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Bioactivity and chemical characterization in hydrophilic and lipophilic compounds of *Chenopodium ambrosioides* L.

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Abstract

The bioactive properties (antioxidant and antitumour activities, and hepatotoxicity) of the infusion and methanolic extracts of *Chenopodium ambrosioides* L., a plant commonly used in Portuguese folk medicine, were compared. The chemical composition in hydrophilic (sugars, organic acids and phenolic compounds) and lipophilic (fatty acids and tocopherols) fractions were determined. In general, the infusion revealed higher antioxidant activity, while the methanolic extract was the only one showing antitumour effects against colon, cervical and hepatocellular carcinoma cell lines. No toxicity in non-tumour cells was observed either for the infusion or the extract. The studied plant proved to be a good source of natural antioxidants and other bioactive compounds, which may have industrial use. As far as we know, this is the first detailed chemical characterization and bioactivity evaluation of *C. ambrosioides* methanolic extract and infusion.

Keywords: Chenopodium ambrosioides L.; Antioxidant activity; Antitumour activity; Chemical compounds

1. Introduction

Oxidative stress is an imbalance between the generation of reactive oxygen species (ROS; which include unstable oxygen radicals such as superoxide radical and hydroxyl radical and non-radical molecules like hydrogen peroxide) and the body's antioxidant defence capacity, having an important role in normal cell functioning. When produced in excess ROS can have harmful effects, affecting cellular lipids, proteins and DNA, leading to their modification, and often destruction, and inhibiting their normal function (Valko et al., 2007; Rosenfeldt et al., 2013). Relevant diseases such as cancer, diabetes, cirrhosis, heart disease or dementia disorders, as well as aging process have been associated with the uncontrolled production of free radicals (Valko et al., 2007; Halliwell, 2012).

Some plants traditionally used have medicinal properties with great potential for therapeutic applications in the treatment of some of the aforementioned diseases, since they are a natural source of bioactive compounds, including antioxidants, such as polyphenols, vitamins, carotenoids, unsaturated fatty acids and sugars, which can be useful for various applications, especially as food additives and in health promotion as ingredients in formulations of functional foods and nutraceuticals (Ramarathnam, Osawa, Ochi, & Kawakishi, 1995; Skerget et al., 2005).

Chenopodium ambrosioides L. (Amaranthaceae; syn: *Dysphania ambrosioides* (L.) Mosyakin & Clemants) is an example of a plant formerly used in Portuguese traditional medicine, normally consumed as infusion of its dried leaves and flowering stems. It is an exotic plant from Central and South America that in former times was introduced by migrants from those countries. Nowadays the species has escaped to wild and can be occasionally found in pathways and near homegardens. It has diverse pharmacological

applications in the treatment of influenza, cold or gastrointestinal and respiratory ailments, as well as vomiting, antihelmintic, healing of skin ulceration caused by *Leishmania* species, anti-inflammatory and antitumor properties (Nascimento et al., 2006; Cruz et al., 2007; Carvalho, 2010; Kamel, El-Emam, Mahmoud, Fouda, & Bayaumy, 2011).

Studies on chemical characterization and bioactivity evaluation of this plant, particularly in the most consumed form (infusion) are scarce. The present work aims to characterize the chemical composition of *C. ambrosioides* in hydrophilic (sugars, organic acids and phenolic compounds) and lipophilic (fatty acids and tocopherols) molecules, as also some bioactive properties (antioxidant and antitumour activities, and hepatotoxicity) of its infusion and methanolic extract.

2. Materials and methods

2.1. Sample

Chenopodium ambrosioides L. (Amaranthaceae) (English names: Epazote, wormseed, Jesuit's tea, Mexican tea; Local names: Té; chá-bravo; chá de Santa Marinha), also known as *Dysphania ambrosioides* (L.) Mosyakin & Clemants, (Amaranthaceae), used to be cultivated in homegardens in Bragança (Northeastern Portugal). Nowadays it is less frequent in gardens and there are some specimens growing wild nearby the local villages. However, if available, inflorescences and upper leaves are still wild gathered, dried and used as herbal infusions. The material was collected in Varge (Bragança) from different plants considering the species availability and local consumers' criteria for medicinal use. A sample was made putting together all the material from several specimens.

Voucher specimens are deposited at the Herbarium of the Escola Superior Agrária de Bragança (BRESA). The samples were lyophilized (FreeZone 4.5, Labconco, Kansas City, MO, USA), reduced to a fine dried powder (20 mesh) and mixed to obtain a homogenate sample.

2.2. Standards and Reagents

Acetonitrile (99.9%), n-hexane (97%) and ethyl acetate (99.8%) were of HPLC grade from Fisher Scientific (Lisbon, Portugal). The fatty acids methyl ester (FAME) reference standard mixture 37 (standard 47885-U) was purchased from Sigma (St. Louis, MO, USA), as also were other individual fatty acid isomers and standards: L-ascorbic acid, tocopherols (α-, β-, γ-, and δ-isoforms), sugars (D(-)-fructose, D(+)-melezitose, D(+)-sucrose, D(+)-glucose, D(+)-trehalose and D(+)-raffinose pentahydrate), organic acids and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid). Phenolic compounds were purchased from Extrasynthèse (Genay, France). Racemic tocol, 50 mg/mL, was purchased from Matreya (Pleasant Gap, PA, USA). 2,2-Diphenyl-1- picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Foetal bovine serum (FBS), L-glutamine, hank's balanced salt solution (HBSS), trypsin-EDTA (ethylenediaminetetraacetic acid), penicillin/streptomycin solution (100 U/mL and 100 mg/mL, respectively), RPMI-1640 and DMEM media were from Hyclone (Logan, USA). Acetic acid, ellipticine, sulphorhodamine B (SRB), trypan blue, trichloroacetic acid (TCA) and Tris were from Sigma Chemical Co. (St Louis, MO, USA). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA). All other chemicals and solvents were of analytical grade and purchased from common sources.

