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A comparison of the bioactivity and phytochemical profile of three different cultivars of globe amaranth: red, white, and pink†

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The phytochemical profiles and bioactivities of red, white and pink globe amaranth (*Gomphrena haageana* K., *Gomphrena globosa* var. albiflora and *Gomphrena* sp., respectively), much less studied than the purple species (*G. globosa* L.), were compared. The chemical characterization of the samples included the analysis of macronutrients and individual profiles of sugars, organic acids, fatty acids, tocopherols, and phenolic compounds. Their bioactivity was evaluated by determining the antioxidant and anti-inflammatory activities; the absence of cytotoxicity was also determined. Red and pink samples showed the highest sugar content. Otherwise, the white sample gave the highest level of organic acids, and together with the pink one showed the highest tocopherol and PUFA levels. Quercetin-3-*O*-rutinoside was the major flavonol in white and pink samples, whereas a tetrahydroxy-methylenedioxyflavone was the major compound in the red variety, which revealed a different phenolic profile. The pink globe amaranth hydromethanolic extract revealed the highest antioxidant activity, followed by those of red and white samples. The anti-inflammatory activity was more relevant in red and pink varieties. None of the samples presented toxicity in liver cells. Overall, these samples can be used in bioactive formulations against inflammatory processes and in free radical production.

1. Introduction

Medicinal plants play a vital role in the health and healing of man, not only in traditional medicine but also as one of the major sources of drugs.[1] Plants synthesize a variety of secondary metabolites, many of which are bioactive and could have commercial interest as pharmaceutical compounds, being capable of protecting against and treating various diseases.[2] Recently, there has been an increasing interest in the therapeutic potential of plants as antioxidants, reducing free radicals that induce tissue injury, and as anti-inflammatories. Although several synthetic drugs are commercially available, their safety and toxicity is a concern, so there is a tendency to substitute them by natural compounds.[3]

Oxidative stress and inflammation play critical roles in the pathogenesis of many diseases, such as cancer, cardiovascular disease, arthritis or obesity.[4] Oxidative stress occurs when the balance between pro-oxidants and antioxidants is disturbed, resulting in tissue accumulation of free radicals and other reactive oxygen species (ROS). If the human body does not eliminate these harmful products, they may cause oxidative damage to functional macromolecules such as DNA, proteins and lipids.[5] Inflammation is one of the body's self-defense systems that is classified as part of our innate immunity. Thus, bacterial or viral infections trigger numerous immunological events, including the production of cytokines, chemokines, and inflammatory mediators such as nitric oxide (NO), prostaglandin E2 (PGE2) or tumor necrosis factor (TNF)-α,[6,7] whose activation is mediated by nuclear factor-kappa B (NF-κB), a transcription factor that regulates the transcription of DNA,[8] as well as the migration and infiltration of leukocytes, the increased expression of surface molecules such as MHC (Major Histocompatibility Complex) molecules, complement receptors, and the release of hydrolytic enzymes.[9]

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Bioactive molecules such as phenolic compounds, quinones, vitamins, coumarins, and alkaloids, are present in a large number of plant species.[10] Phenolic compounds are the most numerous and ubiquitously distributed groups of plant secondary metabolites, presenting a wide range of biological effects mainly related to their antioxidant capacity due to the presence of H-donating hydroxyl groups.[11] It is also strongly suggested in the literature that plant polyphenols inhibit the inflammation process by regulating the production of pro-inflammatory molecules, such as TNF-α,[12] leukocyte adhesion, and NO, all produced during inflammatory reactions.[13,14] Inflammatory pathways simultaneously contribute to and are regulated by oxidative stress. In fact, NO reacts with free radicals, such as superoxides, to produce highly damaging peroxynitrites, which can oxidize low-density lipoproteins that lead to irreversible damage in cell membranes. Hence, inhibition of the production of such pro-inflammatory molecules (NO and TNF-α) is expected to have a therapeutic value as antioxidant agents and against inflammatory diseases.[13,15]

Gomphrena sp. is a comestible and commercial ornamental plant commonly known as globe amaranth or bachelor button that belongs to the family *Amaranthaceae*.[16] Plants of this family are particularly predominant in South America, consisting of approximately 120 species, which are employed in folk medicine in the treatment of several diseases due to their biological activities, including antimicrobial,[17] antioxidant, cytotoxic,[18] and hypotensive activities,[19] and they also possess nutritive value.[20]

Recent studies have focused mainly on the most common cultivar, purple globe amaranth, dealing with its phytochemical composition,[20,21] antimicrobial, antioxidant and cytotoxic activities, and cardiovascular effects,[18,22,23] as well as its medicinal benefits.[24] Nevertheless, to the best of our knowledge, other *Gomphrena* species are still poorly or not studied and, since the consumption data indicate that these plants are widely employed around the world for various purposes, especially their traditional use as infusions in order to treat throat disorders, hence it seems of great interest to explore their bioactive potential.

Therefore, the aim of this study was to compare the phytochemical profile and bioactive properties of different varieties of globe amaranth (red, white and pink), and contribute to the characterization of the less studied *Gomphrena* species.

2. Materials and methods

2.1 Samples

Three different cultivars (red, white and pink) of *Gomphrena* species, commonly known as globe amaranth, were obtained from "Cantinho das Aromáticas", organic farms from Vila Nova de Gaia (Portugal), as dry flower material (ESI†). Red, white and pink dried flower samples corresponded to *Gomphrena haageana* K., *Gomphrena globosa* var. albiflora and *Gomphrena* sp., respectively.

