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Fractionation of Buckwheat Seed Phenolics and Analysis of Their Antioxidant Activity

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Key words: buckwheat, phenolic compounds, flavonoids, Sephadex LH-20, antioxidant activity

Five fractions of phenolic compounds were obtained from the extract of common buckwheat seed (*Fagopyrum esculentum* Moench) using Sephadex LH-20 column chromatography with methanol as a mobile phase. The total phenolics content ranged from 19.8±1.5 (fraction I) to 164±2.2 mg (+)-catechin eq/g (fraction IV). The profiles of phenolic acids and flavonoids in the fractions were analysed using RP-HPLC-DAD. The antioxidant activity was tested as $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ scavenging activity and capability to reduce the Fe(III)/2,4,6-Tris(2-pyridyl)-*s*-triazine complex to the ferrous form. Results were expressed as Trolox equivalent antioxidant capacity (TEAC), IC_{50} and ferric reducing antioxidant power (FRAP) values, respectively. The highest antioxidant activity was noted for fraction IV that was predominated by flavones. TEAC, IC_{50} and FRAP values were: 1.47±0.01 mmol Trolox eq/g, 0.058±0.003 mg/assay and 2.18±0.05 mmol Fe(II)/g, respectively. Rutin constituted 77.7% of the compounds identified in fraction III. The antiradical activity and reducing capability of this fraction were lower compared to fraction IV, but significantly higher than in fractions I and II. The main phenolic compounds of fractions I and II were phenolic acids (caffeic, 5-*O*-caffeoylquinic and *p*-coumaric). The antioxidant activity of fraction V was similar to that of fraction III.

INTRODUCTION

Buckwheat belongs to the *Polygonaceae* family, but its seeds (more precisely: achenes) are usually classified among the cereal grains because of their similar usage. Two buckwheat species are commonly cultivated, namely: common buckwheat (*Fagopyrum esculentum*) and tartary buckwheat (*Fagopyrum tataricum*). The nutritive value of buckwheat seeds is significantly higher than that of cereal crops. They contain 48.7––55.2% of soluble carbohydrates, *ca*. 3% of lipids, 12.7–17.8% of dietary fiber and *ca*. 12% of protein with a high biological value and high levels of most essential amino acids [Eggum *et al.*, 1981]. Buckwheat seeds are also a source of many bioactive constituents including phenolic compounds, inositols, tocopherols, carotenoids, phytosterols, squalene, vitamins, glutathione and melatonin [Giménez-Bastida *et al.*, 2015]. Owing to the presence of compounds with biological activity, buckwheat has been recently gaining a growing interest as a potential functional food [Zhang *et al.*, 2012; Giménez--Bastida *et al.*, 2015]. Ample studies have been conducted on traditional buckwheat products (groats cooked as porridge and flour used in the preparation of pancakes, noodles, *etc*.), as well as on the development of novel products based on or with the addition of buckwheat (sprouts, biscuits and snacks, buckwheat-enhanced wheat bread, *etc*.) in terms of their effect upon human health [Wójtowicz *et al.*, 2013; Giménez-Bastida *et al.*, 2015]. The antioxidant, anti-inflammatory, antiobesity, blood cholesterol decreasing, mammary gland cancer inhibiting, and hyperglycemia prevalence lowering activities of buckwheat have already been reported in literature [Zhang *et al.*, 2012].

The antioxidant potential of buckwheat seeds is determined mainly by phenolic compounds [Holasova *et al.*, 2002]. Buckwheat seeds contain low molecular weight (LMW) phenolics – phenolic acids and flavonoids [Verardo *et al*., 2010; Inglett *et al*., 2011] and condensed tannins with high molecular weights (HMW) [Watanabe *et al.*, 1997; Karamać, 2007]. The predominating LMW phenolic compound is rutin (quercetin-3-*O*-rutinoside) [Zielińska *et al.*, 2007; Ölschläger *et al.*, 2008; Kiprovski *et al.*, 2015]. In a study by Jiang *et al.* [2007], rutin contribution in seeds of *F. esculentum* reached 0.02% and was found to play a significant role in the antioxidant capacity of buckwheat seeds. These authors demonstrated a strong correlation between rutin content assayed for seeds of various cultivars/accessions and their antioxidant capacity (r^2=0.976). A statistically significant correlation (r^2=0.987) was also reported by Holasova *et al.* [2002] who compared rutin content with antioxidant capacity of seeds and other aerial parts of buckwheat. In contrast, Oomah & Mazza [1996] suggested that