2.3. Evaluation of bioactive properties

2.3.1. Samples preparation. The methanolic extract was obtained from the lyophilized plant material. The sample (1 g) was extracted by stirring with 25 mL of methanol (25 ºC at 150 rpm) for 1 h and subsequently filtered through Whatman No. 4 paper. The residue was then extracted with 25 mL of methanol (25 ºC at 150 rpm) for 1 h. The combined methanolic extracts were evaporated at 40 ºC (rotary evaporator Büchi R-210, Flawil, Switzerland) to dryness.

The infusion was also obtained from the lyophilized plant material. The sample (1 g) was added to 200 mL of boiling distilled water and left to stand at room temperature for 5 min, and then filtered under reduced pressure. The obtained infusion was frozen and lyophilized.

Methanolic extract and infusion were redissolved in *i)* methanol and water, respectively (final concentration 2.5 mg/mL) for antioxidant activity evaluation, or *ii)* water (final concentration 8 mg/mL) for antitumour activity evaluation. The final solutions were further diluted to different concentrations to be submitted to distinct bioactivity evaluation in *in vitro* assays. The results were expressed in *i)* EC_{50} values (sample concentration providing 50% of antioxidant activity or 0.5 of absorbance in the reducing power assay) for antioxidant activity, or *ii)* GI_{50} values (sample concentration that inhibited 50% of the net cell growth) for antitumour activity. Trolox and ellipticine were used as positive controls in antioxidant and antitumour activity evaluation assays, respectively.

2.3.2. Antioxidant activity. DPPH radical-scavenging activity was evaluated by using an ELX800 microplate reader (Bio-Tek Instruments, Inc; Winooski, VT, USA), and calculated as a percentage of DPPH discolouration using the formula: [(A_{DPPH}-

$A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution containing the sample at 515 nm, and A_{DPPH} is the absorbance of the DPPH solution. Reducing power was evaluated by the capacity to convert Fe^{3+} into Fe^{2+}, measuring the absorbance at 690 nm in the microplate reader mentioned above. Inhibition of β-carotene bleaching was evaluated though the β-carotene/linoleate assay; the neutralization of linoleate free radicals avoids β-carotene bleaching, which is measured by the formula: β-carotene absorbance after 2h of assay/initial absorbance) × 100. Lipid peroxidation inhibition in porcine (*Sus scrofa*) brain homogenates was evaluated by the decrease in thiobarbituric acid reactive substances (TBARS); the colour intensity of the malondialdehyde-thiobarbituric acid (MDA-TBA) was measured by its absorbance at 532 nm; the inhibition ratio (%) was calculated using the following formula: $[(A - B)/A] \times 100\%$, where A and B were the absorbance of the control and the sample solution, respectively (Pinela et al., 2012).

2.3.3. Antitumour activity. Five human tumour cell lines were used: MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer), HCT-15 (colon carcinoma), HeLa (cervical carcinoma) and HepG2 (hepatocellular carcinoma). Cells were routinely maintained as adherent cell cultures in RPMI-1640 medium containing 10% heat-inactivated FBS (MCF-7, NCI-H460 and HCT-15) and 2 mM glutamine or in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin (HeLa and HepG2 cells), at 37 ºC, in a humidified air incubator containing 5% CO_2. Each cell line was plated at an appropriate density (7.5×10^3 cells/well for MCF-7, NCI-H460 and HCT-15 or 1.0×10^4 cells/well for HeLa and HepG2) in 96-well plates and allowed to attach for 24 h. Cells were then treated for 48 h with various extract concentrations. Following this incubation period, the adherent cells were fixed

by adding cold 10% trichloroacetic acid (TCA, 100 µL) and incubated for 60 min at 4 ºC. Plates were then washed with deionised water and dried; sulphorhodamine B solution (0.1% in 1% acetic acid, 100 µL) was then added to each plate well and incubated for 30 min at room temperature. Unbound SRB was removed by washing with 1% acetic acid. Plates were air dried, the bound SRB was solubilised with 10 mM Tris (200 µL) and the absorbance was measured at 540 nm in the microplate reader mentioned above (Guimarães et al., 2013).

2.3.4. Hepatotoxicity. A cell culture was prepared from a freshly harvested porcine liver obtained from a local slaughter house, and it was designed as PLP2. Briefly, the liver tissues were rinsed in hank's balanced salt solution containing 100 U/mL penicillin, 100 µg/mL streptomycin and divided into 1×1 mm^3 explants. Some of these explants were placed in 25 cm^2 tissue flasks in DMEM medium supplemented with 10% fetal bovine serum, 2 mM nonessential amino acids and 100 U/mL penicillin, 100 mg/mL streptomycin and incubated at 37 ºC with a humidified atmosphere containing 5% CO_2. The medium was changed every two days. Cultivation of the cells was continued with direct monitoring every two to three days using a phase contrast microscope. Before confluence was reached, cells were subcultured and plated in 96-well plates at a density of 1.0×10^4 cells/well, and cultivated in DMEM medium with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin (Abreu et al., 2011).

