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PHENOLIC PROFILE AND ANTIOXIDANT PROPERTIES OF DRIED BUCKWHEAT LEAF AND FLOWER EXTRACTS

Article Highlights

- Buckwheat leaf and flower extracts were obtained by different extraction procedures
- Rutin and chlorogenic acid were identified as most abundant phenolic compounds
- Some of the extracts were as efficient as BHT in β -carotene bleaching test
- The extracts demonstrated strong ability to inhibit the destruction of erythrocytes
- The extracts prolonged the beginning of the oxidation process in sunflower oil

Abstract

Due to a high content of rutin (2-10%), dried buckwheat leaf and flower (DBLF) formulations were shown to be efficient in the treatment of vascular diseases. In order to find a cost effective way for the extraction of antioxidants, the effects of ethanol/water ratio and temperature on the extraction efficiency of phenolic compounds and the mechanisms of antioxidant action of the extracts were tested. Extraction with ethanol/water mixture (80:20, v/v) for 24 h at room temperature, after the mixture was just brought to boil was demonstrated to be an efficient and cheap way for obtaining a high yield of rutin (49.94 ± 0.623 mg/g DBLF). The most abundant phenolic compounds in DBLF extracts were rutin and chlorogenic acid. Flavonoids, especially rutin, were shown to be the most responsible for the antioxidant activity in all investigated lipid model systems, acting as free radical scavengers, electron-donating substances and chelators of iron ions. In β -carotene bleaching tests, the extracts with the highest activity were as efficient as BHT (butylated hydroxytoluole). Regarding the results of antihemolytic and Schaal oven tests, the extracts demonstrated remarkable ability to inhibit the oxidative destruction of erythrocytes and to prolong the beginning of the oxidation process in sunflower oil.

Keywords: dried buckwheat leaf and flower, rutin, antioxidant activity, lipid oxidation, extraction.

Common buckwheat (*Fagopyrum esculentum* Moench) is a highly nutritious pseudocereal known as a very rich source of antioxidants, especially rutin [1]. The nutritive profile and antioxidant potential of buckwheat seeds has been extensively investigated [2-4], and buckwheat seeds can now be regarded a “functional food” [5]. However, recent studies indicate that the highest concentration of rutin, up to 10%, is accu-

mulated in leaves and blossoms of the buckwheat plant [1,6].

Plant phenolic compounds are well known as highly effective free radical scavengers and antioxidants [7]. The role of an antioxidant in a food product is related to its ability to inhibit or stop rancidity and/or deterioration of the nutritional quality [8]. Also, many natural antioxidants are supposed to have protective effects against chronic diseases, mainly by scavenging oxygen radicals, which can deteriorate biological membranes.

Fagopyri herba is a herbal drug derived from dried areal tissues of common buckwheat (*Fagopyrum esculentum* Moench) and has been used in the treatment of vascular diseases [9]. Rutin (quercetin-3-

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-*O*-rutinoside), the dominant flavonol glycoside in DBLF, has been reported to possess antioxidant activity, to antagonize the increase of capillary fragility associated with haemorrhagic disease, to reduce high blood pressure [10], to decrease the permeability of the blood vessels, to have an anti-oedema effect, and to reduce the risk of atherosclerosis [11].

Since DBLF can be considered as an ingredient for designing functional food products, the objective of this research was to find a cost effective way for the extraction of phenolic compounds from DBLF in order to obtain the highest antioxidant activity of the extracts. Water and ethanol/water mixtures were chosen as nontoxic and environmentally friendly solvents, which have been shown to be effective in the extraction of quercetin glycosides [12]. Besides the phenol profiling, the aim of this work was to test the mechanisms of antioxidant action of the obtained extracts in chosen model systems to be able to predict their possible use.

EXPERIMENTAL

Materials

DBLF (dried areal parts of *Fagopyrum esculentum* Moench collected during the flowering season) was obtained from the Institute for Medicinal Plants Research "Dr Josif Pančić" (Belgrade, Serbia) where a herbarium voucher specimen (No. 31210911) was deposited. For replicates, three packages of herbal drug were provided, each containing 500 g of material.