2.2 Standards and reagents

Acetonitrile 99.9%, *n*-hexane 95% and ethyl acetate 99.8% were of HPLC grade from Fisher Scientific (Lisbon, Portugal). Fatty acid 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, trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), L-ascorbic acid, tocopherol, sugar and organic acid standards. Racemic tocol, 50 mg mL^{-1}, was purchased from Matreya (Pleasant Gap, PA, USA). Phenolic standards were from Extrasynthèse (Genay, France). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Dulbecco's modified Eagle's medium, Hank's balanced salt solution (HBSS), fetal bovine serum (FBS), L-glutamine, trypsin-EDTA, penicillin/streptomycin solution (100 U mL^{-1} and 100 mg mL^{-1}, respectively) were purchased from Gibco Invitrogen Life Technologies (Paisley, UK). Sulforhodamine B, trypan blue, trichloroacetic acid (TCA) and Tris were purchased from Sigma Chemical Co. (Saint Louis, MO, USA). RAW264.7 cells were purchased from ECACC ("European Collection of Animal Cell Culture") (Salisburg, UK), lipopolysaccharide (LPS) from Sigma and DMEM medium from HyClone. The Griess Reagent System Kit was purchased from Promega, and dexamethasone was purchased from Sigma. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

2.3 Nutritional composition

2.3.1 Nutritional value. The samples were analyzed for chemical composition (protein, fat, carbohydrates and ash) using the AOAC procedures.[25] The crude protein content of the samples (N × 6.25) was estimated by the macro-Kjeldahl method; crude fat was determined using a Soxhlet apparatus by extracting a known weight of the sample with petroleum ether; the ash content was determined by incineration at 600 ± 15 °C. Total carbohydrates were calculated by difference and total energy was calculated according to the following equation: Energy (kcal) = 4 × (g protein + g carbohydrates) + 9 × (g fat).

2.3.2 Sugars. Free sugars were determined *via* high performance liquid chromatography coupled to a refraction index detector (HPLC-RI), after an extraction procedure previously described by the authors[26] using melezitose as the internal standard (IS). Equipment consisted of an integrated system with a pump (Knauer, Smartline system 1000, Berlin, Germany), a degasser system (Smartline manager 5000), an auto-sampler (AS-2057 Jasco, Easton, MD, USA) and an RI detector (Knauer Smartline 2300). Data were analyzed using Clarity 2.4 Software (DataApex, Prague, Czech Republic). The chromatographic separation was achieved with a Eurospher 100-5 NH_2 (Knauer) column (5 μm, 4.6 × 250 mm) operating at 35 °C (7971 R Grace oven). The mobile phase was acetonitrile/deionized water, 70 : 30 (v/v) at a flow rate of 1 mL min^{-1}. The compounds were identified by chromatographic comparisons with authentic standards. Quantification was performed using

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the internal standard method and sugar contents were further expressed in g per 100 g of dry weight.

2.3.3 Organic acids. Organic acids were determined following a procedure previously described by the authors.[27] 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, 4.6 × 250 mm) thermostated at 35 °C. Elution was performed with sulphuric acid 3.6 mM using a flow rate of 0.8 mL min^{-1}. Detection was carried out in a PDA, using 215 nm and 245 nm (for ascorbic acid) as preferred wavelengths. The organic acids found were quantified by a comparison of the area of their peaks with calibration curves obtained from commercial standards of each compound. For quantitative analysis, calibration curves were prepared from different standard compounds: oxalic acid ($y = 10^7x + 96\,178$; $R^2 = 0.999$); malic acid ($y = 952\,269x + 17\,803$; $R^2 = 1$); fumaric acid ($y = 172\,760x + 52\,193$; $R^2 = 0.999$). The results were expressed in g per 100 g of dry weight.

2.3.4 Tocopherols. Tocopherols were determined following a procedure previously described by the authors.[26] Analysis was performed by HPLC (equipment described above), and a fluorescence detector (FP-2020; Jasco) programmed for excitation at 290 nm and emission at 330 nm. Chromatographic separation was achieved with a Polyamide II (YMC Waters, Milford, MA, USA) normal-phase column (5 μm, 4.6 mm × 250 mm), operating at 35 °C. 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^{-1}. The compounds were identified *via* chromatographic comparisons with authentic standards. Quantification was based on the fluorescence signal response of each standard, using the IS (tocol) method and by using calibration curves obtained from commercial standards of each compound. The results were expressed in mg per 100 g of dry weight.

2.3.5 Fatty acids. Fatty acids were determined by gas–liquid chromatography with a flame ionization detection (GC-FID)/capillary column as described previously by the authors.[26] 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 °C) and a Macherey-Nagel column (30 m × 0.32 mm i.d. × 0.25 μm df, Bethlehem, PA, USA). The oven temperature program was as follows: the initial temperature of the column was 50 °C, held for 2 min, then a 30 °C min^{-1} ramp to 125 °C, a 5 °C min^{-1} ramp to 160 °C, a 20 °C min^{-1} ramp to 180 °C, a 3 °C min^{-1} ramp to 200 °C, and a 20 °C min^{-1} ramp to 220 °C held for 15 min. The carrier gas (hydrogen) flow-rate was 4.0 mL min^{-1} (0.61 bar), measured at 50 °C. Split injection (1 : 40) was carried out at 250 °C. 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, Prague, Czech Republic) and expressed in relative percentage of each fatty acid.

2.4 Non-nutrient composition

2.4.1 Extraction procedure. The dry material was used to prepare hydromethanolic extracts by adding 25 mL of methanol : water (80 : 20 v/v) to 1 g of each sample. The extraction was carried out by stirring at 150 rpm for 1 h and subsequently filtering through Whatman no. 4 paper. The residue was then extracted with an additional 25 mL of methanol : water (80 : 20 v/v) for another hour under the same conditions. The combined extracts were evaporated at 40 °C in a rotary evaporator (Büchi R-210, Flawil, Switzerland), frozen and lyophilized (FreeZone 4.5, Labconco, Kansas City, MO, USA).