2.4. Chemical composition in hydrophilic compounds

2.4.1. Sugars. Free sugars were determined by high performance liquid chromatography coupled to a refraction index detector (HPLC-RI). Dried sample powder (1.0 g) was spiked with the melezitose as internal standard (IS, 5 mg/mL), and was extracted with

40 mL of 80% aqueous ethanol at 80 ºC for 30 min. The resulting suspension was centrifuged (Centurion K24OR refrigerated centrifuge, West Sussex, UK) at 15,000*g* for 10 min. The supernatant was concentrated at 60 ºC under reduced pressure and defatted three times with 10 mL of ethyl ether, successively. After concentration at 40 ºC, the solid residues were dissolved in water to a final volume of 5 mL and filtered through 0.2 µm nylon filters from Whatman (Pinela et al., 2012). The equipment of analysis consisted of an integrated system with a pump (Knauer, Smartline system 1000, Brelin, Germany), degasser system (Smartline manager 5000), auto-sampler (AS-2057 Jasco, Easton, MD) and an RI detector (Knauer Smartline 2300). Data were analysed using Clarity 2.4 Software (DataApex). The chromatographic separation was achieved with a Eurospher 100-5 NH_2 column (4.6 × 250 mm, 5 mm, Knauer) operating at 30 ºC (7971 R Grace oven). The mobile phase was acetonitrile/deionized water, 70:30 (*v/v*) at a flow rate of 1 mL/min. The compounds were identified by chromatographic comparisons with authentic standards. Quantification was performed using the internal standard method and sugar contents were further expressed in g per 100 g of dry weight (dw).

2.4.2. Organic acids extraction and analysis. Organic acids were determined using ultra-fast liquid chromatography coupled to a photodiode array detector (UFLC-PDA). Samples (~2 g) were extracted by stirring with 25 mL of meta-phosphoric acid (25ºC at 150 rpm) for 45 min and subsequently filtered through Whatman No. 4 paper. Before analysis, the sample was filtered through 0.2 µm nylon filters (Barros, Pereira, Ferreira, 2013). The analysis was performed using a Shimadzu 20A series UFLC (Shimadzu Corporation, Kyoto, Japan). Separation was achieved on a SphereClone (Phenomenex, Torrance, CA, USA) reverse phase C_{18} column (5 µm, 250 mm × 4.6 mm i.d.)

thermostatted at 35 ºC. The elution was performed with sulphuric acid (3.6 mM) using a flow rate of 0.8 mL/min. Detection was carried out in a PDA, using 215 and 245 nm (for ascorbic acid) as preferred wavelengths. The organic acids found were quantified by comparison of the area of their peaks recorded at 215 and 245 nm with calibration curves obtained from commercial standards of each compound: ascorbic acid (y=8E+07x+55079; R^2=1); citric (y=1E+06x+4170.6; R^2=1); fumaric acid (y=172760x+52193; R^2=0.999); malic acid (y=952269x+17803; R^2=1); oxalic acid (y=1E+07x+96178; R^2=0.999); quinic acid (y=601768x+8853.2; R^2=1). The results were expressed in g per 100 g of dry weight (dw).

2.4.3. Phenolic compounds extraction and analysis. The previously described methanolic extract and infusion were dissolved in water:methanol (80:20, *v/v*) and water, respectively (final concentration 1 mg/mL) and analysed using a Hewlett-Packard 1100 chromatograph (Hewlett-Packard 1100, Agilent Technologies, Santa Clara, CA, US) with a quaternary pump and a diode array detector (DAD) coupled to an HP Chem Station (rev. A.05.04) data-processing station. A Waters Spherisorb S3 ODS-2 C_{18}, 3 µm (4.6 mm × 150 mm) column thermostatted at 35 °C was used. The solvents used were: (A) 0.1% formic acid in water, (B) acetonitrile. The elution gradient established was isocratic 15% for 5 min, 15% B to 20% B over 5 min, 20-25% B over 10 min, 25-35% B over 10 min, 35-50% for 10 min, and re-equilibration of the column, using a flow rate of 0.5 mL/min. Double online detection was carried out in the DAD using 280 nm and 370 nm as preferred wavelengths and in a mass spectrometer (MS) connected to HPLC system via the DAD cell outlet.

MS detection was performed in an API 3200 Qtrap (Applied Biosystems, Darmstadt, Germany) equipped with an ESI source and a triple quadrupole-ion trap mass analyzer

that was controlled by the Analyst 5.1 software. Zero grade air served as the nebulizer gas (30 psi) and turbo gas for solvent drying (400 ºC, 40 psi). Nitrogen served as the curtain (20 psi) and collision gas (medium). The quadrupols were set at unit resolution. The ion spray voltage was set at -4500V in the negative mode. The MS detector was programmed for recording in two consecutive modes: Enhanced MS (EMS) and enhanced product ion (EPI) analysis. EMS was employed to show full scan spectra, so as to obtain an overview of all of the ions in sample. Settings used were: declustering potential (DP) -450 V, entrance potential (EP) -6 V, collision energy (CE) -10V. EPI mode was performed in order to obtain the fragmentation pattern of the parent ion(s) in the previous scan using the following parameters: DP -50 V, EP -6 V, CE -25V, and collision energy spread (CES) 0 V. Spectra were recorded in negative ion mode between *m*/*z* 100 and 1000.