Butylated hydroxytoluole (BHT), 1,1-diphenyl-2-picrylhydrazyl (DPPH), ethylene diamine tetraacetic acid disodium salt dihydrate (EDTA), 3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine (ferrozine), ferrous sulfate heptahydrate, linoleic acid (99%), potassium ferricyanide, sodium carbonate, Tween 40, trichloroacetic acid (TCA), Folin-Ciocalteu's reagent, standard substances including gallic acid, protocatechuic acid, caffeic acid, vanillic acid, chlorogenic acid, syringic acid, ferulic acid, rutin, myricetin, rosmarinic acid, *trans* cinnamic acid, naringenin, luteolin, kaempferol, and apigenin were obtained from Sigma (Sigma-Aldrich GmbH, Sternheim, Germany). Quercetin was a product of J. T. Baker (Deventer, the Netherlands), while high-performance liquid chromatography (HPLC) grade methanol, formic acid (HPLC grade) and ethanol 96% were purchased from Merck (Darmstadt, Germany). Water was purified using Millipore Elix 10 UV water purification system (Molsheim, France), and ultrapure water used for

HPLC mobile phase preparation was obtained using Simplicity UV, Millipore (Molsheim, France).

Preparation of extracts

DBLF (2 g) was mixed either with 50 mL of water or ethanol/water mixtures (50:50 and 80:20, *V/V*). Maceration was performed for 24 h at room temperature followed by extraction in an ultrasonic bath (10 min at room temperature). Corresponding extracts brought to boil before the maceration were also prepared. The extracts were filtered through the filter paper (Whatman, Grade 4 Chr, UK) and stored at -4 °C (up to two days) until further use. Preparation of the extracts for HPLC-MS/MS analyses included additional evaporation to dryness and redissolving in an appropriate mobile phase.

Total flavonoid content

Colorimetric aluminum chloride method was used for determination of total flavonoid content [13], which is based on the formation of a complex flavonoid-aluminium. The probes were prepared by mixing 5 mL of extract, 1 mL of distilled water, and 2.5 mL of AlCl₃ solution (26.6 mg AlCl₃·6H₂O and 80 mg CH₃COONa dissolved in 20 mL distilled water). A blank probe was prepared by replacing AlCl₃ solution with distilled water. The absorbance of probes and blank probe were measured immediately at 430 nm. Rutin was used as a standard and results were expressed as rutin equivalents (RE, g RE per 100 g of sample). Absorption readings at 415 nm were taken against a blank sample (reaction mixture without AlCl₃). Rutin (2.5 to 50 µg/mL) was used for the calibration curve construction.

Identification of phenolic compounds by LC-MS/MS

Rapid resolution liquid chromatography with mass selective detection was performed using an Agilent Technologies 1200 Series liquid chromatograph coupled with Agilent Technologies 6410 triple quadrupole (QQQ) mass spectrometer. An Eclipse XDB-C18, 1.8 µm, 4.6 mm×50 mm column (Agilent) was used for separation of 18 phenolic compounds. The solvent gradient program was created by varying the proportion of solvent A (0.1 vol.% formic acid in water) and solvent B (methanol). The following gradient mode was used for phenolic acids identification: initial 10% B; 0-2 min, 10-43% B; 2-7 min, 43% B; 7-9.5 min, 43-100% B; 9.5-10.5 min, 100% B with flow rate of 1 mL/min. Identification of flavonoids was performed using the following gradient mode: initial 4% B; 0-1.5 min, 4-4.5% B; 1.5-4 min, 4.5-10% B; 4-11 min, 10% B; 11-18 min, 10-100% B, with flow rate of 1.2 mL/min. Injection volume was 1 µL. The eluted

components were ionized in negative electrospray ionization (ESI) mode, using nitrogen as nebulizer (pressure of 50 psi) and drying gas (temperature of 350 °C, flow 8 L/min). Extracts used for LC-MS/MS quantification were dissolved in starting mobile phase solvent to the concentration of 0.2 mg/mL. All used standards were dissolved in methanol to prepare stock solutions of 1 mg/mL and the mix of stock solutions was prepared, with concentration of each compound being 100 µg/mL. Analyses were performed in MRM (multiple reaction monitoring) mode. Compound-specific, optimized MS/MS parameters are given in Table 1.