2.4.2 Analysis of phenolic compounds. The previously described hydromethanolic extracts were dissolved in water : methanol (80 : 20, v/v) to a final concentration of 20 mg mL^{-1} and analysed using a Hewlett-Packard 1100 chromatograph (Hewlett-Packard 1100, Agilent Technologies, Santa Clara, CA, USA) 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 thermostated 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, and 35–50% for 10 min, and re-equilibration of the column was performed using a flow rate of 0.5 mL min^{-1}. 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 a HPLC system *via* the DAD cell outlet.[21]

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 analyser that was controlled by the Analyst 5.1 software. Zero grade air served as the nebulizer gas (30 psi) and turbo gas was used for solvent drying (400 °C, 40 psi). Nitrogen served as the curtain (20 psi) and collision gas (medium). The quadrupoles were set at unit resolution. The ion spray voltage was set at −4500 V 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 a sample. The settings used were: declustering potential (DP) −450 V, entrance potential (EP) −6 V, collision energy (CE) −10 V. The 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 −25 V, and collision energy spread (CES) 0 V. Spectra were recorded in the negative ion mode between *m*/*z* 100 and 1500.

The phenolic compounds were identified by comparing their retention time, and UV-vis and mass spectra with those obtained from standard compounds, when available. Otherwise, peaks were tentatively identified from the information obtained from their mass spectra and data reported in the literature. For quantitative analysis, a calibration curve for each available phenolic standard was constructed based on the UV

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signal: *p*-coumaric ($y = 884.6x + 184.49$; $R^2 = 0.999$); kaempferol-3-*O*-glucoside ($y = 288.55x - 4.0503$; $R^2 = 1$); kaempferol-3-*O*-rutinoside ($y = 239.16x - 10.587$; $R^2 = 1$); isorhamnetin-3-*O*-glucoside ($y = 218.26x - 0.98$; $R^2 = 1$); isorhamnetin-3-*O*-rutinoside ($y = 284.12x + 67.055$; $R^2 = 0.999$); quercetin-3-*O*-glucoside ($y = 363.45x + 117.86$; $R^2 = 0.999$), quercetin-3-*O*-rutinoside ($y = 281.98x - 0.3459$; $R^2 = 1$). For the detected phenolic compounds for which a commercial standard was not available, quantification was performed through the calibration curve of other compounds from the same phenolic group. The results were expressed in mg per g of the lyophilized extract.

2.5 Antioxidant activity evaluation

For the antioxidant activity assays, the lyophilized hydromethanolic extracts were dissolved in methanol : water (80 : 20 v/v) and concentrated at 10 mg mL^{-1}. For the different assays, these extracts were then subjected to further dilutions from 10 mg mL^{-1} to 0.02 mg mL^{-1}.

The 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. The 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 through 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 2 h 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 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 solutions, respectively.[21] The results were expressed in EC_{50} values (sample concentration providing 50% of antioxidant activity or 0.5 of absorbance in the reducing power assay). Trolox was used as the positive control.

2.6 Anti-inflammatory activity evaluation

2.6.1 Cell treatment. For the anti-inflammatory activity assay, the lyophilized hydromethanolic extracts were dissolved in water, and concentrated at 8 mg mL^{-1}. For the different assays, the extracts were then subjected to further dilutions from 8 mg mL^{-1} to 0.125 mg mL^{-1}.

The mouse macrophage-like cell line RAW264.7 was cultured in a DMEM medium supplemented with 10% heat-inactivated foetal bovine serum, 100 U per mL penicillin and 100 mg per mL streptomycin and was incubated at 37 °C under a humidified atmosphere containing 5% CO_2. For each experiment, cells were detached with a cell scraper. Under our experiment on cell density (5×10^5 cells per mL), the proportion of dead cells was less than 1%, according to Trypan blue dye exclusion tests.

Cells were seeded in 96-well plates at 150 000 cells per well and allowed to attach to the plate overnight. Then, the cells were treated with different concentrations of each of the extract for 1 h. Dexamethasone (50 μM) was used as a positive control for the experiment. The following step was stimulation with LPS (1 μg mL^{-1}) for 18 h. The effect of all the tested samples in the absence of LPS was also evaluated, in order to observe if they induced changes in NO basal levels. In negative controls, no LPS was added. Both extracts and LPS were dissolved in supplemented DMEM.

2.6.2 Nitric oxide determination. For the determination of nitric oxide, the Griess Reagent System kit (Promega) was used, which contains sulfanilamide, NED and nitrite solutions. A reference curve of the nitrite was prepared in a 96-well plate as described in the instructions thereof. One hundred microliters of the cell culture supernatant were transferred to the plate in duplicate and mixed with sulfanilamide and NED solutions, 5–10 minutes each, at room temperature. The nitrite produced was determined by measuring the optical density at 515 nm, in the microplate reader referred above, and compared to the standard calibration curve.

2.7 Hepatotoxicity evaluation

The effect of the samples on the growth of porcine liver primary cells (PLP2), established by the group, was evaluated by the sulforhodamine B (SRB) colorimetric assay with some modifications as described by Abreu *et al.*[28] Briefly, the liver tissues were rinsed in Hank's balanced salt solution containing 100 U per mL penicillin and 100 μg per mL streptomycin and divided into 1 × 1 mm^3 explants. Some of these explants were placed in 25 cm^3 tissue flasks in DMEM supplemented with 10% fetal bovine serum, 2 mM nonessential amino acids and 100 U per mL penicillin, and 100 mg per mL streptomycin and incubated at 37 °C under a humidified atmosphere containing 5% CO_2. The medium was changed every 2 days. Cultivation of the cells was continued with direct monitoring every 2–3 days using a phase contrast microscope. Before confluence, cells were sub-cultured and plated in 96-well plates at a density of 1.0×10^4 cells per well, and cultivated in DMEM medium with 10% FBS, 100 U per mL penicillin and 100 μg per mL streptomycin. Cells were treated for 48 h with the different diluted sample solutions and the SRB assay was performed. The results were expressed in GI_{50} values (sample concentration that inhibited 50% of the net cell growth). Ellipticine was used as the positive control.