The phenolic compounds present in the samples were characterised according to their UV and mass spectra and retention times compared with standards when available. For the quantitative analysis of phenolic compounds, a 5-level calibration curve was obtained by injection of known concentrations (2.5-100 μg/mL) of different standards compounds: *p*-coumaric (y=884.6x+184.49; R^2=0.999); ferulic acid (y=505.97x-64.578; R^2=0.999); isorahmetin-3-O-rutinoside (y=327.42x+313.78; R^2=0.999); luteolin-6-*C*-glucoside (y=508.54x-152.82; R^2=0.997); luteolin-7-*O*-glucoside (y=80.829x-21.291; R^2=0.999); kaempferol-3-*O*-glucoside (y=288.55x-4.05; R^2=1); kaempferol-3-*O*-rutinoside (y=239.16x-10.587; R^2=1); quercetin-3-*O*-glucoside (y=253.52x-11.615; R^2=0.999) and quercetin-3-*O*-rutinoside (y=281.98x-0.3459; R^2=1). The results were expressed in mg per 100 g of dry weight (dw).

2.5. Chemical composition in lypophilic compounds

2.5.1. Fatty acids. Fatty acids were determined by gas-liquid chromatography with flame ionization detection (GC-FID)/capillary column, after trans-esterification procedure. Fatty acids (obtained after Soxhlet extraction) were methylated with 5 mL of methanol:sulphuric acid:toluene 2:1:1 (*v:v:v*), during at least 12 h in a bath at 50 ºC and 160 rpm; then 3 mL of deionised water were added, to obtain phase separation; the FAME were recovered with 3 ml of diethyl ether by shaking in vortex , and the upper phase was passed through a micro-column of sodium sulphate anhydrous, in order to eliminate the water; the sample was recovered in a vial with Teflon, and before injection the sample was filtered with 0.2 µm nylon filter from Whatman (Pinela et al., 2012). The analysis was carried out with a DANI model GC 1000 instrument equipped with a split/splitless injector, a flame ionization detector (FID at 260 ºC) and a Macherey–Nagel (Düren, Germany) column (50% cyanopropyl-methyl-50% phenylmethylpolysiloxane, 30 m × 0.32 mm i.d. × 0.25 µm d_f). The oven temperature program was as follows: the initial temperature of the column was 50 ºC, held for 2 min, then a 30 ºC/min ramp to 125 ºC, 5 ºC/min ramp to 160 ºC, 20 ºC/ min ramp to 180 ºC, 3 ºC/min ramp to 200 ºC, 20 ºC/min ramp to 220 ºC and held for 15 min. The carrier gas (hydrogen) flow-rate was 4.0 mL/min (0.61 bar), measured at 50 ºC. Split injection (1:40) was carried out at 250 ºC. Fatty acid identification was made by comparing the relative retention times of FAME peaks from samples with standards. The results were recorded and processed using the CSW 1.7 Software (DataApex 1.7) and expressed in relative percentage of each fatty acid.

2.5.2. Tocopherols. Tocopherols were determined by HPLC (equipment described above), and a fluorescence detector (FP-2020; Jasco). BHT solution in hexane (10 mg/mL; 100 µL) and IS solution in hexane (tocol; 50 µg/mL; 400 µL) were added to the

sample prior to the extraction procedure. The samples (~500 mg) were homogenized with methanol (4 mL) by vortex mixing (1 min). Subsequently, hexane (4 mL) was added and again vortex mixed for 1 min. After that, saturated NaCl aqueous solution (2 mL) was added, the mixture was homogenized (1 min), centrifuged (5 min, 4000*g*) and the clear upper layer was carefully transferred to a vial. The sample was re-extracted twice with hexane. The combined extracts were taken to dryness under a nitrogen stream, redissolved in 2 mL of n-hexane, dehydrated with anhydrous sodium sulphate, filtered through 0.2 µm nylon filters from Whatman, transferred into a dark injection vial prior to the analysis (Pinela et al., 2012). The fluorescence detector was programmed for excitation at 290 nm and emission at 330 nm. The chromatographic separation was achieved with a Polyamide II (250 mm × 4.6 mm i.d.) normal-phase column from YMC Waters operating at 30 ºC. The mobile phase used was a mixture of n-hexane and ethyl acetate (70:30, *v/v*) at a flow rate of 1 mL/min, and the injection volume was 20 µL. The compounds were identified by chromatographic comparisons with authentic standards. Quantification was based on calibration curves obtained from commercial standards of each compound using the IS methodology. The results were expressed in mg per 100 g of dry weight (dw).

2.6. Statistical analysis

Three samples were used and all the assays were carried out in triplicate. The results are expressed as mean values and standard deviation (SD). The results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test with $\alpha = 0.05$. This treatment was carried out using SPSS v. 18.0 program.

3. Results and Discussion

3.1. Evaluation of bioactive properties

The results obtained in the evaluation of the bioactive properties (antioxidant and antitumour activities, and hepatotoxicity) of the infusion and the methanolic extract of *C. ambrosioides* are given in **Table 1**. The infusion gave higher DPPH scavenging activity and β-carotene bleaching and TBARS inhibitions than the methanolic extract. The latter revealed higher reducing power. The essential oil extracted from the leaves of *C. ambrosioides* (Kumar, Kumar, Dubey, & Tripathi, 2007) was also reported to show powerful antioxidant activity. To the best of our knowledge, no reports are available on the infusion or methanolic extract of the aforementioned plant.

The effects of *C. ambrosioides* methanolic extract and infusion on the growth of five human tumour cell lines (MCF-7, NCI-H460, HCT-15, HeLa and HepG2), represented as the concentrations that caused 50% of cell growth inhibition (GI_{50}), are also summarized in **Table 1**. The infusion of *C. ambrosioides* did not show any antitumour potential; however, the methanolic extract presented some activity on HCT-15, HeLa and HepG2 cell lines. It should be highlighted that no hepatotoxicity in non-tumour cells was observed for any of the samples (GI_{50} > 400 μg/mL). Trolox and ellipticine were used as positive controls of antioxidant and antitumour activities evaluation assays, respectively, but comparison with the samples should be avoided, because they are individual compounds and not mixtures.