Quantification of phenolic compounds by HPLC-DAD

A liquid chromatograph (Agilent 1200 series), equipped with a DAD detector and an Eclipse XDB-C18, 1.8 µm, 4.6 mm×50 mm column (Agilent) was used for quantification of identified phenolic compounds in DBLF extracts. A single rapid resolution HPLC method suitable for the determination of 17 phenolic compounds, developed and validated as previously reported by Mišan *et al.* [14], was used. In brief, solvent gradient was performed by varying the proportion of solvent A (methanol) to solvent B (1 vol.% formic acid in water) as follows: initial 10% A; 0-10 min, 10-25 % A; 10-20 min, 25-60 % A; 20-30 min, 60-70 % A at a flow-rate of 1 mL/min. The total running time and post-running time were 45 and 10 min, respectively. The column temperature was 30

°C. The injected volume of samples and standards was 5 µL and it was done automatically using an autosampler. The spectra were acquired in the range of 210-400 nm and chromatograms plotted at 280, 330 and 350 nm with a bandwidth of 4 nm, and with reference wavelength/bandwidth of 500/100 nm.

DPPH radical scavenging activity

A modified method of Hatano *et al.* [15] was used to determine effect of different extracts on scavenging of 1,1-diphenyl-2-picrylhydrazyl (DPPH*) radicals. 0.1 mL of examined extract previously diluted to obtain at least four different concentrations (0.1 to 5 mg plant material/mL), 1.0 mL of DPPH* (90 µmol/L) and 2.9 mL of methanol were shaken vigorously and left to stand in the dark for 60 min. The absorbance was measured at 515 nm (Cintra 101, GBC scientific, UV/Vis) against the control (above mentioned mixture without extract). Results were expressed as the concentration (mg plant material/mL) of the extract leading to 50% reduction of the initial DPPH* concentration (IC_{50} value). BHT in the concentration range 0.006-0.600 mg/mL was used as a control.

Chelating activity on Fe²⁺

Chelating activity of the examined extracts on Fe²⁺ was measured according to the method of Decker and Welch [16]. Plant extracts were dissolved in ethanol, in an appropriate manner to obtain a series of dilutions (10 to 40 mg plant material/mL).

Table 1. Retention time, MRM transition, collision energy (CE), and fragmentor voltage of 18 phenolic compounds

Phenolic acid	Time, min	MRM (<i>m/z</i>)	CE / eV	Fragmentor voltage, V
Quinic acid	0.612	191>127; 109	12;12	99
Gallic acid	1.399	169>125	8	103
Protocatechuic acid	1.787	153>109	8	67
p-Hydroxybenzoic acid	4.787	137>93	8	82
Chlorogenic acid	6.612	353>191	20	113
Vanillic acid	7.135	167>123; 152	4;8	93
Caffeic acid	7.615	179>107; 135	16;8	73
Syringic acid	9.873	197>106; 121	16;8	73
Ferulic acid	14.254	193>134; 149; 178	4;4;4	119
Sinapic acid	14.569	223>208; 164	4;0	118
Rosmarinic acid	14.569	359>161;179	40;56	170
Flavonoids				
Catechin	1.070	289>245; 203; 137	8;12;16	134
Epicatechin	1.830	289>109	20	134
Rutin	3.192	609>179; 300	40;36	118
Myricetin	3.528	317>109; 137; 151	32;28;20	144
Quercetin	4.578	301>121; 151; 179	28;16;12	135
Naringenin	4.761	271>151; 119	8;20	93
Kaempferol	6.246	285>151; 93; 117	16;36;40	155

The IC_{50} value (mg/mL) was defined as the concentration of extract that chelates 50% of Fe^{2+} under the experimental conditions. It was obtained by interpolation from linear regression analysis. EDTA in the concentration range 0.006–0.600 mg/mL was used as a control.

