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ORIGINAL PAPER

Antioxidant Properties and Preliminary Evaluation of Phytochemical Composition of Different Anatomical Parts of Amaranth

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Abstract Antioxidant properties of amaranth extracts isolated sequentially by acetone and methanol/water from defatted plant leaves, flowers, stems and seeds were assessed by $ABTS^{+\bullet}$, $DPPH^{\bullet}$, ORAC and total phenols content (TPC) assays. In addition, antioxidant properties of solid plant material were evaluated by the direct QUENCHER method using the same assays. Leaves and flowers of amaranth as well as their extracts possessed the highest antioxidant activities. Radical scavenging capacity in $ABTS^{+\bullet}$ assay for leaves, flowers, stems and seeds evaluated by QUENCHER method were 144.24±2.41, 112.33±7.45, 19.05±1.13 and 21.82±1.06 μmol trolox equivalents in 1 g of dry weight, respectively. On-line HPLC-$DPPH^{\bullet}$ assay was used to determine the activity of separated compounds and it was observed that rutin was the main radical scavenger in amaranth extracts. Preliminary screening of extract composition was performed by UPLC/ESI-QTOF-MS and rutin, nicotiflorin, isoquercitrin, 4-hydroxybenzoic and *p*-coumaric acids were identified by measuring their accurate mass and retention time.

Keywords *Amaranthus* · Antioxidant activity · Radical scavenging · Total phenolic content · Rutin

Abbreviations

AAPH	2,2′-azobis (2-methylpropionamidine) dihydrochloride
ABTS	2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid)
ASE	Accelerated solvent extraction
DPPH	2,2-diphenyl −1-picrylhydrazyl radical
FA	Flowers acetone extract
FM	Flowers methanol/water extract
HPLC	High performance liquid chromatography
LA	Leaves acetone extract
LM	Leaves methanol/water extract
ORAC	Oxygen radical absorbance capacity
RSC	Radical scavenging capacity
SA	Stems acetone extract
SDA	Seeds acetone extract
SDM	Seeds methanol/water extract
SM	Stems methanol/water extract
TEAC	Trolox equivalent antioxidant capacity
Trolox	6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid
TPC	Total phenolic content
UPLC/ESI-QTOF-MS	Ultra high performance liquid chromatography - electrospray ionisation quadrupole time of flight - mass spectrometry

Introduction

Amaranthus spp. is a promissing source of valuable nutritional components: the seeds contain highly unsaturated oil, squalene, tocopherols and gluten-free proteins and therefore is a good substitute for cereals [1, 2], while the leafy vegetables are rich in dietary antioxidants and other microconstituents and have been used as a stir-fry vegetable, in soups and other foods [3]. The leaves of amaranth possess high antioxidant activity comparing with many other traditional green leafy vegetables [4], they are also a good source of iron and provitamin A; therefore

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their inclusion into the diet may help overcoming various nutritional problems [5–7].

Antioxidant properties of amaranth were studied by using various methods, mainly $DPPH^{\bullet}$ and $ABTS^{+\bullet}$ scavenging, ferric reducing antioxidant power (FRAP) assays and TPC measured with Folin-Ciocalteu reagent [8–20]. However, in many cases the results are difficult to compare due to different extraction, sample preparation and antioxidant activity evaluation procedures. Literature survey shows that there is a need of more systematic studies of amaranth antioxidant properties in order to comprehensively evaluate their beneficial properties in human nutrition [20].

The purpose of this study was to evaluate antioxidant properties of seeds, leaves, flowers, and stems of locally grown *Amaranthus hybridus*. All anatomical parts of amaranth were evaluated by applying radical scavenging and antioxidant activity determination methods, such as $ABTS^{\bullet+}$, $DPPH^{\bullet}$ scavenging, ORAC and TPC. The extracts were prepared using different polarity solvents, while the whole plat material was also assessed by using a direct QUENCHER antioxidant activity determination procedure. Such approach is expected to provide more comprehensive data on antioxidant properties of amaranth and to make preliminary prognosis for their health benefits.

