Phenolic acids in seeds and products obtained from *Amaranthus cruentus*

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Summary

Several classes of chemical substances like phenolic acids, squalene, tocopherols or sterols are found in non-saponifiable lipid fraction of amaranth seeds. The aim of this study was to investigate phenolic acids and total phenolic compounds contents in seeds of amaranth (*Amaranthus cruentus).* Several amaranth-based products like expanded seeds ("popping") and flakes were also investigated. The total content of phenolics in seeds was in range between 272.6 mg·kg^{-1} and 615.3 mg·kg^{-1} dry matter (d.m.). The DPPH free radical-scavenging ability of the seeds ranged from 11 060 μmol·kg^{-1} to 15 330 μmol·kg^{-1} d.m. Phenolic acid composition was determined by high performance liquid chromatography-based method. The total content of phenolic acids ranged from 286 mg·kg^{-1} to 384 mg·kg^{-1} in seeds. Seven different phenolic acids were identified. Ferulic, vanillic and *p*-hydroxybenzoic acids were the major phenolic acids determined in amaranth seeds. There was a significant difference in phenolic acids in popping and flakes, but not in case of ferulic acid. In this experiment, the cultivation area affected the sum of phenolic acids contents, showing significant differences in the areas of Nowy Gaj, Laziska and Piaski Górne. Results showed that differences in individual phenolic acids contents are statistically random and not connected with cultivation area.

Keywords

Amaranthus cruentus; antioxidant activity; flakes; phenolic acids; popping

Amaranth *(Amaranthus cruentus),* commonly known as "Szarłat" in Poland, is one of the oldest crop plants in the world. Its nutritional value was appreciated by the ancient Inca, Maya and Aztec civilizations [1]. Currently, amaranth is cultivated in many countries of both Americas, south-eastern Asia and in Africa. Since early 1990s, plantations have also been established in many European countries, including Poland.

Field experiments conducted in seven farms throughout Poland proved that amaranth can be cultivated in various climatic and soil conditions typically found in Poland, with a seed yield from 1.8 t·ha^{-1} to 3.5 t·ha^{-1} [2]. Initially, native South American varieties of Amaranth were cultivated. However. They were later replaced by a Polish cultivar Rawa, which is better fitted to the climatic and soil conditions of Poland [3]. Amaranth seeds are most frequently used for production of expanded seeds ("popping") and flakes. These products are gluten-free and have high nutritional value, which makes them valuable for consumers suffering from celiac disease [4–6].

Amaranth seeds are a rich source of biologically active substances that, due to their chemistry, show significant antioxidant activities. Several classes of chemical substances like phenolic acids, squalene, tocopherols or sterols are found in non-saponifiable lipid (NSL) fraction of amaranth seeds [6–9]. These compounds are also found in amaranth-based products in various quantities [10].

Phenolic acids are one of the most important group of antioxidants that are found in cereals [11]. These compounds fulfill various important functions in plants biochemical mechanisms, e.g.

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act as substrates for biosynthesis reactions (e.g. caffeic acid is a precursor of lignins). They also protect the plant against harmful effects of ultraviolet irradiation [12]. Contents of polyphenols and phenolic acids in plants is however strongly influenced by many factors, such as climatic and agro-technical factors, level of ripeness, time of harvest and storage conditions.

The common occurrence of polyphenols and phenolic acids in plants makes them inseparable components of the human diet. Consumption of food rich in polyphenols has a favourable effect on reduction of cardiovascular disease development risk. The influence of these compounds on the lipemia and inflammatory states is yet not clear. Significantly higher effects are observed in regards to functions concerning the endothelium of blood vessels and hemostasis [13].

The scope of this research was to identify and quantify phenolic acids in seeds of *Amaranthus cruentus* and products obtained from them. Additionally, antioxidant activities of amaranth seeds originating from different areas of cultivation were also assessed.

MATERIAL AND METHODS

Sample preparation

Seeds of *Amaranthus cruentus* from 2006 and 2007 crops cultivated in Lubuskie, Małopolskie and Lower Silesia Provinces of Poland were used (Tab. 1). Seed samples were subsequently used for production of several amaranth-based products i.e. "popping" and cereal flakes. Popping and flakes were produced in "Szarłat" company (Łomża, Poland). Composite sample obtained by mixing seed samples in equal proportions were used in the production process. For analytical methods involving the extraction step, a 10 g sub-sample was grounded for 1 min with a laboratory mill (IKA Werke, Staufen, Germany).