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the antioxidant activity of buckwheat seeds is independent of rutin content. Zielińska *et al*. [2010] demonstrated a low contribution of rutin in the antioxidant potential of buckwheat seed products (flour, hull, groats and sprouts) and concluded that the focus on rutin as the main antioxidant in buckwheat can be overestimated. For whole common buckwheat seeds, the contribution of rutin in the antioxidant capacity was estimated at 25% [Zielińska *et al*., 2012]. Because lipophilic antioxidants, *e.g.* tocopherols and carotenoids, were reported to insignificantly participate in the antioxidant capacity of buckwheat [Holasova *et al.*, 2002], rather other phenolic compounds determine the antioxidant potential of its seeds, including proanthocyanidins (condensed tannins). The content of extractable flavan-3-ols oligomers in common buckwheat seeds reached 118 mg/100g [Hellström *et al.,* 2009] and was significantly higher than that of rutin. Simultaneously, proanthocyanidins are characterized by a higher antioxidant activity compared to the LMW phenolics [Watanabe *et al.,* 1997; Karamać, 2010]. In turn, information on the effect of buckwheat seed phenolic acids and flavonoids other than rutin on the antioxidant potential is sparse. Considering the above, the aim of this study was to apply Sephadex LH-20 column chromatography to separate fractions of LMW compounds from seeds of common buckwheat as well as to determine profiles of phenolics of these fractions and their antioxidant activity.

MATERIAL AND METHODS

Material

The study was conducted using whole seeds (achenes) of common buckwheat (*Fagopyrum esculentum* Moench) of Kora cultivar obtained from the Plant Breading Station in Olsztyn (Poland).

Extraction of phenolic compounds

Buckwheat seeds were ground in a laboratory mill. Flour (3×20 g) was suspended in 80% acetone (v/v) in the ratio of 1:8 (w/v), and extraction was conducted for 15 min at 60°C. After cooling, the supernatant was separated and the residue was extracted twice more. Acetone from combined filtrates was evaporated under vacuum at 40°C (Rotavapor R-200, Büchi Labortechnik, Switzerland). The resultant aqueous residue was lyophilized for ~48 h at -70°C and 0.013 mbar (Lyph Lock 6 freeze dry system, Labconco, USA). The extract was stored at -20°C until used.

Fractionation of extract of phenolic compounds

The extract (2 g) was dissolved in 6 mL of methanol and loaded onto a chromatographic column packed with Sephadex LH-20 (i.d.=3 cm, H=40 cm). Methanol was used a mobile phase. Separation was carried out with the use of a peristaltic pump (LKB 2132 MicroPerpex, Pharmacia LKB, Sweden) and a fraction collector (RediFrac, Pharmacia). Fractions with the volume of 4.5 mL were collected after discarding the void volume of the eluate V_0=98 mL. Absorbance of individual fractions was read at two wavelengths – 280 and 350 nm (spectrophotometer DU-7500, Beckman Instruments, USA). Based on the chromatogram plotted from the separation, the individual fractions were combined into five major fractions, that were dried by methanol evaporation under vacuum at 40°C.

Total phenolics content

The content of total phenolics of major fractions was determined using Folin-Ciocalteu's reagent [Naczk & Shahidi, 1989]. Fractions were dissolved in methanol in concentrations of 1 mg/mL (I and II) or 2 mg/mL (III-V). Solutions of the fractions (0.25 mL) were vortexed (Vortex Genie2, Scientific Industries, USA) with 0.25 mL of Folin–Ciocalteu's reagent (diluted two-fold), 0.5 mL of a saturated solution of sodium carbonate and 4 mL of water. After 25 min, the samples were centrifuged (MPW-210, MPW Med. Instruments, Poland) for 5 min at 5000×*g*. Absorbance of supernatants was read at λ=725 nm using a DU-7500 spectrophotometer. Results were expressed as mg of (+)-catechin equivalents per g of fraction based on the standard curve for (+)-catechin (r=0.996).

