

The Obtaining and Characterization of a Rich-phenolic Extract from *Amaranthus hypochondriacus* L.

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*In this paper we report the obtaining and characterization of a rich-phenolic extract from *Amaranthus hypochondriacus* L. (Amaranthaceae). The extraction solvent was evaluated on the basis of phenolic content measured by UV/Vis spectrophotometry and HPLC analysis. The extract was characterized using UV/Vis and FT-IR spectrophotometry and HPLC. The extract has a rich content of phenolic compounds, the most abundant being catechin, chlorogenic acid, cyanidin-3-glucoside, luteolin glucoside, apigenin 7-O-glucoside and rutin.*

Keywords: grain amaranths, *Amaranthus hypochondriacus*, Amaranthaceae, phenolic content, flavonoid content, HPLC

Amaranthus hypochondriacus L. is one of the grain amaranths (besides *A. cruentus* L. and *A. caudatus* L.), a stress-tolerant species belonging to a genus of plants that also includes ornamental species, herbs used as fodder or noxious weeds [1]. It is one of the few edible dicots using a C4 photosynthetic pathway [2] and it has been employed as a grain species on the territory of current Mexico since pre-Columbian times, having high protein content (13%-19%), superior to that of many cereals [3]. Unlike cereals, amaranth proteins consist mainly of globulins and albumins, with little or no prolamin proteins, the latter, usually richly present in cereals, being problematic for persons with coeliac disease [4]. Proteins of amaranth grains contain 47.6% essential amino acids [5], are highly bioavailable, with a quality comparable to that of animal proteins [6]; in one analysis, of several sources tested, they were found to be the richest in methionine, lysine and arginine [4, 7, 8]. Protein hydrolysates or isolates of amaranth grains have been recently claimed to have angiotensin I-converting enzyme-inhibitory properties [8-10], (*in vitro*) anti-inflammatory activity by inhibition of the NF- κ B signaling pathway [11], anti-diabetic effects through inhibition of dipeptidyl peptidase IV [12], liver protecting effects [13], potential anti-allergic [14], antimycotical/antifungal [15, 16] and antitumour [17] properties, and to mitigate acrylamide formation in foods [18]. Amaranth seed proteins in general [19, 20] and those of *A. hypochondriacus* [21, 22] in particular, have been shown in non-clinical studies to have beneficial effects on serum and liver lipid metabolism. The amount of lipids in amaranth grains is 2-3 times higher than in cereals, they have a high proportion of unsaturated fatty acids (mainly linoleic and oleic) and amaranth lipids are rather stable to oxidation, presumably due to a high tocopherol content [23]. Several proteinase [4, 24] and amylase [25] inhibitors, lectins [26, 27], squalene [28], triterpenic saponins [29], polyphenols and flavonoids (rutin, nicotiflorin) [30-34] were

also reported in the seeds. Starch granules from grains are unusually small [35] and their particularities have been described in the literature [36].

Unlike grains, the rest of the species organs have been little investigated so far. As other *Amaranthus* species, *A. hypochondriacus* biosynthesizes betalains, nitrogen-containing hydrosoluble pigments specific for the families of the core Caryophyllales [37] (betacyanins and betaxanthins [38], mainly amaranthine and iso-amaranthine [39]). Among flavonoids, quercetin and its glycosides have been identified in leaves (mainly rutin in younger leaves, additional ones – isoquercetin, hyperoside [40] - in older specimens) [39, 41-43] seemingly one of the richest of the *Amaranthus* species in rutin (234.54 mg/g) [39], but kaempferol-3-rutinoside has also been reported at much lower levels (less than one-hundredth of the rutin amount) [42]. Other polyphenols identified in leaves are ellagic, salicylic, syringic, gallic, vanilic, ferulic, *p*-coumaric, sinapic [40], gentisic and 2,4-dihydroxybenzoic acids [39], the most abundant being the ellagic and sinapic acids [40]. A high level of ascorbic acid was identified in foliage (288 mg/100 g) [39]; elevated amounts of proteins (29.5% in at first harvest, 22.7% at second harvest), beta-carotene (24.1 mg%), and several minerals were reported in aerial parts [44] (but seeds seem to be rather poor in minerals, when compared with those of other *Amaranthus* species [45,46]).