Reducing power

The reducing power of the examined extracts, in series of different concentrations (0.100 to 15 mg plant material/mL), was determined according to the method of Oyaizu [17]. This method is based on the reduction of Fe^{3+} to Fe^{2+} and measuring absorbance (at 700 nm) of the Perl's Prussian blue complex. The IC_{50} value (mg/mL) was defined as an effective concentration of extract at which the absorbance of reaction mixture reaches 0.5 for reducing power. It was obtained by interpolation from linear regression analysis. BHT in the concentration range 0.006–0.600 mg/mL was used as a control.

β -Carotene bleaching method

Antioxidant activity (AA) of the series of examined extracts (1 to 40 mg plant material/mL) was determined using β -carotene bleaching method, an *in vitro* assay which measures oxidative loss of β -carotene in β -carotene/linoleic acid emulsion. Thermal autoxidation at 50 °C was performed for 2 h. The degradation rate of β -carotene was calculated according to first-order kinetics and the AA was expressed as percent of inhibition relative to the control [18]. The IC_{50} value (mg/mL) was defined as the concentration at which AA was 50% under the experimental conditions, and it was obtained by interpolation from linear regression analysis. BHT in the concentration range 0.2–2 mg/mL was used as a control.

Antihemolytic activity

Antihemolytic activity of the extracts was determined using the method of Ko *et al.* [19], which was further optimized for DBLF extracts as previously described by Šarić *et al.* [20]. A solution of hydrogen peroxide (0.0625 vol.%) in phosphate-buffered saline (PBS) solution was used instead of concentrated solution, and incubation time was set to 2 h instead of 4 h from the original method. The extent of the hemolysis in every sample was calculated as: $100(A_{\text{sample}}/A_{\text{total hemolysis}}) = \% \text{ of hemolysis}$. Ascorbic acid solution in the concentration range 0.005–0.500 mg/mL was used as a control. Since the percentage of hemolysis was calculated for different sample concentrations (1 to 40 mg plant material/mL), these values were plotted against sample concentrations and, using linear regression, IC_{50} values (concentration of the inves-

tigated extract at which 50% of hemolysis inhibition is achieved) of every investigated extract, as well as the ascorbic acid solution, were calculated.

Schaal oven test

The Schaal oven test at 70 °C was conducted to evaluate the antioxidant effectiveness of ethanolic extracts of DBLF in retarding the rancidity of commercially available refined sunflower oil during 12 days of storage. For that purpose, 5 g of oil was mixed with 20 mass% tested extracts and spread in 1 cm layer in glass containers. The test was carried out in the dark and oxidative changes were monitored gravimetrically. Experiments were also carried out with synthetic antioxidant, BHT at 10 ppm level and the control sample with no added antioxidants. The extracts were evaporated to dryness and redissolved in methanol. A control sample was prepared by using the same amount of methanol used to dissolve the antioxidant (BHT) and the extracts. In order to monitor the kinetics of oxidation process [21], first derivatives of samples weight gain were calculated using the software SciDAVis 0.2.4. [22].

Statistical analysis

Apart from the extraction procedures and Schaal oven test, which were done in duplicate, all analyses were performed in triplicate, and the mean values with the standard deviations are reported. Analysis of variance and Duncan's multiple range tests were used. Statistical data analysis software Statistica (StatSoft, Inc. 2011) [23] was used for analysis. P values < 0.05 were regarded as significant.

RESULTS AND DISCUSSION

Referring to Kim *et al.* [24] who tested different solvents in order to find the most effective way for the extraction of rutin from buckwheat, the use of aqueous ethanol and acetone (both 50 vol.%) as extraction solvents produced the highest yields of rutin. Kim *et al.* [24] also found that the extraction temperatures in the range 60–80 °C and the extraction time from 0.5 to 1 h were optimal for achieving a high recovery yield of rutin. On the other hand, Hinneburg and Neubert [12] suggested that an extract with good antioxidant activity, a high content of phenolics, and a low content of the phototoxic fagopyrin can be yielded by agitated maceration with 30% ethanol at 60 °C for 2 h.