2.8 Statistical analysis

For all the experiments, three samples were analyzed and all the assays were carried out in triplicate. The results are expressed as mean values ± standard deviation (SD). The differences between the different samples were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference *post hoc* test with $\alpha = 0.05$, coupled with

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Welch's statistic. This analysis was carried out using the SPSS v. 22.0 program.

3. Results and discussion

3.1 Nutritional composition

The results obtained for macronutrients are presented in Table 1. Carbohydrates were the major macronutrients found in all the samples (85.6 to 88.2 g per 100 g), with slightly higher amounts in red and white globe amaranth, followed by ash and protein. Pink globe amaranth contained the highest levels of ash (7.5 g per 100 g) and fat (1.20 g per 100 g) whereas red and white globe amaranth showed a slightly higher energy (381 and 380 kcal per 100 g, respectively), in agreement with their higher levels of carbohydrates.

The chemical composition of the samples in hydrophilic (sugars and organic acids) and lipophilic (fatty acids and tocopherols) compounds is shown in Table 1. Fructose, glucose and sucrose were found in all the samples, with red and pink globe amaranth revealing higher total sugar contents (2.47 and 2.40 g per 100 g, respectively). The levels of individual sugars were similar in the three samples, with fructose being slightly more abundant in red globe amaranth (0.76 g per 100 g) and glucose in pink globe amaranth (1.66 g per 100 g). In a recent study carried out by Pereira *et al.*,[29] the infusions obtained from these same samples of *Gomphrena* showed carbohydrate concentrations below the detection limit, which could be explained by the low levels present in the original plant material.

Regarding organic acids, white globe amaranth revealed the highest total amount (1.32 g per 100 g), with a significant contribution of oxalic acid (1.16 g per 100 g), which was also the prevailing organic acid in the other samples; red globe amaranth presented a higher concentration of malic acid (0.20 g per 100 g) and was also revealed to possess fumaric acid, although in a very low concentration (0.007 g per 100 g).

Regarding tocopherols, white and pink globe amaranth showed similar levels of γ-tocopherol (1.04 and 1.09 mg per 100 g) and total tocopherols (1.37 and 1.38 mg per 100 g, respectively). α-Tocopherol was found in higher concentrations in red globe amaranth (0.55 mg per 100 g) that was the only sample where δ-tocopherol was not detected.

Up to 20 fatty acids were identified in the studied samples, with prevalence of saturated fatty acids (SFA) and polyunsaturated fatty acids (PUFA) over monounsaturated fatty acids (MUFA). Red globe amaranth revealed the highest percentages of SFA (58.5%), with the main contribution of palmitic (C16:0; 34.6%) and stearic (C18:0; 8.1%) acids. MUFA were predominant in pink globe amaranth (8.2%) that presented oleic (C18:1n9; 7.1%) and eicosenoic (C20:1; 0.30%) acids, whereas PUFA prevailed in white (45.9%) and pink (45.6%) amaranth due to the significant contributions of linoleic (C18:2n6; 31.9 and 30.2%, respectively) and α-linolenic (C18:3n3; 13.7 and 15.07%, respectively) acids.

Table 1 Nutritional value and nutrients in the globe amaranth cultivars

	Red	White	Pink
Ash (g per 100 g dw)	5.4 ± 0.2^{c}	6.1 ± 0.3^{b}	7.5 ± 0.4^{a}
Protein (g per 100 g dw)	5.9 ± 0.3^{a}	5.6 ± 0.2^{a}	5.70 ± 0.01^{a}
Fat (g per 100 g dw)	0.50 ± 0.03^{c}	0.80 ± 0.02^{b}	1.20 ± 0.06^{a}
Carbohydrates (g per 100 g dw)	88.2 ± 0.3^{a}	87.5 ± 0.3^{a}	85.6 ± 0.2^{b}
Energy (kcal per 100 g dw)	381 ± 1^{a}	380 ± 1^{a}	376 ± 1^{b}
Fructose (g per 100 g dw)	0.76 ± 0.01^{a}	0.53 ± 0.03^{b}	0.57 ± 0.02^{b}
Glucose (g per 100 g dw)	1.58 ± 0.04^{ab}	1.52 ± 0.07^{b}	1.66 ± 0.09^{a}
Sucrose (g per 100 g dw)	0.13 ± 0.02^{b}	0.17 ± 0.01^{a}	0.17 ± 0.03^{a}
Total sugars (g per 100 g dw)	2.47 ± 0.06^{a}	2.22 ± 0.04^{b}	2.40 ± 0.04^{a}
Oxalic acid (g per 100 g dw)	0.82 ± 0.01^{c}	1.16 ± 0.01^{a}	0.95 ± 0.02^{b}
Malic acid (g per 100 g dw)	0.20 ± 0.01^{a}	0.16 ± 0.03^{b}	0.14 ± 0.01^{c}
Fumaric acid (g per 100 g dw)	0.0070 ± 0.0002	nd	nd
Total organic acids (g per 100 g dw)	1.03 ± 0.02^{c}	1.32 ± 0.01^{a}	1.09 ± 0.01^{b}
α-Tocopherol (mg per 100 g dw)	0.55 ± 0.03^{a}	0.28 ± 0.02^{b}	0.23 ± 0.01^{c}
γ-Tocopherol (mg per 100 g dw)	0.50 ± 0.04^{b}	1.04 ± 0.06^{a}	1.09 ± 0.05^{a}
δ-Tocopherol (mg per 100 g dw)	nd	0.05 ± 0.01	0.06 ± 0.01
Total tocopherols (mg per 100 g dw)	1.05 ± 0.07^{b}	1.37 ± 0.08^{a}	1.38 ± 0.03^{a}
C16:0 (palmitic acid; %)	34.6 ± 0.4	25.7 ± 0.4	25.3 ± 0.1
C18:0 (stearic acid; %)	8.1 ± 0.1	5.93 ± 0.09	4.65 ± 0.02
C18:1n9 (oleic acid; %)	5.38 ± 0.02	4.91 ± 0.09	7.1 ± 0.3
C18:2n6 (linoleic acid; %)	23.6 ± 0.3	31.9 ± 0.3	30.2 ± 0.2
C18:3n3 (α-linolenic acid; %)	10.8 ± 0.9	13.7 ± 0.8	15.07 ± 0.05
C22:0 (behenic acid; %)	3.94 ± 0.02	5.65 ± 0.02	5.08 ± 0.03
SFA (%)	58.5 ± 0.5^{a}	48.9 ± 0.5^{b}	46.1 ± 0.4^{c}
MUFA (%)	6.0 ± 0.1^{b}	5.2 ± 0.1^{c}	8.2 ± 0.3^{a}
PUFA (%)	35.5 ± 0.5^{b}	45.9 ± 0.6^{a}	45.6 ± 0.1^{a}