3.2. Chemical composition in hydrophilic compounds

The chemical composition of the samples in sugars and organic acids was also analyzed and the results are shown in **Table 2**. The sugars found were fructose, glucose, sucrose and trehalose, sucrose being the most abundant.

Oxalic, quinic, malic, ascorbic, citric and fumaric acids were also identified and quantified (**Table 2)**, being oxalic acid the most abundant organic acid. Some organic acids (*e.g.*, citric acid) have been reported as having antioxidant capacity (Hraš, Halodin, Knez, & Bauman, 2000).

Phenolic compounds found in *C. ambrosioides* are presented in **Table 3** and **Figure 1**. Thirty-five compounds were detected, eight of which were phenolic acid derivatives (hydroxycinnamic acid derivatives). Among them, five compounds (peaks 1-3, 5 and 9) were *p*-coumaric acid derivatives identified according to their UV spectra and pseudomolecular ion. Peak 9 was identified as *trans p*-coumaric acid by comparison of its UV spectrum (λ_{max} 312 nm) and retention time with a commercial standard. Peak 1 was identified as a *p*-coumaroyl pentoside acid according to its pseudomolecular $[M-H]^-$ ion (*m/z* at 295) and the release of fragments at *m/z* 163 [*p*-coumaric acid-H]$^-$ (-132 mu, pentose) and *m/z* 119 (loss of 132+44 mu, pentose + CO_2). Peaks 2, 3 and 5 presented pseudomolecular ions $[M-H]^-$ at *m/z* 287 and 387 releasing the same fragment ions at *m/z* 163 and 119, which allowed assigning them to *p*-coumaroyl acid derivatives, although their precise identities could not be established. The other three phenolic acid derivatives were identified as ferulic acid derivatives based on the observation of the ions at *m/z* 193 ([ferulic acid-H]$^-$) and 149 ([ferulic acid-CO_2-H]$^-$). Peak 14 could be identified as free ferulic acid by comparison of its UV spectrum (λ_{max} 326 nm) and retention time with a commercial standard. Peak 4 was associated to a feruloyl pentoside acid based on its molecular ion fragmentation pattern similar to peak 1, whereas no precise identity could be established for peak 6.

The remaining phenolic compounds corresponded to flavone and flavonol derivatives, most of them derived from quercetin (λ_{max} around 354 nm and MS^2 fragment at *m/z* 301;

12 compounds) and kaempferol (λ_{max} around 346 nm and MS^2 fragment at *m/z* 285; 11 compounds) (**Table 3**). Quercetin 3-*O*-rutinoside (peak 17), quercetin 3-*O*-glucoside (peak 21) and kaempferol 3-*O*-rutinoside (peak 24) were positively identified according to their retention, mass and UV-vis characteristics by comparison with commercial standards.

Peak 10 ($[M-H]^-$ at *m/z* 609) could be interpreted as a quercetin *O*-diglycosides in which each of the sugar moieties are located at different positions on the aglycone, owing to the observation of fragments derived from the loss of each sugar residue. However, it might also be rationalised as a quercetin *O*-rhamnosyl-glucoside, in which the fragment at *m/z* 447 would correspond to the loss of the terminal glucose of the dissacharide, whereas that at *m/z* 463 might be rationalised as produced by an internal rearrangement in the sugar moieties following the loss of the internal dehydrated glucose/pentose and further linkage of the terminal rhamnose to the aglycone (Ma, Cuyckens, Heuvel, & Claeys, 2001). In that case, the greater abundance of the Y_0 ion (*m/z* at 301; aglycone) than Y_1 ion (*m/z* at 447; breakdown of the interglycosidic linkage) might support the existence of a 1,2 interglycosidic linkage (Cuyckens, Rozenberg, Hoffmann, & Claeys, 2001), which allow the identification of peak 10 as quercetin 3-*O*-neohesperidose.

Peaks 15 and 20, both with a pseudo molecular ion $[M-H]^-$ at *m/z* 579 releasing fragments at *m/z* 447 (-132 mu; pentiosyl residue) and 301 (-132-146 mu; loss of pentosyl+ rhamnosyl residues), could be assigned as quercetin *O*-rhamnosyl-pentosides in which the pentose is the terminal unit owing to the lack of a fragment at *m/z* 433, which should result from the loss of the rhamnose residue if both sugars were located at different positions on the aglycone. The observation that $Y_0 > Y_1$ ion in the case of peak 20 might point to a 1,2 interglycosidic linkage, whereas a 1,6 linkage might exist in peak 15 where $Y_1 > Y_0$ (Cuyckens et al., 2001). Peaks 29 and 30, both possessing a

pseudo molecular ion $[M-H]^-$ at *m/z* 623 and releasing fragments at *m/z* 447 (-176 mu; loss of a glucuronyl residue) and 301 (-176-146 mu; loss of glucuronyl+rhamnosyl residues) should correspond to quercetin *O*-rhamnosyl-glucuronides. Furthermore, as mentioned above, in peak 30 a 1,2 interglycosidic linkage could be observation ($Y_0 > Y_1$), whereas a 1,6 linkage might exist in peak 29 ($Y_1 > Y_0$). Peak 34 can be assigned to an acetyl derivative of peak 30 owing to its pseudomolecular ion ($[M-H]^-$ at *m/z* 665) 42 mu higher than that peak.

The pseudomolecular ion of peak 19 ($[M-H]^-$ at *m/z* 593) is coherent with a quercetin derivative bearing two rhamnosyl residues. In principle, it can be supposed that each sugar is located at different positions on the aglycone as suggested by the formation of a fragment ion at *m/z* 447 from the loss of one of the rhamnosyl moieties, although the possibility that they constituted a disaccharide cannot be disregarded, either.