Materials and Methods

Materials

Amaranthus hybridus plant material was collected in July 2011 in Dotnuva region (Lithuania) in a flowering state; the plants were kindly donated by Prof. A. Svirskis. The leaves, flowers and stems were separated and dried at ambient conditions in the dark. The seeds were obtained from Peckus farm (Alytus region, Lithuania). Dried samples were kept in the dark before further handling. Voucher specimens have been deposited in the Department of Food Technology of Kaunas University of Technology, Lithuania; their numbers AH2011/L, AH2011/S, AH2011/F, AH2011/S.

The solvents used for extraction were of analytical grade. Rutin hydrate (95 %), $DPPH^{\bullet}$, $ABTS^{\bullet+}$, Trolox, microcrystalline cellulose (20 μm), fluorescein sodium salt, AAPH, Folin–Ciocalteau's reagent solution, gallic acid and HPLC grade solvents used for chromatographic analysis were obtained from Sigma-Aldrich Chemie (Steinheim, Germany).

Sample Preparation and Extraction

Dried leaves, flowers and stems were ground by ultra centrifugal mill ZM 200 (Retsch, Haan, Germany) using 0.2 mm hole size sieve. The seeds were powdered in a laboratory mill (Miag, Braunschweig, Germany). All ground samples were additionally sieved by using 0.23 mm sieve. ASE was performed at 70 °C temperature and 10.3 MPa pressure during 25 min in a Dionex ASE 350 system (Dionex, Sunnyvale, CA) from 20 g of material mixed with diatomaceous earth (4:1) and placed in a 66 ml stainless-steel cells. The extraction was performed sequentially using the solvents of increasing polarity, hexane, acetone and a mixture of methanol/water (70:30, v/v). Hexane was used to remove lipophilic substances, which were not used in further analysis. The following extracts were obtained: FA, FM, LA, LM, SA, SM, SDA, SDM. Organic solvents were removed in a rotary vacuum evaporator at 40 °C while water was evaporated in the freeze dryer. The extracts after solvent evaporation were kept under nitrogen flow for 20 min and stored in dark glass bottles at −18 °C.

Measurements of Antioxidant and Radical Scavenging Activity

$ABTS^{\bullet+}$ Cation Radical and Stable $DPPH^{\bullet}$ Radical Scavenging Assays

TEAC assay was used to determine RSC of amaranth extracts as described previously [21] with slight modifications. The working solution of $ABTS^{\bullet+}$ was produced by reacting 7 mM ABTS stock solution with 2.45 mM potassium persulphate; two stock solutions were mixed in equal quantities and kept for 14–16 h. The working solution further was diluted with a mixture of ethanol:water (50:50) to obtain the absorbance of 0.70±0.02 at 734 nm. Plant extracts or Trolox solutions (3 μl) were reacted with 300 μl of the $ABTS^{\bullet+}$ solution during 30 min and the absorbance was read at 734 nm in a FLUOstar Omega reader (BMG Labtech, Offenburg, Germany). A series of trolox solutions (150–1500 μM) were used for calibration. The percentage RSC of $ABTS^{\bullet+}$ was calculated by the formula: $[(Abs_{control}\text{-}Abs_{sample})/(Abs_{control})]\times 100$, where $Abs_{control}$ and Abs_{sample} are the absorbances of $ABTS^{\bullet+}$ in control mixture with methanol and the extract, respectively. The TEAC values were calculated from the calibration curve and the RSC values were expressed in μmol Trolox equivalents (TE) per g dry weight (DW) plant material and extract (μmol TE/g). $DPPH^{\bullet}$ assay is based on radical by the antioxidant, which results in a decrease in absorbance at 515 nm [22]. Briefly, 3 μl of extracts or trolox solutions were mixed in the microplate wells with 300 μl of $DPPH^{\bullet}$ solution. The measurements were performed after 30 min at 515 nm wavelength using a FLUOstar Omega reader. Plant extracts were diluted with methanol to the final concentration of 1–3 %. A series of trolox solutions (50–1000 μM) were used for calibration and the final results were expressed in μmol TE/g.

