deposited at the herbarium PACA/IAP-UNISINOS, RS, Brazil, register number PACA 103073, PACA 103235, PACA 103233, PACA 103236 and PACA 103234 for plants **I**, **II**, **II**, **IV** and **V**, respectively.

Extracts preparation and chemical analysis

Dried and powdered leaves of each plant (50 g) were extracted with ethanol (3 × 300 mL) at room temperature. The solvent was removed under reduced pressure to give the crude extracts. Phytochemical tests for sterols/triterpenes, phenolic compounds, tannins, saponins, and alkaloids were carried out according to usual methodology [16].

Quantitative determination of total soluble phenols

The extracts, dissolved in methanol, were analyzed for their total soluble phenolic content according to the Folin-Ciocalteau colorimetric method [17], using gallic acid as reference. The results were expressed as mg of gallic acid equivalents (GAE) per g of extract or fraction (mg of GAE/g). The analyses were performed in triplicate.

Radical scavenging activity using the DPPH method

The antiradical activity of extracts was determined using the stable 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) [18]. The test was performed in 96-well microplates. Fifty μL of a 250 μM DPPH solution in MeOH was added to a range of solutions of different concentrations (7 serial 3-fold dilutions to give a final range of 100 to 1.6 μg mL^{-1}) of extracts to be tested in MeOH (10 μL). Absorbance at 517 nm was

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determined 30 min after the addition of each of the compounds tested, and the percentage of activity was calculated. The pure compounds quercetin, vitexin, caffeic acid, chlorogenic acid from Sigma-Aldrich were also tested and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) from Sigma-Aldrich was used as positive control. All samples were tested in triplicate. The antioxidant activity of each sample was expressed as the SC_{50} value, which is the concentration in μg mL^{-1} of each extract that scavenged 50% of the DPPH radicals. All of the results are expressed as the mean of 3 different trials.

Evaluation of antioxidant capacity by ORAC assay

The antioxidant capacity of the ethanolic extract by oxygen radical absorbance capacity (ORAC) kinetic assay, was carried out according to the method established by Ou and co-workers [19,20], with modifications [4]. The data were expressed as μmoles of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) equivalents (TE) per g of the the extract on a dry weight basis (μmol of TE/g) and as relative Trolox equivalent for pure compounds. Quercetin, vitexin, caffeic acid and chlorogenic acid from Sigma-Aldrich were also tested. The analyses were performed in triplicate.

HPLC-DAD/ESI-MS analyses

Crude extracts of Amaranthaceae plants were diluted in a solution containing 50% (v/v) chromatographic grade methanol and 50% (v/v) deionized water and 0.5% ammonium hydroxide. In the fingerprinting ESI-MS analysis, the general conditions were: source temperature 100°C, capillary voltage 3.0 kV and cone voltage 30 V. For measurements in the negative ion mode, ESI(-)-MS, 10.0 μL of concentrated NH_4OH was added to the sample mixture having a total volume of 1000 μL yielding 0.1% as a final concentration. ESI-MS was performed by direct infusion with a flow rate of 10 μL min mL^{-1} using a syringe pump. Structural analysis of single ions in the mass spectrum of each extract was performed by ESI-MS/MS. The ion with the *m/z* of interest was selected and submitted to 15-45 eV collisions with argon in the collision quadrupole. The compounds were identified by comparison of their ESI-MS/MS fragmentation spectra with literature data [4,8,21-23]. HPLC analyses were conducted using an RP-18 column (Lichrospher, 5 μm, 225/4.6 mm, Merck). The mobile phase consisted of a linear gradient combining solvent A (acetonitrile) and solvent B (water/acetic acid, 99:1, v/v, pH 2.88) as follows: 15% A (15 min), 15-20% A (7 min), 20% A (5 min), 20-40% A (5 min), 40% A (5 min), 40-15% A (3 min). The analyses were carried out in triplicate at a flow rate of 0.8 mL/min and an injection volume of 20 μL. The UV-DAD detector was set to record between 200 and 600 nm, and the UV chromatograms were measured at 254 and 330 nm. The samples were the crude extract and vitexin standard (Sigma-Aldrich) at 1 mg/mL.