dw – dry weight; nd – not detected. SFA – saturated fatty acids; MUFA – monounsaturated fatty acids; PUFA – polyunsaturated fatty acids. Only the fatty acids with abundance higher than 5% were presented in the table; the difference to 100% corresponds to other fourteen less abundant fatty acids. In each row different letters mean statistically significant differences ($p < 0.05$).

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3.2 Composition in phenolic compounds

Data (retention time, λ_{max} in the visible region, pseudomolecular ions and main fragment ions observed in MS^2) obtained by HPLC-DAD-ESI/MS regarding phenolic compound identification and quantification in the analyzed samples of globe amaranth are presented in Tables 2 and 3. As an example, the profile of phenolic compounds in pink globe amaranth is shown in Fig. 1.

The same twenty phenolic compounds, all of them flavonoid glycosides, were detected in both pink (*Gomphrena* sp.) and white (*Gomphrena globosa* var. albiflora) globe amaranth, fourteen of which had been already reported in inflorescences of purple globe amaranth (*Gomphrena globosa* L.) previously analyzed in our laboratory,[21] so that the same identities have been assumed. The remaining six compounds (*i.e.*, 1, 5, 8, 15, 16 and 17 in Table 2) have been assigned based on their mass

Table 2 Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, identification and quantification of phenolic compounds in white and pink globe amaranth (mean ± SD)

Peak	Rt (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (*m/z*)	Main MS^2 fragments (*m/z*)	Tentative identification	Quantification (mg g^{-1} extract) White	Pink
1	16.7	354	741	609(8), 301(40)	Quercetin 3-*O*-(2-pentosyl,6-rhamnosyl)-hexoside	0.12 ± 0.01	0.12 ± 0.01
2	17.9	354	595	301(100)	Quercetin 3-*O*-(6-pentosyl)-hexoside	0.12 ± 0.01	0.15 ± 0.01
3	18.8	354	609	301(100)	Quercetin 3-*O*-rutinoside	5.21 ± 0.01	4.93 ± 0.10
4	19.1	340	725	593(10), 285(40)	Kaempferol 3-*O*-(2-pentosyl,6-*O*-rhamnosyl)-hexoside	0.92 ± 0.03	1.22 ± 0.05
5	19.7	356	593	285(100)	Kaempferol 3-*O*-(6-rhamnosyl)-hexoside	0.36 ± 0.01	0.44 ± 0.02
6	20.2	356	463	301(100)	Quercetin 3-*O*-glucoside	0.71 ± 0.01	0.73 ± 0.02
7	21.1	352	579	447(10), 285(35)	Kaempferol 3-*O*-(2-pentosyl)-hexoside	0.20 ± 0.01	0.22 ± 0.01
8	21.6	358	505	301(100)	Quercetin *O*-acetylhexoside	tr	0.018 ± 0.003
9	22.3	350	593	285(100)	Kaempferol 3-*O*-rutinoside	3.27 ± 0.03	3.31 ± 0.01
10	23.3	352	623	315(100)	Isorhamnetin 3-*O*-rutinoside	0.71 ± 0.02	0.75 ± 0.01
11	23.9	350	447	285(100)	Kaempferol 3-*O*-glucoside	0.47 ± 0.02	0.52 ± 0.02
12	24.9	358	477	315(100)	Isorhamnetin 3-*O*-glucoside	0.31 ± 0.01	0.36 ± 0.03
13	26.0	346	477	315(100)	Isorhamnetin *O*-hexoside	0.39 ± 0.01	0.39 ± 0.01
14	26.7	346	489	285(100)	Kaempferol *O*-acetylhexoside	0.25 ± 0.01	0.29 ± 0.01
15	28.5	340	563	285(100)	Kaempferol *O*-rhamnosyl-pentoside	0.15 ± 0.01	0.19 ± 0.01
16	29.3	276, 340	607	313(100)	Gomphrenol 3-*O*-(2-pentosyl)-hexoside	0.32 ± 0.01	0.36 ± 0.03
17	30.7	280, 334	649	313(100)	Gomphrenol 3-*O*-(2-pentosyl,6 acetyl)-hexoside	0.21 ± 0.01	0.31 ± 0.03
18	31.8	338	639	463(39), 301(30)	Quercetin *O*-glucuronide-*O*-hexoside	0.037 ± 0.001	0.08 ± 0.01
19	32.3	278, 342	475	313(100)	Gomphrenol 3-*O*-hexoside	0.39 ± 0.01	0.40 ± 0.01
20	33.9	276, 340	517	313(100)	Gomphrenol 3-*O*-(6-acetyl)-hexoside	0.84 ± 0.03	0.84 ± 0.01
					Total phenolic compounds	14.99 ± 0.14	15.62 ± 0.20

Table 3 Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, identification and quantification of phenolic compounds in the red variety of globe amaranth (mean ± SD)