ORAC Assay

The advantage of ORAC assay is that it uses a biologically relevant radical source [23]. The reaction was carried out in

75 mM phosphate buffer (pH 7.4); a stock solution of fluorescein was prepared according to Prior et al. [23], the samples were prepared by dissolving plant extracts in methanol. Prepared samples or trolox (25 μl) and fluorescein (120 μl; 14 μM) solutions were placed in the 96 wells black opaque microplates with transparent flat-bottom. The microplates were sealed and incubated for 15 min at 37 °C. After incubation AAPH solution as a peroxyl radical generator (25 μl; 240 mM) was added manually with a multichannel pipette. The microplate was immediately placed in the FLUOstar Omega fluorescent reader. The plate with the samples was shaken prior to each reading. Fluorescence measurements (excitation wavelength 485 nm; emission wavelength 510 nm) were read every 66 s, in total 90 cycles. Raw data were analyzed using software Mars (BMG Labtech GmbH, Offenburg, Germany). Fluorescein and AAPH solutions were prepared fresh daily. Aqueous solutions of trolox were used for calibration (12–200 μM). Antioxidant curves (fluorescence versus time) were normalized and the area under the fluorescence decay curve (AUC) was calculated as $AUC = 1 + \sum_{i=1}^{i=120} \frac{f_i}{f_0}$, where f_0 is the initial fluorescence at 0 min and f_i is the fluorescence at time i. The final ORAC values were calculated by using a regression equation between the trolox concentration and the net area under the curve (AUC). The antioxidant activity was expressed in μmol trolox equivalent antioxidant capacity per g DW plant material and extract (μmol TE/g).

Measurement of Total Phenols Content (TPC)

The TPC was determined in the extracts using the method of Singleton and Rossi [24] with slight modifications. Ten μl of appropriate dilutions of the extracts or gallic acid solutions were oxidized with 190 μl Folin-Ciocalteau's reagent solution in deionized water (1:13). The reagents were mixed, allowed to stand for 3 min and then neutralized with 100 μl of 7 % Na_2CO_3. The mixture was vortexed for 90 min and the absorbance was measured at 765 nm in the FLUOstar Omega reader. The TPC was calculated using gallic acid calibration curve and expressed in mg gallic acid equivalents per g DW plant material and extract (mg GAE/g).

Assessment of Antioxidant Capacity by QUENCHER Assay

The measurements of the total antioxidant capacity using modified $ABTS^{\bullet+}$, $DPPH^{\bullet}$, ORAC and TPC methods were applied directly to the solid particles of amaranth as described by Pastoriza et al. [25]. All assays were carried in the same way as described for the extracts isolated with solvents. In $ABTS^{\bullet+}$ scavenging assay 10 mg of the powdered sample were weighed in a testing tube and diluted with 40 μl of methanol. The reaction was started by adding 5 ml of $ABTS^{\bullet+}$ reagent. The mixture was vortexed for 2 min, centrifuged at 10500*g* for 3 min, and 300 μl of optically clear supernatant was transferred to the microplate. The $DPPH^{\bullet}$ scavenging assay was performed similarly to the $ABTS^{\bullet+}$ assay. In ORAC assay, 10 mg of the powdered sample was transferred to a test tube and the reaction was started by adding 5 ml of fluorescein. The mixture was kept at 37 °C for 15 min and then 175 μl of prepared solution was transferred to the microplate and 25 μl of AAPH solution added. For TPC, 10 mg of the sample were transferred to test tube with 2.9 ml Folin-Ciocalteau's reagent solution. The reagents was mixed and allowed to stand for 3 min. Then the mixture was neutralized with 2.1 ml of 7 % Na_2CO_3, vortexed for 77 min and centrifuged at 10500*g* for 3 min; the absorbance was measured at 765 nm.