Cytotoxicity assay

It was used the U251 (glioma, CNS), UACC-62 (melanoma), MCF-7 (breast), NCI-ADR/RES (ovarian-resistant), 786-0 (kidney), NCI-H460 (lung, no small cells), PC-3 (prostate), OVCAR-3 (ovarian), HT-29 (colon), and VERO (no cancer) cell lines. The extracts of the five Amaranthaceae plants studied and the compounds detected in the extracts quercetin, vitexin and caffeic acid (from Sigma-Aldrich) were assayed. The assay was done as described previously [24]. Briefly, the cells were distributed in 96 well plates (100 μL cells/well) and exposed to various concentrations of samples (0.25, 2.5, 25.0 and 250.0 μg/mL) in DMSO (0.1%) at 37°C, with 5% of CO_2, for 48 h. The final concentration of DMSO did not affect the cell viability. A 50% trichloroacetic acid solution was added and after incubation for 30 min at 4°C, the cells were washed and dried. Cell proliferation was determined by spectrophotometric quantification (at 540 nm) of the cellular protein content using sulphorodamine B. The experiments were carried, at least, in triplicate and the concentration necessary to total growth inhibition (TGI) was calculated in μg/mL. Doxorubicin was used as positive control.

Statistical analysis

Data are reported as mean (%RSD, relative standard deviation) of triplicate determinations. The statistical analyses were carried out using the Microsoft Excel 2010 software package (Microsoft Corp., Redmond, WA).

Results

Antioxidant analysis

Some of the samples exhibited antioxidant activity that was concentration-dependent in DPPH assays, with SC_{50} values varying from 12.5 to 150.2 μg/mL. In DPPH assays the highest antioxidant activity was exhibited by the ethanol extract of the whole plant of *P. tuberosa* (SC_{50}=11.5 μg/mL), followed by extracts of *F. tomentosa* (SC_{50}=43.6 μg/mL), and *A. Philo Xero ides* (SC_{50}=150.2 μg/mL). In comparison with pure compounds, the SC_{50} of the extract of *P. tuberosa* was more active than chlorogenic acid and similar to that of caffeic acid (Table 1). Moreover, in the ORAC-FL kinetic assay, the extracts showed good antioxidant capacity with values between 291.0 and 6403.0 μmol of Trolox equivalents per gram of extract (μmol of TE/g). In ORAC-FL assays, the highest antioxidant activity was exhibited by the ethanol extract of the whole plant of *F. tomentosa*, followed by extracts of *P. tuberosa*, *A. philoxeroides* and *A. hirtula*. The ethanolic extract of *A. praelonga* showed small antioxidant capacity in both the DPPH and ORAC-FL assays (Table 1).

Thus, the results documented in this study demonstrate that some of the extracts analyzed showed antioxidant capacity (measured by DPPH and ORAC assays) and this activity correlates with the total phenolic content (measured by FCR assay). Moreover, the isolated compounds caffeic acid, quercetin and vitexin showed considerable antioxidant capacity (Table 1) and were detected in some of the studied species.

Sample Ethanol extract	Phenol content[a] (mg of GAE/g)[b]	DPPH assay, SC_{50}[a] (μg/mL)[c]	ORAC assay[a] (μmol of TE/g)[d]
Alternanthera philoxeroides (I)	<3.6	150.2 (1.2)	1019.2 (2.4)
Alternanthera hirtula (II)	3.6 (1.2)	>200.0	897.0 (1.5)
Alternanthera praelonga (III)	<3.6	>200.0	291.0 (2.2)
Froelichia tomentosa (IV)	20.0 (2.5)	43.6 (2.1)	6403.0 (2.6)
Pfaffia tuberosa (V)	17.2 (1.4)	11.5 (1.8)	1830.2 (2.4)
Quercetin	-	8.3 (2.1)	5.6 (0.9)[e]
Vitexin	-	18.5 (3.1)	1.0 (1.2)[e]
Caffeic acid	-	11.2 (2.4)	2.9 (2.0)[e]
Chlorogenic acid	-	12.8 (1.5)	2.6 (1.5)[e]
Trolox*	-	3.8 (1.8)	-