Tab. 1. Amaranth seed samples.

Sample	Place of growing (locality/province)	Year of growing
1	Nowy Gaj/Lublin Province	2007
2	Herbdów/Małopolskie Province	2007
3	Łaziska/Lublin Province	2007
4	Piaski/Lublin Province	2007
5	Oleśnica/Lower Silesia Province	2007
6	the vicinity of Lublin/Lublin Province	2007
7	Piaski Górne/Lublin Province	2006

Determination of polyphenols

The total content of phenolic compounds was determined spectrophotometrically, using the method with Folin-Ciocalteu reagent [14]. The procedure involved extraction of phenolic compounds with 20% water : methanol solution. Sample (2 g) was extracted three times with 10 ml of solvent solution. Combined extracts were concentrated using a rotary evaporator type R210 (Büchi Labortechnik, Flawil, Switzerland) to approximate volume of 10 ml. A volume of 0.25 ml of the extract was transferred into a measuring flask and evaporated to dryness under a gentle stream of nitrogen. A volume of 0.25 ml of Folin-Ciocalteu reagent and 0.5 ml of 14% sodium carbonate (Na_2CO_3) water solution were added to the flask. Flask was filled up with distilled water to a final volume of 5 ml. After 30 min, absorbance at 720 nm was measured using a SP6-500 UV spectrophotometer (Pye Unicam, Cambridge, United Kingdom). Measurements were carried out against a blank sample. The content of phenolic compounds was calculated using (+)-catechin calibration curve.

Determination of phenolic acids

Total phenolic acids content was determined according to the method described by MATTILA and KUMPULAINEN [15], with modifications proposed by MATTILA et al. [16]. A 0.5–0.8 g sample was homogenized with 7 ml of a mixture of methanol and butylated hydroxyanisole (BHA, $2\,g \cdot l^{-1}$) obtained from POCH (Gliwice, Poland), and 10% acetic acid (85 : 15), by Ultra Turrax T25 homogenizer (IKA Werke). The homogenized sample was ultrasonicated for 30 min and made to a volume of 10 ml with distilled water. A volume of 12 ml of distilled water, containing 1% ascorbic acid and 0.415% EDTA, and 5 ml of 10 $mol \cdot l^{-1}$ NaOH were then added to the test tube, sealed and stirred for 16 h at 20 °C using a magnetic stirrer. The solution was then adjusted to pH 2 with concentrated HCl and the liberated phenolic acids were extracted with 15 ml of a mixture of cold diethylether and ethylacetate (1 : 1), centrifuged at 620 ×*g* (Rotofix 32; Hettich Zentrifugen, Tuttlingen, Germany) and the organic layer was recovered. The extraction step was repeated twice and the organic layers were combined. After alkaline hydrolysis, acid hydrolysis was performed by adding 2.5 ml of concentrated HCl into the test tube and subsequent incubation of the sample in a water bath at 85 °C for 30 min. The sample was then cooled down, and the sample further processed in the same manner as after alkaline hydrolysis. The organic layers from alkaline and acid hydro-

lyses were combined, evaporated to dryness, redissolved in 2 ml of methanol, filtered and analysed by high performance liquid chromatography (HPLC).

The analytical HPLC system consisted of an Agilent 1100 Series chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with a diode array detector. The HPLC pumps, autosampler, column oven and diode array system were monitored and controlled by HP ChemStation computer programme (Agilent Technologies). Protocatechuic acid, *p*-hydroxybenzoic acid and vanillic acid were determined at 254 nm, syringic acid, *p*-coumaric acid, *m*-coumaric acid, *o*-coumaric acid and *trans*-cinnamic acid at 280 nm, and caffeic acid, ferulic acid and sinapic acid at 329 nm. Separations were performed on a 150 × 4.0 mm i.d., 3 μm, Inertsil ODS-3 column (GL Sciences, Tokyo, Japan) equipped with a C-18 guard cartridge. The temperature of the column oven was set at 35 °C. A gradient elution was employed with a mobile phase consisting of 50 mmol·l^{-1} H_3PO_4 at pH 2.5 (solution A) and acetonitrile (solution B) as follows: isocratic elution 95% A, 0–5 min; linear gradient from 95% A to 85% A, 5–17 min; linear gradient from 85% A to 80% A, 17–40 min, linear gradient from 80% A to 50% A, 40–60 min; isocratic elution 50% A, 60–65 min; linear gradient from 50% A to 95% A, 65–67 min; post-time, 6 min before the next injection. The flow rate of the mobile phase was 0.7 ml·min^{-1} and the injection volume was 10 μl. All monomeric phenolic acids were quantified using the external standard calibration method. All quantifications were based on peak area. Analyses were performed in triplicates.