In all methods, when the samples exerted too high antioxidant activity, they were diluted with microcrystalline cellulose as an inert material. The samples of cellulose-reagent mixture were prepared as control in all measurements using microcrystalline cellulose. Trolox solutions were used to prepare the calibration curve, using microcrystalline cellulose as well. The results are expressed in μmol equivalents of Trolox per g of DW.

On Line HPLC-$DPPH^{\bullet}$ Scavenging Assay

The method was used for the preliminary detection of active compounds present in amaranth extracts. Initial conditions with some modifications were according to Paśko et al. [15]. LC system consisted of Waters 1525 binary pump (Waters, Milford, MA), equipped with a manual 7725i Rheodyne injector (Rheodyne, Rohnert Park, CA), Waters 996 PDA detector. Analysis was performed on a Supelco Discovery® HS C-18, 5 μm, 250×4.6 mm i.d. (Supelco Inc. Bellefonte, PA, USA) column using gradient elution consisting of A - 2.5 % acetic acid and B - acetonitrile. The composition of gradient was set as follow: 0 min 10 % B; 0–5 min, from 10–20 % of B; 5–20 min, from 20–25 % of B; 20–30 min, from 25–45 % of B; 30–40 min, from 45–10 % of B, using the flow rate of 1 ml/min. Twenty μl of the sample was injected, UV detection range was 210–450 nm. The constituents were transferred into a post column reaction coil with circulating $DPPH^{\bullet}$ solution. Two chromatograms were recorded simultaneously: absorbance of effluent before the reaction at 210–450 nm and absorbance at 515 nm after reaction of effluent with $DPPH^{\bullet}$. The $DPPH^{\bullet}$ (6×10^{-5} M) and $NH_4C_2H_3O_2$ (1×10^{-1} M) solution in methanol was freshly prepared before analysis and continuously supplied into a reaction coil (length 15 m, diameter 1.5×0.3 mm) with Agilent 1100 series pump (Agilent Technologies, USA) at a flow rate of 0.5 ml/min. The signals were acquired at 515 nm wavelength by UV–VIS detector SPD-20A (Shimadzu, Kyoto, Japan). Rutin was identified by comparing the retention time with that of the corresponding peak of a standard. The extract

samples were prepared by dissolving them in methanol to a final concentration of 1–3 %.

UPLC/ESI-QTOF-MS analysis

An Acquity UPLC system (Waters, Milford, MA, USA) combined with a Bruker maXis UHR-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) was used. The Acquity UPLC was equipped with a binary solvent delivery system, an autosampler with a 10 μL sample loop, a photodiode array (PDA) detector, a column manager, and a data station running the Compass acquisition and data software. An Acquity BEH C18 column (1.7 μm, 50×2.1 mm, i.d.) was used for separation of compounds at 25 °C. The mobile phase was initially composed of 95 % eluent A - acetic acid (0.4 % v/v acetic acid solution in ultra pure water) and 5 % B (acetonitrile), followed by a gradient 0–8.5 min, from 5–25 % of B; 8.5–10 min, from 25–100 % of B; 10–12 min, from 100 % of B. In the PDA detector the effluent was monitored at 254 nm and further was introduced directly into the UHR-TOF mass spectrometer equipped with an ESI source at the flow rate of 0.3 mL/min. Analysis were achieved using the Compass 1.3 (HyStar 3.2 SR2) software. MS experiments were performed in negative ionization mode. The capillary voltage was +4000 V with the end plate offset −500 V. Nitrogen was used as the drying and nebulizing gases at a flow rate of 10.0 L/min and a pressure of 2.0 bar. It was introduced into the collisional cell as the collision gas. The peaks were identified by the characteristic MS fragmentation patterns and accurate masses.

Statistical analysis

The results are given as mean values and standard deviations, which were calculated from at least three replicate measurements using MS Excel 2003. Analysis of variance (one-way ANOVA) was used to test any differences in antioxidant activities, followed by the Duncans' *post hoc* test to compare the means that showed significant variation ($p<0.05$) (STATISTICA 8.0 software, 2007).