Peak	Rt (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Quantification (mg g^{-1})
1′	15.1	354	799	315(100)	Isorhamnetin-*O*-glucuronyl-deoxyhexosyl-hexoside	0.25 ± 0.00
2′	16.8	354	653	315(100)	Isorhamnetin-*O*-glucuronyl-hexoside	0.83 ± 0.00
3′	17.2	312	163	119(100)	*p*-Coumaric acid	1.00 ± 0.04
4′	18.9	356	609	301(100)	Quercetin-3-*O*-rutinoside	1.27 ± 0.00
5′	19.4	354	639	331(36), 316(16)	Patuletin *O*-deoxyhexosyl-hexoside	0.39 ± 0.03
6′	20.2	358	463	301(100)	Quercetin-3-*O*-glucoside	0.45 ± 0.03
7′	20.6	354	493	331(60), 316(22)	Patuletin *O*-hexoside	0.45 ± 0.00
8′	22.0	336	829	635(22), 513(56), 315 (100), 193(20)	Unknown	nq
9′	25.1	348	681	343(96), 328(51)	Methoxy-trihydroxymethylenedioxyflavone *O*-glucuronyl-hexoside	1.07 ± 0.04
10′	26.4	346	637	329(100)	3,5,3′,4′-Tetrahydroxy-6,7-methylenedioxyflavone-3-*O*-deoxyhexosyl-hexoside	3.83 ± 0.01
11′	27.2	348	767	723(79), 343(98), 328(48)	Malonyl derivative of compound 9	0.83 ± 0.01
12′	28.1	342	825	681(90), 343(36), 328(22)	Derivative of compound 9	0.40 ± 0.01
13′	29.0	338	491	329(56), 179(3)	3,5,3′,4′-Tetrahydroxy-6,7-methylenedioxyflavone-3-*O*-hexoside	0.65 ± 0.01
14′	30.0	346	493	447(60), 328(5), 315(8)	Unknown methylenedioxyflavone	3.03 ± 0.02
					Total phenolic compounds	14.46 ± 0.03

nq – not quantified.

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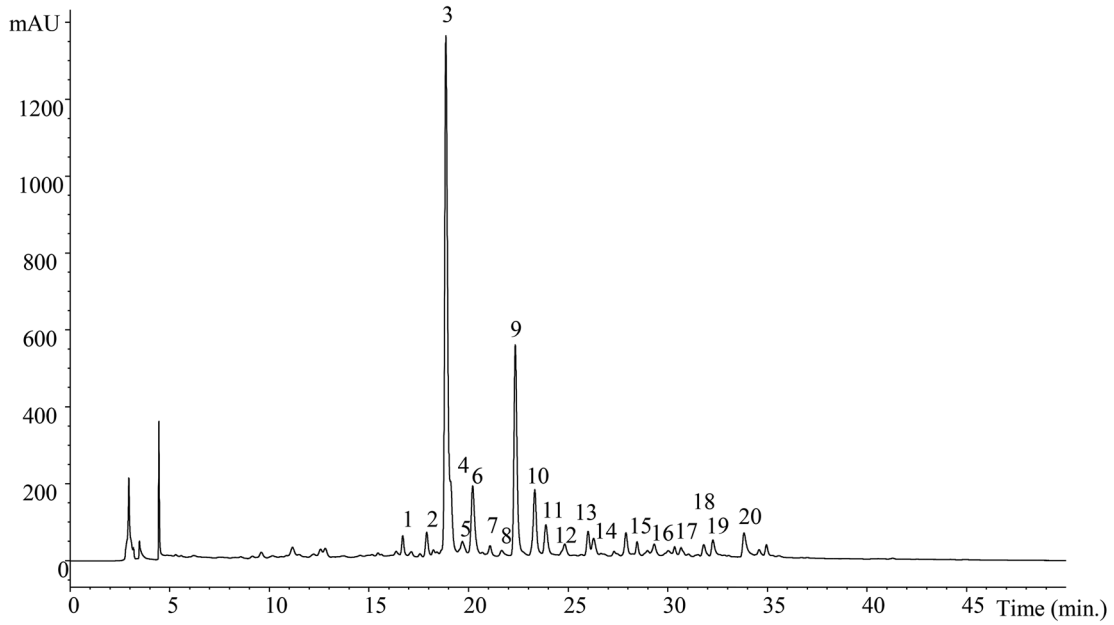

Fig. 1 Phenolic profile of the pink globe amaranth variety recorded at 370 nm.

spectral characteristics. In contrast to purple globe amaranth, no hydroxycinnamoyl derivatives have been found in the samples of white and pink globe amaranth now studied.

Compound 1 presented a pseudomolecular ion $[M - H]^-$ at *m*/*z* 741 releasing fragments at *m*/*z* 609 ($[M - H - 132]^-$, loss of a pentosyl moiety) and 301 (quercetin; further loss of the deoxyhexosylhexoside residue, −308 mu). Although these data do not inform about the nature and substitution position of the sugar moieties, compound 1 was tentatively identified as quercetin 3-*O*-(2-pentosyl,6-*O*-rhamnosyl)-hexoside owing to the previous identification of such a compound in inflorescences of *G. globosa* by Ferreres *et al.*[30]

Compound 5 showed a pseudomolecular ion $[M - H]^-$ at *m*/*z* 593 yielding an MS^2 fragment at *m*/*z* 285 (kaempferol) with the loss of the deoxyhexosylhexoside residue. The compound was identified as kaempferol 3-*O*-rutinoside, which corresponds to peak 9, as confirmed by a comparison with a commercial standard. Buschi & Pomilio[31] in another *Gomphrena* species (*G. martiana*) reported the presence of flavonol 3-*O*-robinosides, whereas Ferreres *et al.*[30] detected a similar compound in *G. globosa* that was identified as kaempferol 3-*O*-(6-rhamnosyl)-hexoside based on mass spectra, without indicating the nature of the hexose. Since no support to the type of sugar substituent can be concluded from the HPLC-DAD-MS analysis performed herein, the same identity as suggested by Ferreres *et al.*[30] was assumed for compound 5. Compound 8 was associated with a quercetin *O*-acetylhexoside according to its pseudomolecular ion $[M - H]^-$ at *m*/*z* 505 and the MS^2 fragment released at *m*/*z* 301 ($[M - H - 42 - 162]^-$, loss of acetyl and hexosyl moieties). Compound 15 ($[M - H]^-$ at *m*/z 563) could correspond to a kaempferol derivative bearing pentosyl and rhamnosyl moieties. Only one MS^2 fragment at *m*/*z* 285 resulting from the loss of a disaccharide was produced, suggesting that both sugars are located on the same position of the aglycone. Therefore, this compound was tentatively assigned as kaempferol *O*-rhamnosyl-pentoside. As far as we know, none of these compounds has been previously identified in *G. globosa*.