HPLC analysis of phenolic compounds

HPLC analysis was conducted using an HPLC system (Shimadzu Co., Japan) consisting of two LC-10AD$_{Vp}$ pumps, SCL-10A$_{Vp}$ system controller, SPD-M10A$_{Vp}$ photo-diode array detector and Class-VP5 chromatography laboratory automated software system. Once dissolved in methanol (5 mg/mL) and filtrated (0.45 μm), the fractions (20 μL) were injected onto a LiChrospher 100 RP-18 column (4×250 mm, particle size 5 μm, Merck, Germany). Phenolic compounds were eluted with a flow rate 1 mL/min in gradient system consisting of solvents A: water-acetonitrile-acetic acid (93:5:2 v/v/v) and B: water-acetonitrile-acetic acid (58:40:2 v/v/v). The gradient profile was: 0–20 min, 0% to 40% B; 20–30 min isocratic 40% B; 30–50 min 40% to 100% B. Between sample injections, the mobile phase A (10 mL) was used for column re-equilibration. The diode array detection was performed by scanning over a wavelength range from 200 to 400 nm. The separated compounds were identified by comparing their retention times and UV spectra with those of standards. Quantification of the identified compounds was achieved based on peak areas of the analyte and corresponding standards. Orientin, homoorientin, vitexin, isovitexin, quercetin, quercitrin, hyperoside, (–)-epicatechin and (–)-epicatechin gallate were purchased at Extrasynthese (France). The other applied reference compounds were obtained at Sigma-Aldrich (USA).

Antiradical activity against ABTS$^{\bullet+}$

The Trolox equivalent antioxidant capacity (TEAC) of the fractions was determined using the ABTS radical cation decolorization assay [Re *et al.*, 1999]. ABTS (96 mg) was activated in a 2.45 mmol/L solution of potassium persulfate for 16 h. The stock solution was diluted with methanol up to a final absorbance of 0.70±0.02 at 734 nm. Fractions were dissolved in methanol in the concentration of 1 or 2 mg/mL. The ABTS$^{\bullet+}$ solution was pipetted in 2-mL doses to test tubes placed in a block heater (TH-24, Meditherm, Poland) warmed to 30°C, and after temperature settling 20 μL of fraction solutions were added. The absorbance was recorded at λ=734 nm after 6 min, strictly. The results were calculated

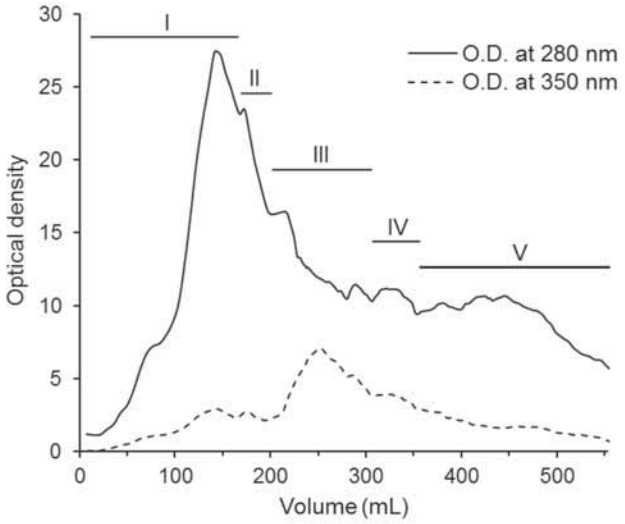

FIGURE 1. Chromatographic Sephadex LH-20 separation of buckwheat seed phenolic extract.

using the standard curve for Trolox (r=0.993) and expressed in mmol of Trolox equivalents per g of fraction.

Antiradical activity against DPPH•

Antiradical activity of fractions against DPPH• was measured according to the method described by Brand-Williams *et al.* [1995]. The samples were dissolved in methanol in concentrations of 1 mg/mL (fractions I and II) or 0.1 mg/mL (fractions III-V). Next, a number of dilutions were obtained from the stock solutions for each fraction. The reaction was run by immediately vortexing 0.1 mL from each fraction dilution with 0.25 mL of 1 mmol/L methanolic solution of DPPH and 2 mL of methanol. After 20 min, absorbance was read at λ=517 nm (Beckman spectrophotometer DU-7500). The percentage of scavenged DPPH• *vs.* concentration of fractions (mg/assay) was plotted. The IC_{50} value, defined as the amount of antioxidant necessary to inactivate 50% of initial DPPH•, was estimated based on the plot.