Results and Discussion

Antioxidant Activity of Amaranth Extracts

There are many assays for the assessment of antioxidant properties of plant extracts, the majority of them are based on electron/hydrogen atom transfer reactions. Huang et al. [26] concluded that ORAC, TPC and one of the electron/hydrogen transfer assays should be recommended for a representative evaluation of antioxidant properties of foods. DPPH$^{\bullet}$ assay is mainly attributed to the electron transfer assays, however the quenching of DPPH$^{\bullet}$ radical to form DPPH-H is also possible. Other electron transfer based methods include the TPC assay by Folin-Ciocalteu reagent and ABTS$^{\bullet+}$ decolorization assay. Following the above mentioned recommendation all these methods were applied for assessing antioxidant potential of amaranth in our study. However, it should be noted that the Folin-Ciocalteu reagent actually measures the reducing capacity of a sample, which is not reflected in the name "total phenolic assay" [26]. Therefore, the TPC value as a measure of the phenolics which has been used in overwhelming majority of previously published articles should be regarded rather conditionally.

Acetone extracts were obtained in remarkably (16–77 times) lower yields comparing to methanol/water extracts (Table 1). It indicates that high polarity compounds are dominant in leaves, flowers and stems after removing their lipophilic fraction with hexane. The leaves contained the highest amount of total soluble compounds isolated with both solvents (20.2 g/100 g DW), followed by flowers (16.46 g/100 g DW), stems (15.34 g/100 g DW) and seeds (9.42 g/100 g DW).

The antioxidant properties were measured for extracts and also calculated for the initial dried amaranth material (Table 1). Both values provide important information, because amaranth may be used for the isolation of bioactive compounds or as a raw material for cooking as well as the ingredient in various foods. There were remarkable variations in the obtained values between different anatomical parts of amaranth, applied solvent and assay procedure. Comparing the extracts it may be observed that the differences between RSC of ABTS$^{\bullet+}$ and DPPH$^{\bullet}$, ORAC and TPC of flowers and leaves were not remarkable for acetone and methanol/water extracts, although in some cases they were significantly different ($p<0.05$), while the extracts from stems and seeds were remarkably weaker radical scavengers and antioxidants except for ORAC value of SA which was similar to that of LA.

The samples of plant materials showed significant differences in antioxidant activities between the extracts isolated with different solvents ($p<0.05$). FA and LA were weaker antioxidants than FM and LM, whereas SA and SDA possessed stronger RSC. For instance, the highest RSC of FM in ABTS$^{\bullet+}$ assay was 406.4 μmol TE/g, that is 1.2 times higher than for FA; in DPPH$^{\bullet}$ assay it was 50.7 μmol TE/g and in ORAC 47.0 μmol TE/g. In general, the values in DPPH$^{\bullet}$ assay were remarkably lower comparing to ABTS$^{\bullet+}$ assay. It may be explained by different reaction kinetics and the peculiarities of the used reagents. It is also interesting to note that the differences between these values are bigger when antioxidant capacity values are higher. A strong correlation between TPC and antioxidant activity was observed: TPC *vs.* ABTS$^{\bullet+}$, $R^2=0.99$: TPC *vs*. DPPH$^{\bullet}$, $R^2=0.998$ and TPC *vs*. ORAC, $R^2=0.988$). Thus, the TPC is a good predictor of the *in vitro* antioxidant activity for amaranth extracts.