[a]Mean value (%RSD, relative standard deviation) of triplicate assays. [b]Total phenolics data expressed as mg of gallic acid equivalents per g (mg of GAE/g). [c]DPPH assay data expressed as SC_{50} (concentration that inhibited 50% of the DPPH radical) in μg per mL. [d]ORAC data expressed as μmol of Trolox equivalents per g (μmol of TE/g). [e]ORAC data expressed as relative Trolox equivalent, mean (%RSD, relative standard deviation) of triplicate assays. *Experimental positive control. -: not evaluated.

Table 1: Total phenol content and antioxidant capacity by the DPPH and ORAC assays of ethanol extracts of Amaranthaceae species.

Cytotoxicity assay

Moreover, we also show that extracts of Amaranthaceae plants were able to inhibit cell growth tumor (Table 2). Our results demonstrated that the extracts of Amaranthaceae, mainly in the plants IV and V, exhibited anti-proliferative activity against human cancer cell lines studied (Total Growth Inhibition, TGI <100 μg/mL) and have no cytotoxicity against VERO cell line (TGI ≥ 125.2 μg/mL). Our results demonstrated that *P. tuberosa* exhibited anti-proliferative activity against all human cancer cell lines studied (Total Growth Inhibition, TGI<100 μg/mL), except for VERO no cancer cell line (TGI=125.2 μg/mL), promissory study indicating a future for the species.

HPLC-DAD/ESI-MS analyses

The ESI-MS fingerprint technique with direct infusion [21-23] was used to characterize the presence of compounds with antioxidant capacity in this work. The extracts were analyzed by direct insertion in the negative ion mode as this method is sensitive and selective for the identification of polar organic compounds with acidic sites, such as phenolic organic acids. Deprotonated forms of the compounds of interest were then selected, dissociated and their ESI-MS/MS were compared with those of standards. The compounds were identified by comparison of their ESI-MS/MS fragmentation spectra with fragmentation spectra of the authentic standard samples and with literature data [4,21]. These analyses showed that some of constituents in the analyzed samples coincided with the mass and ESI-MS/MS fragmentation spectra of phenolic acids and flavonoids, suggesting presence of these compounds in the some of analyzed plants (Table 3; Figures 1 and 2).

To confirm the presence of the flavonoid vitexin, HPLC-UV/DAD analyses of standards samples of flavonoids glycosides and of the crude extract were performed. A peak was produced with a retention time coincident with the standard sample and with the same absorption spectrum in the UV/DAD analysis. Moreover, the identity of vitexin in *Alternanthera* samples plant I and II were confirmed through co-elution with an authentic standard sample (Figure 3).

Discussion

Amaranthaceae has been reported as one of the many vegetables rich in antioxidant [2-4,8]. The study showed that the extracts from Amaranthaceae plants collected in the south of Brazil, present antioxidant activity. In accordance with literature data, samples with values ≥ 1000.0 μmol of TE/g can be considered to have good antioxidant capacity in this assay [19,20].

The isolated compounds caffeic acid, quercetin and vitexin showed considerable antioxidant capacity (Table 1) and were detected in some of the studied species (Table 2), for example in *F. tomentosa,* and *P. tuberosa* that showed the best antioxidant activity in DPPH and ORAC-FL assays.

The differences in the antioxidant capacity of the species studied between the two assays is due to differences in sensitivity of the chemical reactions involved in each test and/or showing correlation with concentration in the antioxidant agents in the matrix: ORAC is a hydrogen atom transfer reaction based assay (HAT) and DPPH is a single electron transfer reaction based assay (ET). It is apparent that the hydrogen atom transfer reaction is a key step in the radical chain reaction. Therefore, the HAT based method is more relevant to the radical chain-breaking antioxidant capacity [20].