FRAP assay

Ferric reducing antioxidant power (FRAP) of fractions was determined using the assay described by Benzie & Strain [1996]. Fractions were dissolved in the concentration of 1 mg/mL (I and II) or 0.25 mg/mL (III-V). FRAP reagent was prepared by mixing the following solutions: 0.3 mol/L acetate buffer with pH 3.6, 10 mmol/L 2,4,6-Tris(2-pyridyl)-*s*-triazine in 40 mmol/L HCl and 20 mmol/L $FeCl_3 \times 6H_2O$ in the ratio of 5:1:1 (v/v/v). This reagent (2.25 mL) was pipetted into test tubes that were heated to 37°C using a block heater (TH-24, Meditherm). Afterwards, 150 μL of a fraction solution and 150 μL of distilled water were added. After 30 min, absorbance was read at λ=593 nm (spectrophotometer Beckman DU-7500). The results were calculated using the standard curve for $FeSO_4 \times 7H_2O$ (r=0.999) and expressed in mmol of Fe(II) per g of fraction.

Statistical analysis

The results of antioxidant assays were expressed as mean values with the standard deviations for at least three replications for each sample. Statistical analysis was performed using GraphPad Prism software (version 6.04 for Windows, GraphPad Software, USA) by the one-way ANOVA. Tukey's multiple comparison test was carried out to compare mean values ($p<0.05$).

TABLE 1. Percent recovery and total phenolic content of buckwheat seed phenolic fractions.

Fraction number	Relative content (%)	Total phenolic content (mg (+)-catechin eq/g)*
I	34.40	19.8 ± 1.5^{e}
II	9.17	70.0 ± 1.4^{d}
III	11.46	151 ± 0.9^{b}
IV	3.56	164 ± 2.2^{a}
V	14.87	141 ± 1.7^{c}

*Data are expressed as mean values ± standard deviations (n=3). Means with different superscript letters (a–e) are significantly different at $p<0.05$.

RESULTS AND DISCUSSION

The chromatogram from the separation of a buckwheat seed extract on a column with Sephadex LH-20 gel was presented in Figure 1. The use of methanol as a mobile phase enabled achieving five fractions (I-V). The maximum optical density at 280 nm was noted when elution volume reached 144 mL (fraction I). In turn, the highest value at 320 nm was determined upon elution of 252 mL (fraction III). The relative content of the analysed fractions in the extract and total phenolics content in these fractions were summarised in Table 1. The greatest part of compounds present in the extract loaded onto the column was recovered in fraction I. It constituted 34.40% of the extract. However, the content of total phenolic compounds in fraction I was very low and reached only 19.8±1.5 mg (+)-catechin eq/g fraction. These results suggest that the more polar substances, such as sugars and organic acids, were eluted with methanol from Sephadex LH-20 gel at the beginning of separation. It is consistent with observations made in other studies where this gel was used for chromatographic separation of extracts from seeds of lentil and sunflower, as well as leaves and branches of *Ostrya japonica* [Amarowicz *et al.*, 2003a; Karamać *et al.*, 2012; Park *et al.*, 2010]. The remaining fractions represented from 3.56% (IV) to 14.87% (V) of the total extract weight (Table 1). The total content of phenolic compounds was similar in fractions III-V (141±1.7 – 164±2.2 mg (+)-catechin eq/g) and slightly lower in fraction II (70.0±1.4 mg (+)-catechin eq/g). The total phenolics content of fractions II-V was comparable with data reported for fractions from rapeseeds [Amarowicz *et al.*, 2003b] and substantially higher compared to that determined for leguminous seeds [Amarowicz *et al.,* 1996; 2003a]. In turn, fractions from sunflower seeds were characterised by a higher content of total phenolics [Karamać *et al*., 2012].

Acetone-water is known to enable the extraction of LMW phenolic compounds, like phenolic acids and flavonoids [Guo *et al.*, 2012], and HMW condensed tannins [Karamać 2007; 2010] from buckwheat seeds. After extract loading onto a column with Sephadex LH-20 gels, the application of methanol or ethanol as a mobile phase allows eluting only LMW com-

TABLE 2. Content of identified phenolic compounds in buckwheat seed fractions (mg/g of fraction).