Compounds 16 and 17 ($[M - H]^-$ at *m*/*z* 607 and 649 mu, respectively) originated a base peak at *m*/*z* 313 mu, which could correspond to gomphrenol (3,5,4′-trihydroxy-6,7-methylenedioxyflavone) previously described in *G. globosa* leaves.[32] Peaks with the same pseudomolecular ions were detected in *G. globosa* inflorescences by Ferreres *et al.*[30] and Silva *et al.*[20] and were suggested to correspond to gomphrenol 3-*O*-(2-pentosyl)-hexoside and gomphrenol 3-*O*-(2-pentosyl,6-acetyl)-hexoside; so, these identities were also tentatively assumed for the compounds detected in our samples. Flavonoids bearing a methylenedioxy group, like gomphrenol (3,5,4′-trihydroxy-6,7-methylenedioxyflavonol), are rare in nature, with a predominance in the genus *Gomphrena*.[33]

Red globe amaranth (*Gomphrena haageana* K.) presented a different phenolic profile (Table 3) when compared with white and pink samples. Fourteen phenolic compounds were detected, from which only two coincided with those observed in the other two *Gomphrena* species, namely quercetin 3-*O*-rutinoside (compound 4′) and quercetin 3-*O*-glucoside (compound 6′). Both flavonols, as well as compound 3′ (*p*-coumaric acid) were positively identified by comparison with commercial standards, having also been previously reported in other globe amaranth varieties.[20,21,30]

Compounds 1′ ($[M - H]^-$ at *m*/*z* 799) and 2′ ($[M - H]^-$ at *m*/*z* 653) would correspond to isorhamnetin derivatives (λ_{max} around 354 nm and the common MS^2 fragment at *m*/*z* 315)

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bearing different numbers of sugar substituents. No information about the identity of the sugar moieties and location onto the aglycone could be obtained, although the fact that only one MS^2 fragment was released in both cases suggested that sugars are attached to a unique position in the form of oligosaccharides. Thus, according to their molecular masses they were assigned as isorhamnetin *O*-glucuronyl-deoxyhexosyl-hexoside and isorhamnetin *O*-glucuronyl-hexoside, respectively.

Compounds 5′ ($[M - H]^-$ at *m*/*z* 639) and 7′ ($[M - H]^-$ at *m*/*z* 493) released a main MS^2 fragment at *m*/*z* 331 from the loss of deoxyhexosyl-hexoside (308 mu) and hexoside (162 mu) moieties, respectively. The ion at *m*/*z* 331 would fit patuletin, whose presence was reported in other species of the genus *Gomphrena*.[33] Thus, the compounds were tentatively identified as patuletin *O*-deoxyhexosyl-hexoside and patuletin *O*-hexoside, respectively. This latter might correspond to patuletin 3-*O*-glucoside described in *G. claussenii* Moq. by Ferreira & Dias.[33]

Compounds 9′–14′ have been assigned as possible methylenedioxyflavonol derivatives, based on their mass spectra and the previous description of similar derivatives in inflorescences of *G. globosa* by Ferreres *et al.*[30] Compound 13′ showed a pseudomolecular ion $[M - H]^-$ at *m*/*z* 491 that released an MS^2 fragment at *m*/*z* 329 (−162 mu; loss of a hexosyl residue), which was assumed to correspond to the deprotonated aglycone matching the structure of a tetrahydroxymethylenedioxyflavone. It was tentatively identified as 3,5,3′,4′-tetrahydroxy-6,7-methylenedioxyflavone-3-*O*-hexoside, as previously described by Ferreres *et al.*[30] Similarly, compound 10′ ($[M - H]^-$ at *m*/*z* 637) releasing a unique MS^2 fragment at *m*/*z* 329 (−308 mu) should correspond to the equivalent deoxyhexosyl-hexoside derivative. Compound 9′ with an ion $[M - H]^-$ at *m*/*z* 681 releasing fragments at *m*/*z* 343 (−338 mu; loss of glucuronyl + hexosyl residues) and 328 (−15 mu; further loss of a methyl residue) might correspond to a methoxy-trihydroxymethylenedioxyflavone *O*-glucuronylhexoside. Compound 11′ ($[M - H]^-$ at *m*/*z* 767) presented a molecular mass 86 mu higher than compound 9′ and the same MS^2 fragments at *m*/*z* 343 and 328, together with another fragment at *m*/*z* 723 (−44 mu; possible loss of a CO_2 group). These characteristics pointed out to a malonyl derivative of compound 9′. Compound 12′ must also be related to compound 9′ owing to the observation of the MS^2 fragments at *m*/*z* 681, 343 and 328, as well as by the existence of similar UV absorption spectra; however, no final structure could be drawn. No identity could be assigned to compound 14′, either, although the presence of a fragment at *m*/*z* 328 suggested that it may also be related to compound 9′, thus also belonging to the group of methylenedioxyflavones. But for compound 13′, reported by Ferreres *et al.*,[30] none of the previous compounds has been described in *G. globosa*, as far as we are aware.

Lastly, the minor compound 8′ presented a MS^2 fragmentation pattern and a UV spectrum that did not allow a tentative identification of its structure.