Phenolic compounds have been presented as important compounds in combating free radical production mainly due to their chemical structure and redox capacity, allowing them to act as reducing agents, hydrogen donating, neutralizing free radicals [25,26], chelating of transition metals and inhibiting lipid peroxidation [27]. In addition, these compounds in biological systems possess pharmacological properties, acting as preventative agents against diseases related to oxidative stress [26,28].

In this study, phytochemical screening showed the presence of triterpenes/sterols and phenolic compounds in all samples. These results are in agreement with previous reports of flavonoids [4,8,11], triterpenes [15] and phenolic compounds [1] in Amaranthaceae species in the genera *Alternanthera, Pfaffia* and *Froelichia* [4,5,8,15,29] and with the HPLC-DAD/ESI-MS profile of these five Amaranthaceae plants studied (Table 3; Figures 1-3). Thus, direct infusion electrospray ionization mass spectrometry (ESI-MS) and HPLC-DAD analyses provided important information about bioactive components present in the Amaranthaceae extracts that are widely reported as potent antioxidants, probably explaining the antioxidant activity of the studied extracts [3-5,8]. The isolated compounds caffeic acid, quercetin and vitexin showed considerable antioxidant capacity (Table 1) and were detected in some of the studied species with similar results documented in the literature data [4,8,26,30].

However, analysis by ESI-MS is dependent on the ionization capacity of these substances in the sample, the concentration of these molecules in the matrix and the complexity of the matrix. Thus, it is not always possible only by this analytical technique has the necessary security to get the identity of all the constituents of a sample. For to extend the analytical security is possible the used of chromatographic techniques associated with universal

	TGI (μg/mL)[b]					
Cell lines[a]	***Alternanthera hirtula* (I)**	***Alternanthera philoxeroides*(II)**	***Alternanthera praelonga* (III)**	***Froelichia tomentosa* (IV)**	***Pfaffia tuberosa* (V)**	***Doxorubicin***
	Ext EtOH	Ext EtOH	Ext EtOH	Ext EtOH	Ext EtOH	
u	>250	>250	43.21	39.92	>250	0.51
m	>250	>250	73.58	13.73	>250	0.29
7	>250	>250	>250	55.60	>250	1.27
4	>250	>250	>250	83.22	>250	4.13
p	>250	>250	>250	31,45	>250	0.78
o	>250	>250	>250	100.8	>250	3.60
h	>250	>250	>250	92.81	>250	>25
k	29.39	9.51	30.52	3.46	0.42	0.05
v	>250	>250	>250	125.2	>250	11.28

[a]Human cancer cell lines: u=UACC-62 (melanoma); m=MCF-7 (mamma); 7=786-O (kidney); 4=NCI-H460 (lung); p=PC-3 (prostate); o=OVCAR-3 (ovary); h=HT-29 (colon); k=K562 (leukemia). Non cancer cell line: v: VERO (epithelial cell from green monkey kidney). [b]TGI: Total Growth Inhibition – concentration that inhibited cell growth by 100%.

Table 2: Concentration of extracts from Amaranthaceae plants able to inhibit total cell growth.