Peak 11 ($[M-H]^-$ at *m/z* 741) can be assigned to a quercetin derivative bearing pentosyl, rhamnosyl and hexosyl residues, based on the loss of 440 u (132+146+162 u) to yield the corresponding aglycone (*m/z* at 301, quercetin). The fact that the three moieties were lost simultaneously suggested that they might constitute a trisaccharide *O*-linked to the aglycone. Similarly, peak 12 would be associated to a quercetin *O*-disaccharide consisting of a pentose and a hexose.

Peak 8 ($[M-H]^-$ at *m/z* 755) would correspond to a quercetin derivative possessing two rhamnosyl and one glucosyl moieties. The observation of a fragment at *m/z* 609 from the lost of a rhamnosyl residue (-146 mu) points to this sugar is located on the aglycone in a position different to the other two sugars that should constitute a disaccharide. The presence of quercetin 3-*O*-rutinoside (peak 17) as majority flavonoid in the plant might suggest that peak 8 could be quercetin 3-*O*-rutinoside-*O*-rhamnoside.

Similar reasoning as for the quercetin derivatives has been applied for assigning the identities of kaempferol (peaks 13, 16, 18, 22, 23, 25, 26, 31, 32 and 35) and isorhamnetin derivatives (peaks 27, 28 and 33), as indicated in **Table 3**.

Finally, peak 7 ($[M-H]^-$ at *m/z* 579) was assigned to a flavone, luteolin *C*-hexoside-*O*-pentoside, based on its fragmentation. Thus, the ion at *m/z* 447 could be interpreted as the loss of the pentosyl moiety (-132 mu) and a fragment of 120 mu characteristic of the cleavage of pyran ring in the more strongly linked *C*-hexoses, whereas the ion at *m/z* 417 might correspond to the loss of the hexosyl moiety and a fragment of 30 mu resulting from CH_2O functional group of the hexose, also observed in the case of *C*-hexoses (Abad-Garcia, Garmon-Lobato, Berrueta, Gallo, & Vicente, 2008). The fragments ions at *m/z* 447 and 285 would correspond to the respective losses of the pentosyl and hexosyl moieties, respectively.

Flavonoids were the major phenolic compounds present in this sample (768 mg/100 g dw), being quercetin (46.98%) and kaempferol derivatives (45.91%) the most abundant. Quercetin 3-*O*-rutinoside was the compound found in the highest amount (205 mg/100 g dw, peak 17), followed by kaempferol dirhamnoside-*O*-pentoside (96 mg/100 g dw, peak 25). Phenolic acids were 6.58% of the total phenolic compounds in this sample and *trans p*-coumaric acid was the most abundant one (25.65 mg/100 g dw, peak 9). Herbal infusions are frequently used in traditional medicine due to their beneficial activities and among their constituents, special relevance has been given to phenolic compounds, which often exhibit high antioxidant capacity being able to counteract oxidative stress (Mejía, Songa, Hecka, Vinicio, & Ramírez-Mares, 2010; Pereira, Marcias, Perez, Marin & Cardoso, 2013). They act as antioxidants through various mechanisms, including hydrogen donating reactions, metal chelation, and up-regulation or protection of antioxidant defenses (*e.g.* intracellular glutathione levels) (Pereira et al.,

2013). In particular, *C. ambrosioides* infusion is a rich source of diverse polyphenols that could contribute to the mentioned activity.

3.3. Chemical composition in lipophilic compounds

The results of lipophilic compounds (fatty acids and tocopherols) are shown in **Table 4**. Up to 26 fatty acids were identified and quantified. Polyunsaturated fatty acids (PUFA) predominated over saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA). α-Linolenic (C18:3n3; 48.54%) and linoleic (C18:2n6; 19.23%) acids contribute to the high levels of PUFA observed (68.44%). Linoleic acid is the most prominent PUFA in the Western diet and previous studies showed health benefits under the prevention of cancer diseases (Whelan, 2008).

α-Tocopherol was, by far, the most abundant tocopherol in *C. ambrosioides* (199.37 mg/100 g dw from a total tocopherols amount of 202.34 mg/100 g dw; **Table 4**). Tocopherols are very important natural antioxidants in plant foods, especially those that are rich in PUFA. Their effectiveness as antioxidants depends not only on their reactivity against harmful radicals, but also the relatively stable nature of his radical due to relocation of the unpaired electron on the ring cromanol (Kagan et al., 2003).

Overall, *C. ambrosioides* infusion revealed, in general, higher antioxidant activity, while the methanolic extract was the only one showing antitumour effects against colon, cervical and hepatocellular carcinoma cell lines. Neither the infusion nor the extract reveal toxicity for non-tumour cells. Bioactive compounds such as some sugars and organic acids, phenolic compounds, unsaturated fatty acids and tocopherols were identified and quantified in *C. ambrosioides*. As far as we know, this is the first detailed

chemical characterization of *C. ambrosioides* and bioactivity evaluation of its methanolic extract and infusion.

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Table 1. Bioactive properties of the methanolic extract and infusion of wild *Chenopodium ambrosioides*.