In the present paper we analyzed the solvent influence on the extraction of phenolic compounds and the obtaining of a rich phenolic extract.

Experimental part

All chemicals and solvents were obtained commercially with the highest purity. The UV-VIS determinations were performed on a Halo DB-20-220; Dynamica, Austria UV-Vis spectrophotometer. The HPLC analysis was performed on an HPLC-DIONEX system with DAD detector, equipped with a P580 pump. Fourier Transform Infrared (FT-IR)

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spectra were recorded using a JASCO FT/IR-4200 spectrometer with an ATR PRO450-S accessory, on a spectral range of 4000-400 cm^{-1} and a resolution of 4 cm^{-1} .

Extract preparation

Obtaining of the extractive solutions

Amaranthus hypochondriacus was harvested in July 2015 from Moara Domneasca, Ilfov county, Romania, during the flowering period. A voucher specimen is available in the drug collection of the Department of Botany and Cell Biology, Carol Davila University of Medicine and Pharmacy (no. 9/2015). After harvesting, the plant material was dried at room temperature ($25 \pm 4^\circ\text{C}$), and stored in paper bags until further processing.

Leaves of *Amaranthus hypochondriacus* were ground (mesh 14) and extracted with 20 parts of solvent (water, ethanol 50% (v/v) and ethanol) under reflux for 30 min. The extractive solutions were then filtered on cotton and on Whatman filter paper. The extractive solutions were subjected to phytochemical analysis.

Obtaining of the rich phenolic extract

Because both flavonoids and total phenolics were extracted in higher concentrations in ethanol 50%, this solvent was used for the obtaining of the phenolic-rich extract. The extract was obtained from 10 g of ground plant material (leaves; mesh 14). The plant material was extracted twice with 10 parts of solvent in order to achieve the ratio used at the obtaining of the extractive solutions. The extraction was performed under the reflux for 30 min/extraction. After cooling the mixture of plant material and extractive solution, the latter was separated by filtering on cotton and Whatman paper. The extractive solutions obtained from the two steps of the extraction phase were combined and concentrated at 40°C using a rotary evaporator (RVO 004, Czech Republic) to a volume of approximately 20 mL. The concentrated extractive solution was then lyophilized at -55°C using a freeze-dryer (CoolSafe ScanVac 55, Denmark). After the weighing, the total dry extract was conditioned in brown glass flasks at room temperature in a desiccator.

Phytochemical determinations

Total polyphenol analysis

Total phenolic content was determined according to the Folin-Ciocalteu method described in the literature with slight modifications [47-49]. Aliquots of 0.5 mL of the test solutions were mixed with 0.6 mL of Folin-Ciocalteu reagent and 15% Na_2CO_3 aqueous solution and completed with the extraction solvent to 10 mL. The samples were maintained at $50 \pm 0.5^\circ\text{C}$ for 15 min in the dark using a water bath (Memmert WNB10, Germany). The absorbance of the samples was measured at $\lambda = 750 \text{ nm}$. The calibration curve was prepared with gallic acid (Sigma-Aldrich, USA) using the condition described above. Total polyphenolic content was calculated using the regression parameters of the calibration curve. All determinations were performed in triplicate.

Total flavonoid content

Total flavonoid content was measured according to the method described in the literature with slight modifications [48, 50, 51]. Aliquots of 1.0 mL of the test solutions were mixed with 10% AlCl_3 aqueous solution, 1M CH_3COOK and then completed with distilled water to 10 mL. Samples were incubated at room temperature for 45 min protected from light. The absorbance of the samples was measured at $\lambda = 429 \text{ nm}$ using the same equipment presented above.

The calibration curve was prepared using quercetin (Sigma-Aldrich, USA) in the same conditions as described above. Total flavonoid content was calculated using the regression parameters of the calibration curve. All determinations were performed in triplicate.

Data analysis

The results were expressed as mg quercetin equivalents (Q equiv.) and mg gallic acid equivalents (GA equiv.), respectively, per 100 mg dry plant material (DP) for the extractive solutions and per 100 mg dry extract (DM) (mean \pm SD). The 95% confidence intervals (CI95%) of the means were also determined. Statistical significance of differences among means was assessed by ANOVA test, followed by Tukey Honestly Significant Difference test. P values below 0.05 were considered statistically significant. All calculations were performed using GraphPad Prism version 5.0 software (USA).