The values of RSC and antioxidant activity in plant dry material highly depend on extract yield. Actually, these values

Table 1 The yields and antioxidant characteristics of different amaranth anatomical parts extracted by organic solvents $ABTS^{\bullet+}$, $DPPH^{\bullet}$, ORAC expressed in μmol TE/g extracts and μmol TE/g DW plant material); TPC expressed in mg GAE/g extract and mg GAE/g DW plant material)

Samples	Yield % w/w	$ABTS^{\bullet+}$		$DPPH^{\bullet}$		ORAC		TPC	
		Extract	DW	Extract	DW	Extract	DW	Extract	DW
FA	0.76 ± 0.04^{ab}	334.5 ± 5.28^{d}	2.5^{bc}	28.2 ± 0.48^{b}	0.21^{a}	32.9 ± 0.18^{e}	0.25^{ab}	27.3 ± 1.31^{g}	0.21^{b}
FM	15.7 ± 0.6^{e}	406.4 ± 5.02^{f}	64.0^{e}	50.7 ± 0.68^{g}	7.98^{d}	47.0 ± 0.71^{g}	7.40^{d}	33.3 ± 1.86^{d}	5.2^{e}
LA	1.2 ± 0.08^{b}	336.0 ± 5.14^{d}	4.0^{c}	28.6 ± 0.9^{b}	0.33^{a}	24.7 ± 0.89^{b}	0.29^{ab}	24.8 ± 1.97^{f}	0.29^{b}
LM	19.0 ± 0.93^{f}	395.3 ± 9.37^{e}	75.1^{f}	46.9 ± 0.55^{f}	8.91^{e}	41.4 ± 1.26^{f}	7.86^{d}	32.3 ± 1.81^{d}	6.1^{f}
SA	0.64 ± 0.05^{ab}	47.2 ± 3.68^{c}	0.30^{ab}	16.2 ± 0.12^{e}	0.1^{a}	24.5 ± 0.89^{b}	0.16^{a}	9.2 ± 0.65^{d}	0.06^{a}
SM	14.7 ± 0.85^{d}	40.5 ± 2.47^{b}	5.94^{d}	10.7 ± 0.52^{c}	1.57^{c}	4.79 ± 1.28^{c}	0.70^{b}	7.3 ± 0.41^{a}	1.1^{d}
SDA	0.12 ± 0.01^{a}	32.1 ± 0.93^{a}	0.03^{a}	14.6 ± 0.65^{d}	0.01^{a}	15.3 ± 0.89^{d}	0.02^{a}	10.6 ± 0.34^{e}	0.01^{a}
SDM	9.3 ± 0.44^{c}	30.1 ± 1.54^{a}	2.79^{d}	9.5 ± 0.73^{a}	0.88^{b}	4.0 ± 0.39^{a}	0.37^{ab}	8.1 ± 0.33^{ab}	0.8^{c}

Values represented as mean ± standard deviation ($n=3$); different superscript letters within the same column indicate significant differences (one way ANOVA and Duncans' test, $p<0.05$)

DW dry weight; *FA* flower acetone extract; *FM* flower methanol/water extract; *LA* leaf acetone extract; *LM* leaf methanol/water extract; *SA*: stem acetone extract; *SM* stem methanol/water extract; *SDA* seed acetone extract; *SDM* seed methanol/water extract

show how much of TE antioxidants can be isolated with the selected solvent from 1 g of plant DW. So far as the yields of polar extracts were remarkably higher than those of acetone extracts, the total content of isolated antioxidants from 1 g of plant DW by methanol/water was many times higher. The sum of the values obtained for both solvents may be considered as a total amount of TEs present in 1 g of DW plant material. However, it should be noted that hexane which usually extracts very low amount of antioxidants from leafy vegetables, effectively isolates abundant lipohilic fraction from seeds, containing lipid soluble antioxidants, such as tocols and squalene.