	Methanolic extract	Infusion	Positive control*
Antioxidant activity			
DPPH scavenging activity (EC_{50}, mg/mL)	0.62 ± 0.08[a]	0.49 ± 0.02[b]	0.04 ± 0.00
Reducing power (EC_{50}, mg/mL)	0.47 ± 0.03[b]	0.65 ± 0.01[a]	0.03 ± 0.00
β-carotene bleaching inhibition (EC_{50}, mg/mL)	2.53 ± 0.04[a]	2.32 ± 0.37[b]	0.003 ± 0.00
TBARS inhibition (EC_{50}, mg/mL)	0.70 ± 0.29[a]	0.25 ± 0.01[b]	0.004 ± 0.00
Antitumour activity			
MCF-7 (breast carcinoma) (GI_{50}, μg/mL)	>400	>400	0.91±0.04
NCI-H460 (non-small cell lung cancer) (GI_{50}, μg/mL)	>400	>400	1.42±0.00
HCT-15 (colon carcinoma) (GI_{50}, μg/mL)	318.75±13.21	>400	1.91±0.06
HeLa (cervical carcinoma) (GI_{50}, μg/mL)	264.17±10.57	>400	1.14±0.21
HepG2 (hepatocellular carcinoma) (GI_{50}, μg/mL)	287.43±21.99	>400	3.22±0.67
Hepatotoxicity			
PLP2 (GI_{50}, μg/mL)	>400	>400	2.06±0.03

*Trolox and ellipticine for antioxidant and antitumour activity assays, respectively. EC_{50} values correspond to the sample concentration achieving 50% of antioxidant activity or 0.5 of absorbance in reducing power assay. GI_{50} values correspond to the sample concentration achieving 50% of growth inhibition in human tumour cell lines or in liver primary culture PLP2. In each row different letters mean significant differences ($p<0.05$).

Table 2. Chemical composition in hydrophilic compounds- sugars and organic acids- of wild *Chenopodium ambrosioides*.

Free sugars	g/100 g dw	Organic acids	g/100 g dw
Fructose	0.24 ± 0.01	Oxalic acid	5.64 ± 0.30
Glucose	0.46 ± 0.01	Quinic acid	0.97 ± 0.14
Sucrose	1.43 ± 0.12	Malic acid	0.67 ± 0.06
Trehalose	0.91 ± 0.03	Ascorbic acid	0.02 ± 0.00
Total sugars	3.04 ± 0.07	Citric acid	0.26 ± 0.01
		Fumaric acid	0.02 ± 0.00
		Total organic acids	7.58 ± 0.52

dw- dry weight.

Table 3. Retention time (Rt), wavelengths of maximum absorption in the UV-vis region (λ_{max}), pseudomolecular and MS^2 fragment ions (in brackets, relative abundances), identification and quantification of phenolic compounds in wild *C. ambrosiodes*.

Peak	Rt (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Identification	Quantification (mg/100 g dw)
1	6.8	310	295	163(100),119(60)	*p*-Coumaroyl pentoside acid	3.53 ± 0.50
2	7.1	314	278	163(6),119(13)	*p*-Coumaroyl acid derivative	9.75 ± 0.44
3	7.5	328	387	387(100)207(25),163(50),119(37)	*p*-Coumaroyl acid derivative	1.41 ± 0.09
4	8.2	328	325	193(100),149(38)	Feruloyl pentoside acid	2.58 ± 0.27
5	8.8	308	278	163(6),119(13)	*p*-Coumaroyl acid derivative	1.21 ± 0.15
6	14.8	326	473	267(27),193(100)	Ferulic acid derivative	3.51 ± 0.14
7	15.7	332	579	447(15),417(7),327(7),285(100)	Luteolin *C*-hexoside-*O*-pentoside	2.27 ± 0.09
8	15.9	354	755	609(2),301(100)	Quercetin 3-*O*-rutinoside-(1→2)-*O*-rhamnoside	15.23 ± 0.41
9	16.4	312	163	119(100)	*trans p*-Coumaric acid	25.65 ± 0.77
10	16.9	356	609	463(30),447(33),301(36)	Quercetin 3-*O*-neohesperide	7.19 ± 0.32
11	17.2	354	741	301(100)	Quercetin *O*-pentosyl-rhamnosyl-hexoside	27.60 ± 0.31
12	17.6	356	595	301(100)	Quercetin *O*-pentosyl-hexoside	3.55 ± 0.46
13	18.1	348	739	285(100)	Kaempferol *O*-dirhamnosyl-hexoside	20.38 ± 0.74
14	18.3	326	193	149(17),135(100)	Ferulic acid	6.43 ± 0.53
15	18.6	354	579	447(100),301(33)	Quercetin *O*-rhamnosyl-pentoside	8.06 ± 0.87
16	19.0	348	739	593(83),431(17),285(67)	Kaempferol dirhamnoside-*O*-hexoside	4.80 ± 0.49
17	19.4	354	609	301(100)	Quercetin-3-*O*-rutinoside	204.95 ± 6.39
18	19.8	346	725	285(100)	Kaempferol *O*-pentosyl-rhamnosyl-hexoside	31.42 ± 1.36
19	20.2	352	593	447(64),301(100)	Quercetin dirhamnoside	56.63 ± 0.35