Determination of DPPH free radical-capturing ability

The DPPH• (2.2-diphenyl-1-picrylhydrazyl) free radical-capturing ability of amaranth seed phenolics was determined using the method described by MOURE et al. [17]. A volume of 0.4 ml of polyphenolic compounds alcoholic solution obtained by the procedure described above and 2 ml of 0.36 mmol·l^{-1} DPPH• radical in methanol were introduced to the reaction mixture. At the same time, a control containing 0.4 ml of 80% methanol and 2 ml of 0.36 mmol·l^{-1} DPPH• radical in methanol was prepared. Absorbance was measured after 16 min of reaction at 515 nm by ATI Unicam UV2-100 spectrophotometer (ATI Unicam, Cambridge, United Kingdom). Results were expressed as the amount of DPPH• radicals (in micromoles) captured by bioactive components extracted from 1 kg of seeds.

Data analysis

All analyses were performed in three replications. The significance of differences between the mean values was estimated by Duncan's test. Statistical analysis was performed at a significance level of $^*P < 0.05$. The correlation analysis was conducted using Statistica v. 8.0 software (StatSoft, Kraków, Poland).

RESULTS AND DISCUSSION

A significant variability in the total content of phenolic compounds in the examined amaranth seeds samples was observed (Tab. 2). No significant correlations were noted between the content of the compounds and the cultivation area. In seeds from 2007 crops, the total content of phenolic compounds ranged from about 370 mg·kg^{-1} dry matter (d.m.) (Piaski, Lublin Province) to almost 620 mg·kg^{-1} d.m. of seeds (Nowy Gaj, Lublin Province) (Tab. 2). The contents in seeds from Piaski Górne (2006 crop, Lublin Province), after one year storage, were significantly lower (272.6 mg·kg^{-1} d.m. of seeds). These values are similar to data reported by GORINSTEIN et al. [6] who claimed that examined seeds of *Amaranthus cruentus* samples contained approximately 430 mg·kg^{-1} d.m. of phenolic compounds [6]. *Amaranthus hypochondriacus* and *Amaranthus hybridus* showed polyphenol contents of 415 mg·kg^{-1} and 405 mg·kg^{-1} d.m., while the contents in other common cereals varied from 330 mg·kg^{-1} d.m. to 912 mg·kg^{-1} d.m. for rice and wheat grains [6].

The present study revealed that extracts from amaranth seed showed the antioxidative potential, beginning with 11 064 μmol·kg^{-1} d.m. of seeds (Tab. 2). Three of the examined samples

Tab. 2. Total contents of phenolic compounds and anti-radical activity in seeds of *Amaranthus cruentus*.

Sample	Total phenolic compounds [mg·kg^{-1}]	DPPH anti-radical activity [μmol·kg^{-1}]
1	615.3 ± 52.6^{a}	12 701 ± 752^{ab}
2	559.1 ± 32.6^{b}	13 928 ± 155^{ac}
3	514.4 ± 37.9^{bc}	11 064 ± 535^{d}
4	369.2 ± 36.5^{d}	11 423 ± 492^{bd}
5	455.2 ± 20.7^{c}	13 020 ± 2 718^{a}
6	497.8 ± 22.0^{bc}	14 686 ± 995^{c}
7	272.6 ± 32.9^{e}	15 327 ± 445^{c}

Results are the mean value ± standard deviation for three replicates; values marked with different letters statistically different ($^*P \leq 0.05$). Values are expressed per kilogram of dry matter. Samples of seeds are listed in the Tab. 1.