As it was mentioned, antioxidant properties of amaranth seeds and leafy parts were studied previously by using different methods, however the results obtained are difficult to compare. For instance, Paśko et al. [16] compared total antioxidant capacity of two amaranth species with quinoa seeds and sprouts and determined that the seed extract of amaranth possessed lower antioxidant activity than quinoa, whereas the TPC in the whole (not defatted) amaranth seeds in their study was up to four times higher comparing to our results. The antioxidant capacities determined by $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ assays and estimated as TEAC interpolated to 50 % inhibition values ($TEAC_{50}$) in sprout extracts of amaranth [16] were up to four times lower comparing with our results on amaranth leaves. Nsimba et al. [27] evaluated antioxidant capacity and TPC in defatted amaranth seeds and reported that the highest antioxidant capacity possessed *A. cruentus*, which scavenged 85.6 % of $DPPH^{\bullet}$ at the applied concentration. The highest TPC of 133.2 mg/g tannic acid equivalents was found for *A. hypochondriacus* seeds and it is remarkably higher as compared with the defatted seeds analyzed in our study. Oboh et al. [17] reported that polar extracts obtained by soaking *A. cruentus* leaves in water for about 24 h possessed 1.4 times higher TPC than LM analyzed in our study.

Direct Evaluation of Antioxidant Capacity by QUENCHER Method

Some antioxidatively active constituents may be strongly bound to other components in plant material matrix and are not extracted by organic solvents or water. Such compounds may be released in human intestinal tract during digestion. Recently a QUENCHER method was developed, which determines the antioxidant activity of the whole plant material including its insoluble fraction. According to Serpen et al. [28], the values obtained by using QUENCHER method for insoluble food components show a significant antioxidant activity, which in many cases remarkably higher than those obtained by the traditional extraction procedures. They hypothesized that free functional groups on the surface of insoluble particles quench with radicals.

It may be observed that all QUENCHER values for the leaves were higher than for other parts, except for $DPPH^{\bullet}$, when RSC of leaves and flowers were similar (Table 2). The RSC and antioxidant activity of stems and seeds were remarkably lower, which is in agreement with the results obtained for the extracts. However, comparing QUENCHER results with those obtained by analyzing the extracts some interesting observations can be noticed. The sum of RSC of leaves and flowers obtained in $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ assay by analyzing the extracts isolated by both solvents and calculated for 1 g of DW was approximately two times lower than the relevant values obtained by a QUENCHER assay. This difference for seeds was even higher. The sum of values obtained in ORAC assay by analyzing the extracts isolated by both solvents and

Table 2 Antioxidant activity (μmol TE/g DW) and total phenols content (mg GAE/kg DW) of amaranth parts obtained by QUENCHER procedure

Samples	$ABTS^{\bullet+}$	$DPPH^{\bullet}$	ORAC	TPC
Flowers	112.33±7.45[b]	16.73±0.35[c]	35.5±0.71[c]	7.86±0.02[c]
Leaves	144.24±2.41[c]	16.42±0.37[c]	51.3±3.73[d]	10.08±0.02[d]
Stems	19.05±1.13[a]	1.72±0.02[a]	12.2±0.31[a]	1.73±0.02[a]
Seeds	21.82±1.06[a]	3.96±0.03[b]	15.7±1.35[b]	3.70±0.02[b]

Values represented as mean ± standard deviation (n=3); different superscript letters within the same column indicate significant differences (one way ANOVA and Duncans' test, $p<0.05$)

TE trolox equivalents; *DW* dry weight; *GAE* gallic acid equivalents

calculated for 1 g of DW was even more times higher in case of QUENCHER assay; however, the differences in TPC values were less remarkable, except for the seeds. Several reasons may be raised to explain these differences. First of all, as it was already mentioned, some part of antioxidatively active compounds may remain in the matrix after extraction because they are bound to other constituents. Some classes of compounds, *e.g.*, antioxidatively active proteins and carbohydrates may be insoluble in the used solvents while in QUENCHER assay their active sites in the structures may participate in antioxidant processes. Finally, the systems of assay are different and the differences in the assay matrix may influence reaction kinetics. In $DPPH^{\bullet}$ assay, on the contrary to other methods, the seeds were stronger radical scavengers than stems. The defatted seeds contain high amount of proteins and some of them may be antioxidatively active substances.