Class of phenolics	Compounds	Fraction number				
		I	II	III	IV	V
Phenolic acids	5-*O*-Caffeoylquinic acid	2.55	–	1.26	tr*	–
	Caffeic acid	0.49	3.41	1.77	tr*	–
	p-Coumaric acid	0.23	–	–	–	–
	Gallic acid	–	–	–	3.16	–
Flavonols	Rutin	1.82	1.44	57.25	4.84	tr*
	Hyperoside	–	–	–	7.48	2.58
	Quercitrin	–	–	–	3.34	0.54
	Quercetin	–	–	–	0.76	2.78
Flavones	Homoorientin	–	–	0.45	2.96	0.29
	Orientin	–	–	1.14	9.22	0.62
	Vitexin	–	–	8.85	4.85	0.31
	Isovitexin	–	–	–	3.93	0.42
Flavan-3-ols	(+)-Catechin	–	–	–	1.42	–
	(–)-Epicatechin	–	–	1.70	5.60	–
	(–)-Epicatechin gallate	–	–	1.22	0.77	–
	Sum:	5.09	4.85	73.64	48.33	7.54

* trace

pounds. Tannins are adsorbed to Sephadex LH-20 in alcohol [Hagerman, 2002]. In our study, substance recovery from the extract using methanol reached 73.47%. The remaining part was, probably, constituted by HMW proanthocyanidins, that remained in the column.

The low-molecular weight phenolic compounds identified in buckwheat seeds using RP-HPLC-DAD included phenolic acids and flavonoids from the following sub-classes: flavonols, flavones and flavan-3-ols (Table 2). Phenolic acids were predominating compounds in fractions I and II. These identified in fraction I included: 5-*O*-caffeoylquinic (chlorogenic), caffeic and *p*-coumaric acids, with the highest content noted for chlorogenic acid – 2.55 mg/g. Fraction II contained caffeic acid. Both these acids were also detected in fraction III, however the major compound of this fraction was rutin, whose content reached 77.7% of compounds quantified in the fraction. Rutin occurred also in the other fractions but in 12–20--fold lower (I, II and IV) or trace amounts (V). In the pool of phenolics in fraction III, high percentage contribution was also determined for vitexin (11.4%). Fraction IV was characterised by the greatest diversity of phenolic compounds, with flavones found as predominating (21.0 mg/g). The identified compounds of this sub-class included: homoorientin (luteolin-6-*C*-glucoside), orientin (luteolin-8-*C*-glucoside), vitexin (apigenin-8-*C*-glucoside) and isovitexin (apigenin-6-*C*-glucoside). Flavonols were assayed in fraction IV in a slightly lower amount (16.4 mg/g). They included quercetin and its glycoside derivatives: hyperoside (quercetin-3-*O*-galactoside), quercitrin (quercetin-3-*O*-rhamnoside) and rutin (quercetin--3-*O*-rutinoside). Fraction IV contained also flavan-3-ols, mainly (–)-epicatechin – 5.60 mg/g. Among the identified compounds eluted from Sephadex LH-20 as fraction V there were flavonols and flavones. Hyperoside and quercetin were quantified in higher amounts, *i.e.* 2.58 and 2.78 mg/g, respectively. The profile of phenolic compounds identified in fractions I-V was consistent with findings of other authors who analysed seeds of common buckwheat in this respect [Verardo *et al.*, 2010; Inglett *et al.*, 2011; Kiprovski *et al.*, 2015]. By using the RP-HPLC-ESI-MS technique, Kiprovski *et al.* [2015] identified 7 compounds as flavonols, among which they precisely determined 5 structures (rutin, hyperoside, isoquercitrin, quercitrin and kaempferol-3-rutinoside). The same quercetin glycosides were identified by Verardo *et al.* [2010]. In turn, Inglett *et al.* [2011] determined 8 flavonols, out of which they identified: rutin, hyperoside, isoquercitrin and quercetin. Flavones determined in the present study (Table 2) were typical compounds of this group for buckwheat seeds [Watanabe *et al.*, 1997; Zielińska *et al.*, 2007; Verardo *et al.*, 2010]. Dietrych-Szostak & Oleszek [1999] reported that homoorientin, orientin, vitexin and isovitexin were presented in buckwheat hulls, whereas only isovitexin occurred in dehulled seeds. Flavan-3-ols and their derivatives, like glycosides and oligomers (procyanidins and propelargonidins) and galloyol esters, were the most numerous group of phenolic compounds in seeds of common buckwheat [Watanabe, 1998; Ölschläger *et al.*, 2008; Inglett *et al.*, 2011]. Verardo *et al.* [2010] noted on as many as 18 compounds belonging to this group, whereas Ölschläger *et al.*, [2008] determined 13 flavan-3-ols and their derivatives, including mainly di- and trimers. In our study, we identified only monomers of flavan-3-ols (Table 2), however it cannot be excluded that fraction V contained their oligomers. Apart from peaks of the identified compounds, the RP-HPLC chromatogram plotted for fraction V depicted also an additional wide, low peak with a long retention time (41–46 min) and absorbance maximum at *ca.* 280 nm (chromatogram is not shown). It may be speculated that it originated from poorly-separated di- and trimers of flavan-3-ols, that may be eluted with methanol from Sephadex LH-20 at the end of separation [Arfan *et al.,* 2009]. Not all compounds were identified in fraction II either. The analysis of their UV spectra rather excludes that they belong to flavonols and flavones, which allows hypothesizing that these were derivatives of phenolic acids or flavan-3-ols. Derivatives of phenolic acids identified in buckwheat seeds included glycosides of caffeic acid and isomers of chlorogenic acid [Verardo *et al.*, 2010; Kiprowski *et al.*, 2015].