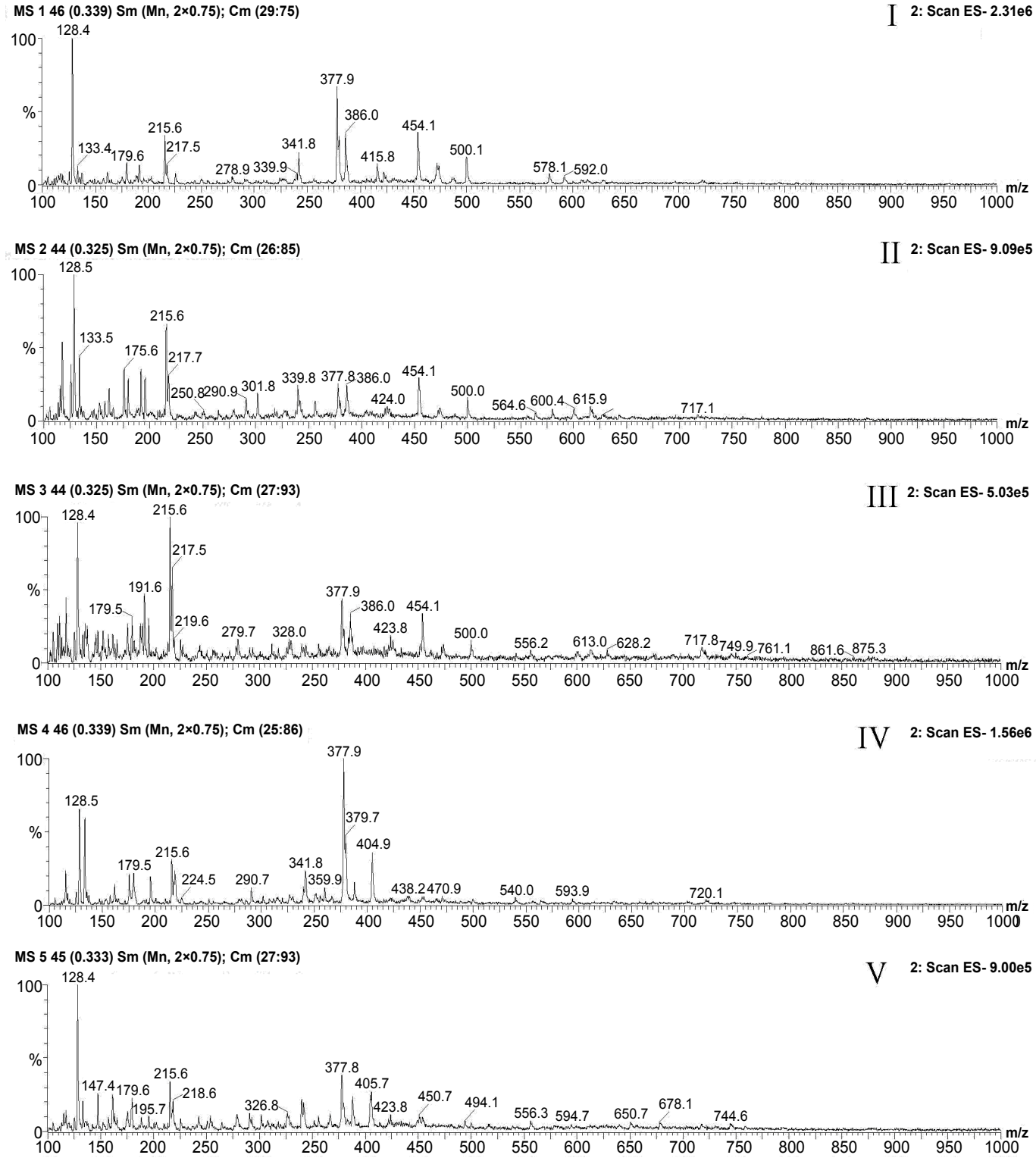

Figure 1: ESI-MS fingerprints of ethanol extracts from five Amaranthaceae whole plants from the south of Brazil (**I**: *Alternanthera philoxeroides,* **II**: *Alternanthera hirtula*, **III**: *Alternanthera praelonga*, **IV**: *Froelichia tomentosa,* and **V**: *Pfaffia tuberosa*).