Quercetin 3-*O*-rutinoside (compound 3) was the major flavonol found in white and pink globe amaranth (Table 2), followed by kaempferol 3-*O*-rutinoside (compound 9), which was also previously reported by us to be the main flavonoid in the purple variety. As for red globe amaranth, the majority compound was compound 10′, a tetrahydroxy-methylenedioxyflavone (Table 3). To our knowledge, this is the first report about the phenolic composition of red, white and pink species of globe amaranth.

3.3 Antioxidant activity

The results of the antioxidant activity, based on radical scavenging and lipid peroxidation inhibition capacities of the hydromethanolic extracts obtained for red, white and pink globe amaranth are presented in Table 4. Among the three studied samples, pink globe amaranth showed the highest antioxidant activity, with the lowest EC_{50} values in all assays (0.25 to 1.02 mg mL^{-1}), followed by red (0.41 to 1.30 mg mL^{-1}) and white (0.57 to 1.47 mg mL^{-1}) globe amaranth. The best results

Table 4 Antioxidant and anti-inflammatory properties, and hepatotoxicity of the hydromethanolic extracts obtained from the globe amaranth cultivars

	Red	White	Pink
Antioxidant activity (EC_{50} values, mg mL^{-1})			
DPPH scavenging activity	1.19 ± 0.06b	1.36 ± 0.03a	1.02 ± 0.01c
Reducing power	0.88 ± 0.01b	1.38 ± 0.03a	0.84 ± 0.02c
β-Carotene bleaching inhibition	1.30 ± 0.04b	1.47 ± 0.04a	0.98 ± 0.06c
TBARS inhibition	0.41 ± 0.01b	0.57 ± 0.01a	0.25 ± 0.03c
Anti-inflammatory activity (EC_{50} values, μg mL^{-1})			
NO production	136 ± 4b	198 ± 5a	133 ± 7b
Hepatotoxicity (GI_{50} values, μg mL^{-1})			
PLP2 growth inhibition	>400	>400	>400

Results of the antioxidant activity are expressed in EC_{50} values: sample concentration providing 50% of the antioxidant activity or 0.5 of absorbance in the reducing power. Results of the anti-inflammatory activity are expressed in EC_{50} values: sample concentration providing 50% of inhibition in the production of NO. Results of hepatotoxicity are expressed in GI_{50} values: sample concentration providing 50% of inhibition of the net cell growth. In each row different letters mean significant differences between samples ($p < 0.05$).

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of the antioxidant activity were obtained in the TBARS assay, where the extracts revealed lipid peroxidation inhibition activity at the lowest concentrations (EC_{50} between 0.25 and 0.57 mg mL^{-1}).

To the best of our knowledge, there are no studies regarding the antioxidant activity of the cultivars studied in the present work, although there are a couple of reports on methanolic extracts[21] and infusions[20] of purple globe amaranth, also from Portugal but from different distributors. Regarding the DPPH scavenging activity of the infusions, Silva *et al.*[20] reported EC_{50} values of 0.47 mg mL^{-1}, whereas the methanolic extract studied by Roriz *et al.*[21] showed a lower antioxidant activity (1.47 to 4.87 mg mL^{-1}) than that achieved with the hydromethanolic extracts of the samples studied in the present work (0.25 to 1.47 mg mL^{-1}).

3.4 Anti-inflammatory activity and hepatotoxicity

In the course of the screening of natural products to find novel anti-inflammatory drugs, the capacity of red, white and pink globe amaranth to inhibit the NO release from macrophages was also tested. As shown in Fig. 2, the hydromethanolic extracts of the samples revealed a dose-dependent anti-inflammatory activity in the range of concentrations checked (up to 400 μg mL^{-1}), with a considerable decrease of NO production even for the low concentrated extracts. Pink and red globe amaranth showed the lowest EC_{50} values (133 and 136 μg mL^{-1}, respectively), with white globe amaranth revealing a slight higher activity (198 μg mL^{-1}). The extracts lack toxicity when tested in the PLP2 cell line (established as primary cultures from pig liver), even at the highest concentration studied (400 μg mL^{-1}) (Table 4). As far as we know, this is the first report on the anti-inflammatory properties of these *Gomphrena* species cultivars and, from the results obtained, they should be considered as potential anti-inflammatory medicines.

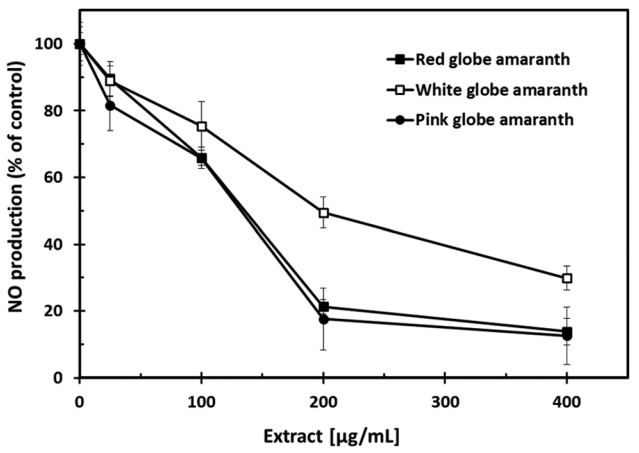

Fig. 2 Anti-inflammatory effect of hydromethanolic extracts of three globe amaranth varieties (red, white and pink). Levels of NO production determined by the Griess assay from culture supernatants of RAW264.7 cells treated with LPS (1 μg mL^{-1}) for 24 h.

4. Conclusion

Overall, the phytochemical profiles and bioactive properties of different cultivars of globe amaranth (red, white and pink) have been compared, so as to contribute to the characterization of these less studied *Gomphrena* species. To the best of the authors' knowledge, this is the first detailed chemical study on the mentioned varieties and data obtained highlight them as sources of bioactive compounds that could be incorporated in functional beverages or foods, as also in other formulations, owing to their anti-inflammatory potential and valuable properties related to oxidative stress.

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