The content of rutin in seeds of common buckwheat was determined at the level of 5.6–43 mg/100 g DW [Kreft *et al.*,

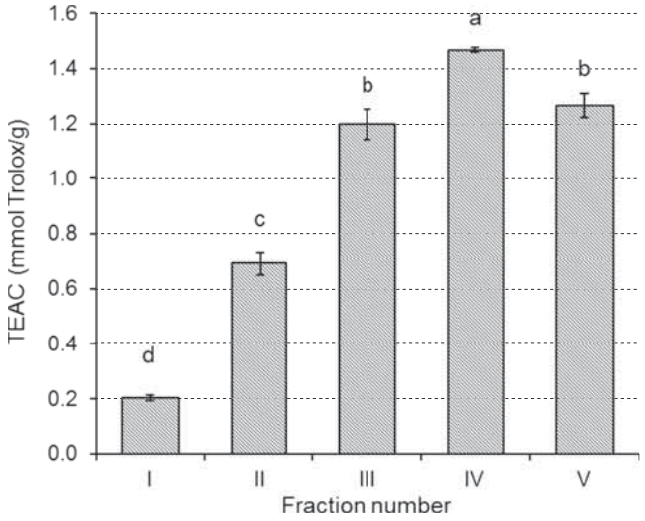

FIGURE 2. ABTS$^{\bullet+}$ scavenging activity of buckwheat seed phenolic fractions expressed as Trolox equivalent antioxidant capacity (TEAC). Data are mean values ± standard deviations (n=4). Values with different letters (a–d) are significantly different at $p<0.05$.

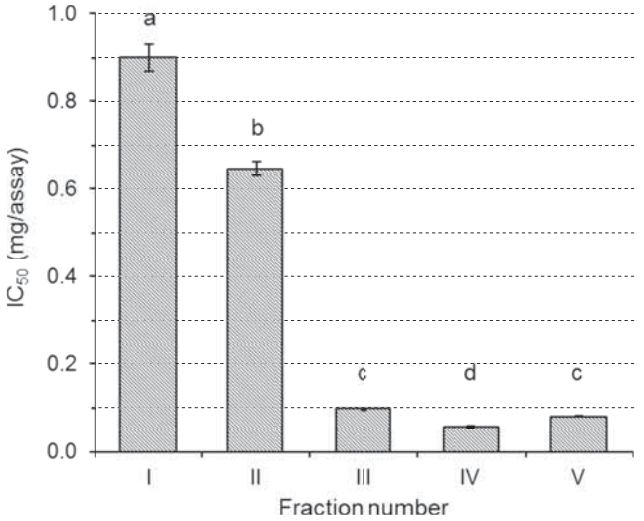

FIGURE 3. DPPH$^{\bullet}$ scavenging activity of buckwheat seed phenolic fractions expressed as IC_{50} values. Data are means ± standard deviations (n=3). Values with different letters (a–d) are significantly different at $p<0.05$.