(2, 6 and 7) were similarly active with the DPPH• radical. In those samples, DPPH anti-radical activity was between 13928 μmol·kg^{-1} and 15327 μmol·kg^{-1} d.m. Slightly lower values were observed with two samples cultivated in Nowy Gaj and Oleśnica (12701 μmol·kg^{-1} d.m. and 13020 μmol·kg^{-1} d.m. of seeds, respectively). The highest DPPH anti-radical activity (15327 μmol·kg^{-1} d.m. of seeds) was demonstrated by seeds cultivated in Piaski Górne, examined after one-year of storage. According to YAWADIO NSIMBA et al. [18], the extract of *Amaranthus cruentus* seeds showed anti-radical activity of 84.0–85.6% DPPH• and 79.5% DPPH• for *Amaranthus hypochondriacus*. SCHOENLECHNER et al. [19] claimed that 0.05% addition of amaranth seed extract was sufficient to inhibit degradation of β-carotene in a model system of β-carotene/linoleic acid. Polyphenols were regarded as the main compounds responsible for antioxidative potential it the mentioned study [19].

Seven different phenolic acids were identified in the analysed samples: caffeic acid, ferulic acid, sinapic acid, *p*-coumaric acid, cinnamic acid, *p*-hydroxybenzoic and vanillic acid (Tab. 3). PAŚKO et al. [20] when analysing *Amaranthus cruentus* seeds, identified gallic acid, *p*-hydroxybenzoic and vanillic acid in Aztek cultivar. The analysed Rawa cultivar additionally contained *p*–coumaric acid, while vanillic acid was not found at all. CHITINDINGU et al. [21], when examining another amaranth species, namely *Amaranthus hybridus*, confirmed the presence of caffeic, protocatechuic and ferulic acids.

The total content of phenolic acids was between 286.67 mg·kg^{-1} (sample 6, vicinity of Lublin) and 384.70 mg·kg^{-1} (sample 1, Nowy Gaj). KLIMCZAK et al. [22], when examining *Amaranthus paniculatus* seeds, obtained a similar sum of phenolic acids, 295.50 mg·kg^{-1}. On the other hand, the same study revealed much lower values for another species, *Amaranthus caudatus*, 106.86 mg·kg^{-1} [22]. According to REPO-CARRASCO-VALENCIA et al. [23], phenolic acid contents varied greatly between various *Amaranthus caudatus* varieties

Tab. 3. Contents of phenolic acids in seeds of *Amaranthus cruentus*.

Sample	Content of phenolic acids [mg·kg^{-1}]							
	Caffeic	Ferulic	Sinapic	*p*-Coumaric	Cinnamic	p-Hydroxybenzoic	Vanillic	Total
1	4.13 ± 0.04^{a}	85.80 ± 1.70^{a}	trace	11.25 ± 0.17^{a}	trace	125.10 ± 2.08^{a}	158.43 ± 0.63^{a}	384.70 ± 2.37^{a}
2	3.45 ± 0.59^{b}	54.30 ± 0.60^{b}	trace	11.48 ± 0.59^{a}	trace	100.44 ± 2.47^{b}	119.60 ± 7.05^{b}	289.28 ± 3.65^{b}
3	3.08 ± 0.01^{c}	66.36 ± 3.65^{c}	trace	11.37 ± 0.16^{a}	trace	141.92 ± 12.09^{c}	149.65 ± 0.16^{c}	372.39 ± 5.96^{c}
4	3.37 ± 0.02^{b}	76.21 ± 4.08^{d}	trace	8.33 ± 0.51^{b}	trace	88.68 ± 2.14^{d}	119.75 ± 1.84^{b}	296.34 ± 6.08^{d}
5	5.51 ± 0.06^{d}	83.81 ± 0.90^{a}	trace	11.14 ± 0.14^{a}	trace	105.36 ± 0.25^{b}	136.58 ± 1.20^{d}	342.40 ± 0.10^{e}
6	4.34 ± 0.08^{a}	70.97 ± 2.47^{e}	trace	8.58 ± 0.34^{b}	trace	93.10 ± 0.05^{d}	109.69 ± 7.85^{e}	286.67 ± 3.54^{d}
7	4.74 ± 0.09^{e}	83.07 ± 2.98^{a}	trace	9.62 ± 0.67^{c}	trace	89.24 ± 1.72^{d}	126.84 ± 1.05^{f}	313.51 ± 0.69^{f}

Results are the mean value ± standard deviation for three replicates; values marked with different letters are statistically different (*$P \leq 0.05$). Samples of seeds are listed in the Tab. 1.

Tab. 4. Contents of phenolic acids in products produced from seeds of *Amaranthus cruentus*.