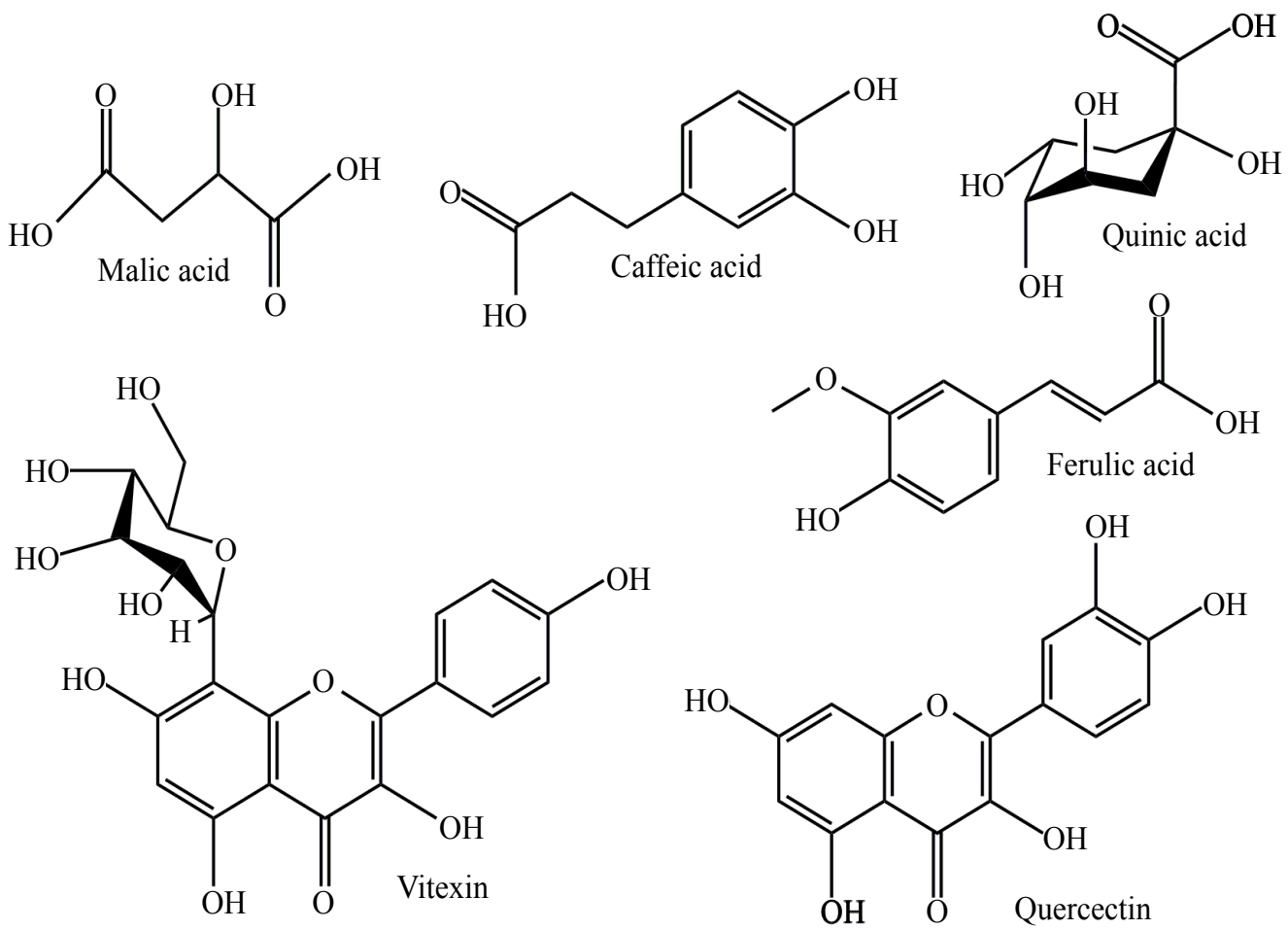

Figure 2: Compounds identified in ethanol extracts from Amaranthaceae plants of the South of Brazil.