Identification of polyphenols by HPLC

Chromatographic conditions

The HPLC analysis was performed using a LiChrosorb RP-8 reversed-phase analytical column 250mm x 4.6mm i.d. 10 μm particle (HiChrom), and operated at 25°C . The mobile phase was a binary gradient: methanol and 0.01M orthophosphoric acid. The linear gradient started at 80% 0.01M orthophosphoric acid and 20% methanol, until 100% methanol, for 80 min. The flow rate was 1 mL/min and the injection volume was 20 μL .

Samples preparation and detection

The detection was performed at 280 nm using a UVD 340U detector. The samples of extractive solutions were mixed with the mobile phase and filtered through a 0.22 μm filter. The total extract was dissolved in mobile phase at a concentration of 10 mg/mL and then filtered through a 0.22 μm filter.

IR analysis of the extract

Fourier Transform Infrared (FT-IR) spectra were performed on the lyophilized extracts according to the method described by Budura E. and Janakiraman N. [52-54]. The FT-IR spectra were recorded using a JASCO FT/IR-4200 spectrometer, on a spectral range of 4000-400 cm^{-1} . The extract was analysed in duplicate and the presented IR spectrum represents the average of the two determinations.

Results and discussions

Polyphenols and flavonoid determination

Extractive solution analysis

Three extractive solutions were obtained from *Amaranthus hypochondriacus* aerial parts using water, ethanol : water 1:1 (v/v) and ethanol. The total phenolic content (TPC) and total flavonoid content (TFC) were determined for each of the three extractive solutions and the results are presented in table 1.

The hydroethanolic extractive solution present the highest TFC and TPC. TFC ranged from 0.154 to 0.547 mg Q equiv./ 100 mg DP, all results being statistically different (ANOVA, $p < 0.0001$; Tukey, $p < 0.05$). TPC ranged from 0.429 to 0.637, mg GA equiv./100 mg DP and statistically different (ANOVA, $p < 0.0001$). However, by applying Tukey post test, no statistical differences were registered between aqueous and hydroethanolic extractive solutions ($p > 0.05$).

Phenolic extract analysis

The dry extract yield was 17.24%. TFC was 1.11 ± 0.0451 with a 95% CI ranging from 0.999 to 1.224 mg Q equiv./

Extraction solvent	TFC (mg Q equiv./100mg DP)	95% CI of TFC (mg Q equiv./100 mg DP)	TPC (mg GA equiv./100 mg DP)	95% CI of TPC (mg GA equiv./100 mg DP)
Water	0.154 ± 0.002	0.149 - 0.159	0.637 ± 0.015	0.599 - 0.675
Ethanol : water 1:1 (v/v)	0.547 ± 0.017	0.505 - 0.589	0.640 ± 0.037	0.547 - 0.733
Ethanol	0.477 ± 0.031	0.399 - 0.557	0.429 ± 0.010	0.403 - 0.455

Table 1
TFC AND TPC FOR THE
AMARANTHUS
HYPOCHONDRIACUS
EXTRACTIVE SOLUTIONS

Values are mean ± standard deviation of triplicate analysis. GA: gallic acid; TFC: total flavonoid content; Q: quercetin; TPC: total phenolic content; DM: dry plant material; CI95%: confidence interval ($\alpha=0.05$) of mean;

Sample	Aqueous extractive solution	Hydroethanolic extractive solution	Ethanolic extraction solution	Rich phenolic extract
Catechin	+	-	+	+
Neochlorogenic acid	+	-	-	-
Chlorogenic acid	+	+	+	+
<i>p</i> -Coumaric acid	-	+	-	-
Ferulic acid	-	+	+	-
Vanilic acid	+	-	+	-
Cyanidin 3 glucoside	+	-	-	+
Luteolin glucoside	+	-	+	+
Apigenin 7-O-glucoside	+	+	+	+
Rutin	+	+	+	+
Quercitroside	+	-	-	-

Table 2
HPLC ANALYSIS OF THE
AMARANTHUS
HYPOCHONDRIACUS
EXTRACTIVE SOLUTIONS
AND RICH-PHENOLIC
EXTRACT

"+" positive identification; "-" the standard was not identified.