2006; Ölschläger *et al.*, 2008; Zielińska *et al.*, 2012]. A wider range of rutin contents, *i.e.* from 3.29 to 151.45 mg/100 g DW seeds, was reported by Kiprovski *et al.* [2015] for twelve European *Fagopyrun esculentum* cultivars. In our study, rutin content in the seeds (calculated as the sum of its content in all fractions considering relative content of the fraction in the extract (Table 1) and extraction yield reaching 4.04%) accounted for 30.3 mg/100 g seeds. Contents of other major phenolic acids and flavonoids were decreasing in the following order: vitexin > chlorogenic acid > hyperoside > caffeic acid > orietin > quercetin > epicatechin (from 4.98 to 1.59 mg/100 g seeds). These values were in agreement with literature data [Zielińska *et al.*, 2007; Ölschläger *et al.*, 2008; Kiprovski *et al.*, 2015].

The antioxidant activity of the fractions separated from seeds of common buckwheat was assayed as the ability to scavenge two synthetic radicals ABTS$^{\bullet+}$ and DPPH$^{\bullet}$, and as the capability to reduce the Fe(III)/2,4,6-Tris(2-pyridyl)-*s*-triazine complex to the ferrous form. The TEAC values expressing the antiradical activity of buckwheat seed fractions against ABTS cation radicals were presented in Figure 2. The highest activity was demonstrated for fraction IV – 1.47±0.01 mmol Trolox/g. The TEAC values noted for fractions III and V were not statistically significantly different ($p>0.05$) and were lower by 14–18% compared to fraction IV. The two remaining fractions were the least active. The capability of buckwheat seed fractions for quenching DPPH$^{\bullet}$ was expressed as the IC_{50} value (Figure 3). Fraction concentration needed to reduce 50% of the DPPH radicals varied from 0.058±0.003 mg/assay (IV) to 0.90±0.03 mg/assay (I). The order of fractions indicating increasing antiradical activity against DPPH$^{\bullet}$ was consistent with that noted for ABTS$^{\bullet+}$ scavenging activity: I < II < III = V < IV. Likewise the antiradical activity, the highest ferric reducing antioxidant power was determined for fraction IV (2.18±0.05 mmol Fe(II)/g) and the lowest one for fraction I (0.18±0.01 mmol Fe(II)/g) (Figure 4). Differences in FRAP values between fractions III and V were small, but statistically significant ($p<0.05$).

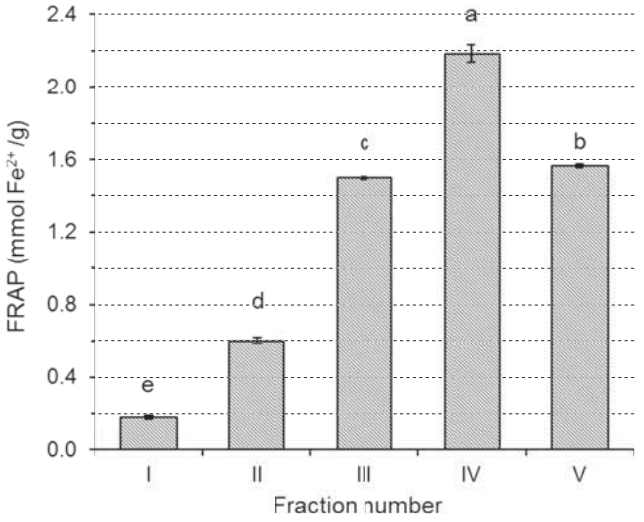

FIGURE 4. Ferric reducing antioxidant power (FRAP) of buckwheat seed phenolic fractions. Data are mean values ± standard deviations (n=4). Values with different letters (a–e) are significantly different at $p<0.05$.

The low antioxidant activity of fractions I and II (Figures 2–3) may be explained rather by a high contribution of non-phenolic compounds (Table 1) than by the low antioxidant activity of compounds present in the fractions. The identified -phenolic acids are characterised by relatively high TEAC values – from 1.26 mmol/L for *p*-coumaric acid to 3.01 mmol/L for gallic acid [Rice-Evans *et al.*, 1996]. The antioxidant activity of fraction III (Figures 2–4) was mainly determined by rutin as a predominating component of the fraction (Table 2). Nevertheless, the antioxidant activity of rutin was quite average compared to other flavonoids [Rice-Evans *et al.*, 1996]. In rutin's structure, the disaccharide residue attached to –OH group blocks the system: 2,3-double bond conjugated with 4-keto and 3-hydroxy groups in the C ring – one of the sites ensuring a high antioxidant activity of flavonoid. Rutin was reported to scavenge ABTS$^{\bullet+}$ and inhibit oxidation of methyl linoleate *ca.*