ESI-MS ions (*m/z*)							
Compound	**Amaranthaceae plants**					**Deprotonated ions $[M-H]^-$ *m/z***	**MS/MS ions *m/z***
	I	**II**	**III**	**IV**	**V**		
Malic acid	+	+	-	+	+	133	15 eV: 133→115
Caffeic acid	+	+	+	+	+	179	15 eV: 179→125
Quinic acid	+	+	+	+	+	191	25 eV: 191→173, 127, 111, 93, 85
Ferulic acid	-	+	+	+	+	195	15 eV: 193→178, 149, 134
Quercetin	-	+	-	+	+	301	15 eV: 301
Vitexin	+/-	+/-	+/-	-	-	431	15 eV: 431

Legend: +: detected; -: not detected; +/-: possible presence (**I**: *Alternanthera philoxeroides*, **II**: *Alternanthera hirtula*, **III**: *Alternanthera praelonga*, **IV**: *Froelichia tomentosa*, and **V**: *Pfaffia tuberosa*)

Table 3: Compounds identified in ethanol extracts from five Amaranthaceae species from the south of Brazil using ESI(-)-MS/MS analyses.

detection systems, resources joining separation of sample constituents and the spectrum peak of each chromatography such as HPLC-UV/DAD and HPLC-MS. In This study was what happened to the vitexin flavonoid that showed low ionization capacity in the analysis of conditions for ESI-MS, leaving doubts as to ion detection with *m/z* 431 in the samples of plants I, II and III, but it was possible to confirm their presence with the analysis by HPLC-UV/DAD with the standard sample authentic employment in plants I and II. The presence of C-glycoside flavones in plants of the genus *Alternanthera* is documented in literature data [4,8]. In the case of the sample of plant III it was not possible to confirm the presence of vitexin flavonoid in the analysis of conditions by ESI-MS and HPLC-UV/DAD, and this flavonoid may be present in the sample but in a concentration below the limit of detection in the HPLC-UV/DAD analysis.

Extracts of Amaranthaceae plants were able to inhibit cell growth tumor. These results indicate good anti-proliferative effect for these plants and suggest the potential of Amaranthaceae plants as a preventive agent against cancer and as source of bioactive compounds [31,32]. The EtOH extracts of *A. hirtula* (I), *A. philoxeroides* (II) and *P. tuberosa* (V) exhibited selective antiproliferative activity for K562 (leukemia) cancer cell line, with TGI varying of 9.51-30.52 µg/mL and no demonstrated toxicity against VERO cell (non cancer cell, epithelial cell from green monkey kidney, TGI>250 µg/mL). On the other hand, the EtOH extract of F. *tomentosa* showed cytotoxicity against all the cell lines tested, including to VERO cell line (TGI=125.2 µg/mL). Flavonoids and phenolic acids were identified in these bioactive extracts and anticancer activities have been reported for these compounds. The antitumor activity of vitexin, quercetin and caffeic acid were previously observed against leukemic cell line [30,33,34]. Zhou et al. documented that vitexin-induced cytotoxic effect is through the induction of apoptosis, which is mediated by activation of caspases