20	20.4	354	579	447(45),301(100)	Quercetin *O*-rhamnosyl-pentoside	1.22 ± 0.03
21	20.8	350	463	301(100)	Quercetin 3-*O*-glucoside	12.91 ± 0.80
22	21.5	348	563	431(53),285(100)	Kaempferol *O*-rhamnosyl-pentoside	4.93 ± 0.08
23	22.4	344	739	593(24),431(24),285(100)	Kaempferol dirhamnoside-*O*-hexoside	11.31 ± 0.44
24	23.1	348	593	285(100)	Kaempferol 3-*O*-rutinoside	74.82 ± 2.29
25	23.5	342	709	563(25),431(63),285(100)	Kaempferol dirhamnoside-*O*-pentoside	95.89 ± 1.64
26	23.9	344	563	431(47),285(100)	Kaempferol *O*-rhamnosyl-pentoside	36.15 ± 1.40
27	24.6	350	607	461(50),315(100)	Isorhamnetin dirhamnoside	tr
28	25.1	352	593	461(80),315(100)	Isorhamnetin *O*-rhamnosyl-pentoside	1.60 ± 0.09
29	26.0	352	623	447(50),301(43)	Quercetin *O*-rhamnosyl-glucuronide	2.48 ± 0.04
30	26.6	352	623	447(33),301(51)	Quercetin *O*-rhamnosyl-glucuronide	33.99 ± 0.28
31	29.6	344	607	459(30),431(20),285(50)	Kaempferol *O*-rhamnosyl-glucuronide	6.54 ± 0.28
32	30.5	346	607	431(100),285(86)	Kaempferol *O*-rhamnosyl-glucuronide	56.08 ± 0.35
33	31.2	350	637	461(100),315(87)	Isorhamnetin *O*-rhamnosyl-glucuronide	0.50 ± 0.00
34	31.7	352	665	623(14),447(35),301(18)	Quercetin (acyl)glucuronide-*O*-rhamnoside	12.53 ± 0.56
35	35.1	344	649	607(6),431(42),285(31)	Kaempferol (acyl)glucuronide-*O*-rhamnoside	35.26 ± 1.43
					Phenolic acids	54.07 ± 1.55
					Flavonoids	768.27 ± 10.70
					Phenolic compounds	822.33 ± 12.25

dw- dry weight; tr- traces

Table 4. Chemical composition in lipophilic compounds of wild *Cl*
ambrosioides.

Fatty acids	Relative percentage	Fatty acids	Relative percentage
C6:0	0.10 ± 0.01	C18:3n3	48.54 ± 0.13
C8:0	0.49 ± 0.02	C20:0	0.83 ± 0.00
C10:0	0.34 ± 0.01	C20:1	0.19 ± 0.01
C12:0	0.17 ± 0.03	C20:2	0.17 ± 0.03
C13:0	0.41 ± 0.01	C20:3n6	0.12 ± 0.01
C14:0	0.48 ± 0.02	C20:4n6	0.04 ± 0.01
C14:1	0.47 ± 0.05	C20:3n3+C21:0	0.29 ± 0.01
C15:0	0.44 ± 0.02	C20:5n3	0.03 ± 0.01
C16:0	14.16 ± 0.03	C22:0	1.85 ± 0.05
C16:1	0.14 ± 0.03	C22:2	0.01 ± 0.00
C17:0	0.32 ± 0.01	C23:0	0.21 ± 0.00
C18:0	1.57 ± 0.08	C24:0	2.52 ± 0.09
C18:1n9	6.90 ± 0.12	SFA	23.87 ± 0.08
C18:2n6	19.23 ± 0.12	MUFA	7.69 ± 0.16
		PUFA	68.44 ± 0.08
Tocopherols	mg/100 g dw		
α-tocopherol	199.37 ± 4.92		
β-tocopherol	0.56 ± 0.00		
γ-tocopherol	2.28 ± 0.10		
δ-tocopherol	0.13 ± 0.00		
Total tocopherols	202.34 ± 5.02		

dw- dry weight. SFA- Saturated fatty acids; MUFA- Monounsaturated fatty a
Polyunsaturated fatty acids.

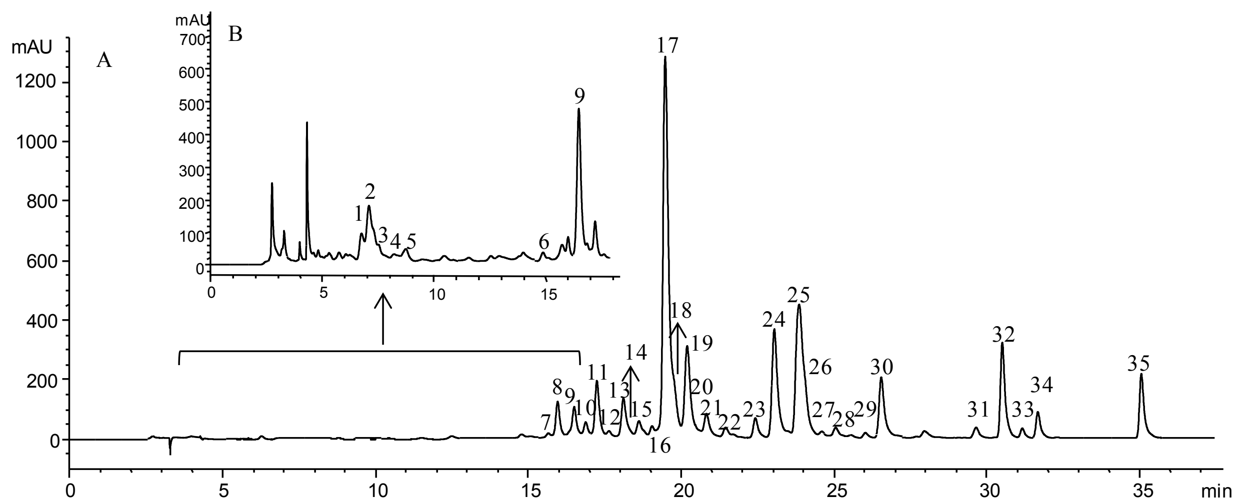

Figure 1. HPLC phenolic profile of *Chenopodium ambrosiodes*, obtained at 370 nm (A) and 280 nm (B) for flavonoids and phenolic acids, respectively.