A strong correlation between TPC and antioxidant activity measured by a QUENCHER method was also observed: TPC *vs*. $ABTS^{\bullet+}$, R^2=0.95; TPC *vs*. ORAC, R^2=0.97. To the best of our knowledge no results have ever been published on evaluating amaranth seeds and leaves using QUENCHER method.

Preliminary Characterization of Amaranth Phytochemicals by Chromatographic Analysis

A reversed-phase HPLC separation coupled with PDA detector and the $DPPH^{\bullet}$ scavenging detector was used for the preliminary screening of antioxidants which may be present in amaranth. Based on negative peak in the chromatogram the main $DPPH^{\bullet}$ scavenger in all extracts was rutin. Rutin concentration was measured from the calibration curve prepared by using different concentrations of a standard: it was in LA 124, in LM 3606, in FA 99, in FM 4072, and in SDM 40 mg/kg DW. In the extracts of stems only traces of rutin were detected. It should be noted that the yield of acetone extracts after ASE with hexane was very low, in most cases less than 1 %. Rutin, as a quercetin glycoside is better soluble in polar solvents such as methanol than in acetone, and most likely acetone extract was saturated by this compound during the first step of extraction. The solubility of rutin in methanol, depending on solvent temperature, was reported approximately 7–14 times higher than in acetone [29]. Rutin as the main amaranth flavonoid was reported previously; the content of this compound in amaranth varied from 0.08 (seeds) to 24.5 g/kg DW (leaves) [30]. Consequently, our results are in the range of previously reported concentrations, although they may highly depend on numerous factors.

For preliminary phytochemical screening of extracts they were analyzed by UPLC-QTOF-MS. The following compounds were identified in leaf and flower extracts by measuring their accurate mass and retention time: rutin, nicotiflorin, isoquercitrin, 4-hydroxybenzoic and *p*-coumaric acids. Detailed information on the identification of amaranth compounds is presented in additional data given in Online Resource 1. It may be clearly observed that rutin is the major quantitatively constituent in amaranth extracts, which is in agreement with other studies on amaranth flavonoids. On the other hand, a large number of recorded peaks on the chromatograms indicate that the extracts are complex mixtures of compounds; however, exact mass data obtained by UPLC-QTOF-MS was not sufficient for their identification, because mass spectra libraries give too many candidate structures for the measured masses. Purification of compounds and analysis by NMR and other spectra methods would be necessary for positive identification of minor amaranth constituents.

Various phenolic compounds and flavonoids were reported previously in amaranth seeds and vegetables. For instance, the sprouts of amaranth contained rutin as the main constituent and gallic, *p*-coumaric and syringic acids as other important constituents [15]. It was also shown that the content of polyphenols in different amaranth seed varieties were influenced by many factors, such as genotype, climatic and environmental conditions, experimental sites and seasons [31]. The content of ferulic, caffeic and *p*-coumaric acids in amaranth seed methanol extracts were higher than in quinoa seeds and soybeans [17]. Rutin, isoquercitrin and nicotiflorin were quantified in amaranth seed flours [32].

Conclusion

All anatomical parts of amaranth possess radical scavenging and antioxidant activities; however, the extracts isolated from defatted leaves and flowers were remarkably stronger antioxidants in $ABTS^{\bullet+}$, $DPPH^{\bullet}$, ORAC and TPC assays compared to defatted stems and seeds. The extracts isolated with polar solvent methanol/water mixture were stronger antioxidants than the extracts obtained with acetone. Antioxidant power of the whole plant material evaluated by QUENCHER method was approximately two times higher than the integrated values obtained for the extracts; it suggests that considerable

amount of antioxidatively active compounds remain in the plant material after extraction. A strong correlation between total polyphenols content and antioxidant activity measured by $ABTS^{\bullet+}$, $DPPH^{\bullet}$ and ORAC was observed. On-line HPLC-$DPPH^{\bullet}$ assay of amaranth extracts showed that rutin was the main radical scavenger in amaranth.

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Conflict of Interest The authors declared that they have no conflict of interest.

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