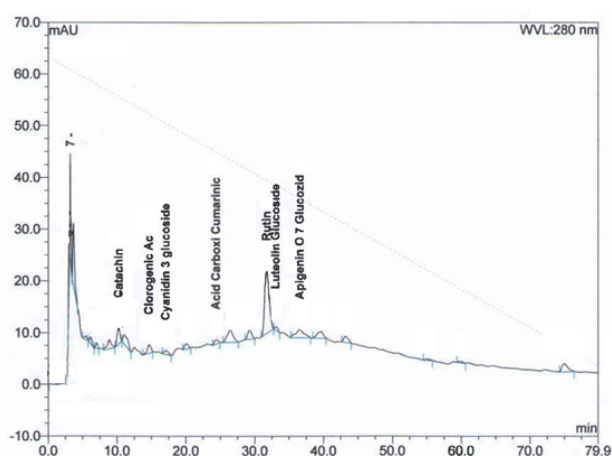


Fig. 1. HPLC chromatogram of the rich phenolic extract from *Amaranthus hypochondriacus*.

100 mg DM, and TPC 1.48 ± 0.4058 with a 95% CI ranging from 0.869 to 2.885 mg GA equiv./ 100 mg DM. Polyphenol compounds have antioxidant properties and potential of use in the prevention and treatment of a variety of conditions known for the involvement of reactive oxygen species in their pathology [55].

HPLC analysis of polyphenols and flavonoids

The results of the HPLC analysis are presented in table 2. All samples contain well - known phenolics. Although *p*-coumaric acid and ferulic acid were identified initially in the extractive solution, the two acids could not be found in the lyophilized extract, presumably because of their thermal degradation.

Cyanidin 3 glucoside, catechin and luteolin glucoside were identified in the dry extract only, probably due to their

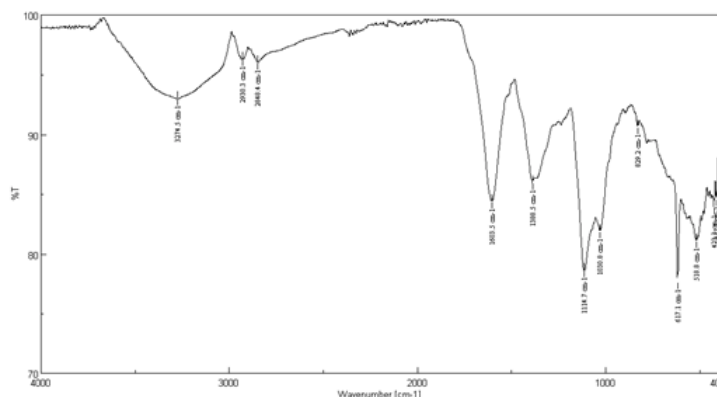


Fig. 2. IR analysis of the rich-phenolic extract from *Amaranthus hypochondriacus*

higher amounts as a result of the concentration process. A representative chromatogram is presented in figure 1.

IR analysis of the extract

Plant extract fingerprinting using the infrared spectra is a rapid and non-destructive investigation, and was used to evaluate the chemical fingerprint of the extract obtained from *Amaranthus hypochondriacus*. The large band close to 3100 cm^{-1} registered for the extract is considered an indicator of polyphenols content. The spectrum is presented in figure 2.

Conclusions

In the present work we have evaluated the extraction of phenolics from aerial parts of *Amaranthus hypochondriacus* L. (Amaranthaceae) using three common solvents: water, hydroethanol 50% and ethanol. Following the results of phenolic compounds determinations, we obtained a rich-

phenolic extract using ethanol : water 1:1 (v/v). The rich-phenolic extract was analyzed using UV/VIS, FT-IR spectroscopy and HPLC. We conclude that *Amaranthus hypochondriacus* L. is a valuable source of phenolic compounds, in the extract being identified both flavonoids and phenolic acids. Based on these study results and on the biological properties of polyphenols, we will further include this extract in specific tests in order to evaluate its biological activity.

The nutritional quality of *Amaranthus* proteins was studied in [56].

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