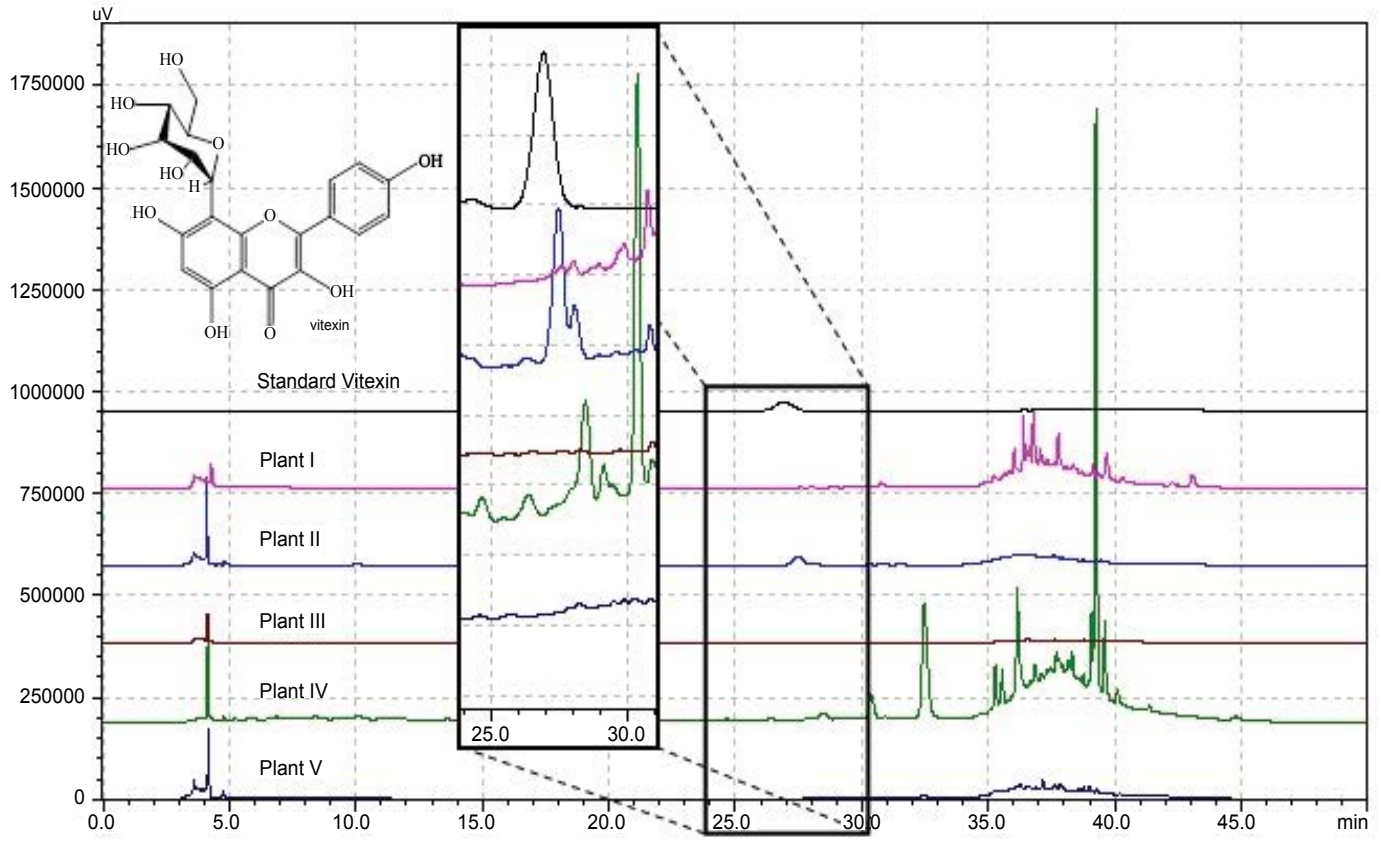

Figure 3: HPLC chromatograms of the ethanol extract for identifying of the flavonoid vitexin: plant **I**: *Alternanthera philoxeroides,* plant **II**: *Alternanthera hirtula,* plant **III**: *Alternanthera praelonga,* plant **IV**: *Froelichia tomentosa,* and plant **V**: *Pfaffia tuberosa*.

[35]. Therefore, the presence of these compounds in these bioactive extracts of Amaranthaceae plants may be responsible, at least in part, for antioxidant and cytotoxic activities observed.

Conclusions

In summary, in this study, ethanolic extracts of five Amaranthaceae plants collected in the south of Brazil showed high levels of phenolic compounds, as well as high antioxidant activity and promising cytotoxic activity against some cancer cell lines and may be considered as promising sources of antioxidant and anti-proliferative compounds, such as phenolic acids and flavonoids. This is the first report of the antioxidant and anti-proliferative activities of the ethanoic extracts form whole plants, suggest that species are a rich source of biologically active compounds. Further studies to confirm the identity of detected compounds and investigating the role of these extracts and constituents in biological systems are necessary to better define their potential therapeutic and preventive uses against ROS-dependent chronic diseases and as a source of cytotoxic agent useful for *in vivo* applications in cancer treatment.

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