Product	Content of phenolic acids [mg·kg^{-1}]							
	Caffeic	Ferulic	Sinapic	*p*-Coumaric	Cinnamic	p-Hydroxybenzoic	Vanillic	Total
Expanded seed	3.55 ± 0.18^{a}	81.9 ± 5.1^{a}	trace	8.96 ± 0.34^{a}	trace	115.3 ± 7.5^{a}	118.2 ± 6.0^{a}	327.93 ± 18.87^{a}
Flakes	4.97 ± 0.15^{b}	85.5 ± 3.4^{a}	trace	11.79 ± 0.93^{b}	trace	140.5 ± 8.6^{b}	155.6 ± 9.7^{b}	398.36 ± 22.63^{b}

Results are the mean value ± standard deviation of three replicates; values marked with different letters are statistically different (*$P \leq 0.05$).

(168–329 mg·kg^{-1} seed). Plant cultivation conditions, like soil or climate, and also genetic factors may influence the contents of phenolic compounds in grains [24, 25].

The results of this study indicate that the content of individual phenolic acids in seeds cultivated in various regions of Poland show high variability. Different contributions of individual compounds to the summary content of phenolic acids was also observed. In the current study, the contents of particular phenolic acids ranged from trace amounts (less than 3.5 mg·kg^{-1}; cinnamic and sinapic acids) to 109–158 mg·kg^{-1} seeds (vanillic acid). Similar contents of vanillic acid are found in rape seeds [26]. The content of *p*-hydroxybenzoic acid in the amaranth seeds varied between 88.68 mg·kg^{-1} (sample 4, Piaski, Lublin Province) and 141.92 mg·kg^{-1} (sample 3, Łaziska, Lublin Province). Ferulic acid was the major hydroxycinnamic acid in amaranth seeds. Seeds of *Amaranthus cruentus* obtained from the plantation located in Małopolskie Province (Herbdów) had lower contents of ferulic acid (54.30 mg·kg^{-1}) compared to other samples (from 66.36 mg·kg^{-1} to 85.80 mg·kg^{-1}). The occurrence of ferulic acid in amaranth seeds might by considered as health beneficial from the dietary point of view, because ferulic acid is regarded as a cholesterol-lowering, anti-thrombosis, anti-inflammatory and anti-cancer factor [27]. Hydroxycinnamic acids, *p*-coumaric, caffeic and sinapic acids, were found in much lower amounts in the analysed samples. It is suspected that one of the natural functions of hydroxycinnamic acids is related to stabilization and protection of grain lipids, which seems to be in particular important when amaranth seeds are used for production of amaranth oil [28].

Amaranth seeds are rarely consumed in their raw form. The most common method of their processing is their expanding and flaking of grains. These processes are categorized as a group of hydrothermal modifications with high temperature and pressure being applied. The application of the mentioned processing improves the digestibility of seeds, however, it often deprives them of their precious bioactive components. Therefore, an additional attempt was made to analyse phenolic acids in amaranth products. **Tab. 4** shows the results of the individual phenolic acids contents in the analysed samples. Compared to the results obtained for raw seeds (presented in Tab. 3), the conclusion can be drawn that the technological processing of amaranth seeds did not reduce the contents of phenolic acids. It seems probable that processing enhanced the extractability of the compounds. The following acids were found in both "popping" and flakes: caffeic, ferulic, sinapic, *p*-coumaric, cinnamic, *p*-hydroxybenzoic and vanillic. GUMUL et al. [29] claimed that, in general, extrusion lowered the content of polyphenols by about 40% in rye seeds, while the highest losses of endogenic phenolic compounds were observed for the extrusion process, 20% of input material (temperature, 120 °C). Similarly to the results obtained for amaranth seeds, sinapic and cinnamic acids were found in trace amounts in both "popping" and flakes, while vanillic and *p*-hydroxybenzoic acids were the most abundant phenolic acids in those products (Tab. 4).

CONCLUSIONS

The *Amaranthus cruentus* seeds analysed in this study were contained significant amounts of phenolic compounds, ranging from 272.5 mg·kg^{-1} to 615.3 mg·kg^{-1}. In amaranthus seeds and products obtained from them (popping and flakes), seven phenolic acids were indentified, among which vanillic, *p*-hydroxybenzoic and ferulic acids dominated. A significant difference in phenolic acids in popping and flakes was found, but not in case ferulic acid. In this experiment, the cultivation area affected the sum of phenolic acids contents, showing significant differences in the areas of Nowy Gaj, Laziska and Piaski Górne. Results show that differences in individual phenolic acids contents are statistically random and not connected with cultivation area.

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