1.7–1.9 times poorer than its aglycone – quercetin [Rice-Evans *et al.*, 1996; Watanabe *et al.,* 1997]. Vitexin present in fraction III (Table 2) rather did not improve its antioxidative activity. Watanabe *et al.* [1997] demonstrated that vitexin isolated from buckwheat hulls showed no peroxyl radical scavenging activity. Its antiradical activity measured in more polar systems against $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ was, respectively, 125 and 440 times lower than that of quercetin [Zielińska & Zieliński, 2011]. The higher antioxidant activity of fraction IV compared to fraction III noted in our study (Figures 2–4) was the resultant of the contents of flavonoids (Table 2) and their individual activities, being usually higher compared to rutin activity. The peroxyl radical scavenging activity of flavonols isolated from buckwheat hulls was decreasing for: quercetin > hyperoside > rutin [Watanabe *et al.,* 1997]. In turn, inhibition of methyl linoleate oxidation by quercetrin was lower than by quercetin but stronger than by rutin [Hopia & Heinonen, 1999]. The antiradical activity of flavones – homoorientin and orientin, was similar or slightly lower than that determined for quercetin in, respectively, DPPH and ABTS tests. In contrast, homoorientin and orientin showed higher capability to scavenge superoxide anion radicals than their aglycone [Zielińska & Zieliński, 2011]. As mentioned above, vitexin was characterised by a very low antioxidant activity. A similar observation was made for isovitexin [Watanabe *et al.* 1997; Zielińska & Zieliński, 2011]. Acc. to a study by Watanabe [1998], (–)-epicatechin in the lipid system was a stronger antioxidant than rutin and only slightly weaker than quercetin. In turn, the TEAC values determined for flavan-3-ols identified in our study (Table 2) were increasing in the following order: (+)-catechin ≈ (–)-epicatechin < epicatechin gallate [Rice-Evans *et al.*, 1996]. In fraction V only quercetin and hyperoside were assayed in slightly higher quantities (Table 2). Their antioxidative activity was high [Watanabe *et al.,* 1997; Rice-Evans *et al.*, 1996], but not high enough to affect the high activity of fraction V (Figures 2–4). As discussed above, fraction V probably contained proanthocyanidins that are characterised by strong antioxidant properties – they are capable of scavenging free radical, display reducing properties and chelate prooxidative metal ions [Watanabe *et al*., 1997; Karamać, 2007, 2010].

CONCLUSIONS

The application of Sephadex LH-20 column chromatography with methanol as the mobile phase for the separation of compounds of common buckwheat seed extract allowed obtaining fractions that contained various classes/subclasses of phenolic compounds. First of all, we managed to separated most of rutin present in the extract (fraction III) from majority of other flavonoids (fraction IV) and phenolic acids (fractions I and II). Such a division of LMW phenolics, identification and quantitative analysis of components of each fraction and determination of the antioxidant activity of the fractions enabled concluding about the contribution of flavonoids in the antioxidant capacity of buckwheat seeds. Fraction III consisting in 77.7% from rutin (Table 2) showed by 18% (ABTS assay), 41% (DPPH assay) and 31% (FRAP assay) lower antioxidant activity than fraction IV containing other flavonoids (Figures 2–4). The content of total phenolics in both fractions was similar (Table 1). Hence, the activity of rutin of buckwheat seeds was lower than that of other flavonoids. However, considering the relative content of fraction III in the extract, that was threefold higher compared to fraction IV (Table 1), it may be concluded that rutin had a greater contribution into the antioxidant potential of common buckwheat seeds than the other flavonoids present in fraction IV. Nevertheless, worthy of notice is that the effect of flavonoids other than rutin on the antioxidant capacity of the seeds was significant and shall not be ignored. Unfortunately, in this study, we did not manage to estimate the contribution of phenolic acids in the antioxidant potential of buckwheat seeds owing to a high content of non-phenolic contaminants in fractions I and II (Table 1) and to incomplete identification of phenolic compounds in fraction II.

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