Due to their low toxicity and supposed efficiency, water and ethanol/water mixtures were chosen for the extraction of phenolic compounds from DBLF in our experiment. Instead of prolonged heating which is energy consuming and may result in destruction of

thermally labile constituents of interest, the effect of short term heating, just to bring the mixture to boil and 24 h maceration afterwards on the extraction efficiency was examined. The following extraction yields were obtained: water extraction = 20.34%; boiling water extraction = 19.72%; ethanol/water (50:50) extraction = 21.17%; boiling ethanol/water (50:50) extraction = 21.28%; ethanol/water (80:20) = 21.28% ; boiling ethanol/water (80:20) extraction = 19.70%.

Total flavonoid content of plant extracts and HPLC identification and quantification of phenolic compounds

Besides the total flavonoid content determination, chemical characterization of the extracts included identification of phenolic compounds by LC-MS/MS and quantification of identified compounds by HPLC/DAD. Eighteen secondary biomolecules were included into the identification (LC-MS/MS) method, based on the availability of reference standards in the laboratory. Method development started with the selection of precursor ions, which was done in MS2Scan mode. The ionisation predominantly resulted in the formation of $[M-H]^-$. To assure high yield of precursor ions while simultaneously preventing in-source fragmentation, fragmentor voltage (V) was optimised for each compound. For this purpose, a standard mixture was analyzed in MS2SIM mode, using fragmentor voltages from 60 to 200 V in 10 V increments. In order to optimise collision energy, the standard mixture was subsequently analysed in Product Ion Scan mode, using $[M-H]^-$ as precursors, optimal fragmentor voltage, and collision cell voltages ranging from 0-50 V (in 10 V increments). Identification of the phenolic constituents of the extracts was based on the comparison of the retention times and ionization patterns in MRM mode with those of the external standards (Table 1). Out of 18 analyzed compounds, the presence of 7 was confirmed in the extracts. After the identification, phenolic compounds were quantified by HPLC/DAD method, which was previously optimized

and validated for the determination of phenolic compounds in crude medicinal plant extracts [14]. Content of phenolic compounds in each extract is presented in Table 2. Referring to the obtained results, the most abundant phenolic compound in DBLF extracts was rutin with the exception of Extract A where chlorogenic acid was present in the highest concentration (Table 2). The highest yield of rutin was obtained by using boiling ethanol/water (80:20, *V/V*) as extracting solvent, while the highest yields of chlorogenic acid were obtained by water extraction. Ethanol/water (50:50) mixture was shown to be the most efficient for the extraction of investigated phenolic acids. According to Hinneburg and Neubert [12], quercetin originates as a product of rutin degradation by flavonol-3-*O*- β -heterodisaccharidase in buckwheat herb or in an extract. Referring to the obtained results, quercetin was present in all extracts at a concentration, which was not highly influenced by the difference in applied extraction procedures.

Our results are in accordance with the findings of Hinneburg and Neubert [12], who reported that the main phenolics of DBLF are rutin, chlorogenic acid, and hyperoside and that rutin content in buckwheat herb can be up to 8%. Besides rutin and quercetin, the other flavonoids like vitexin, isovitexin, orientin and isoorientin, which could exhibit 4-40% of total antioxidant activity, were also reported to be present in green parts of buckwheat [25]. However, due to the lack of external standards, those compounds could not have been quantified by applied HPLC method. Instead, total flavonoid content of each extract was estimated (Table 3). Due to the difference in applied methods, these results differ from the results obtained by HPLC. However, strong positive correlation between HPLC-determined rutin and total flavonoid contents was observed ($r = 0.931$, $P < 0.05$), indicating that rutin is by far the most abundant flavonoid in the extracts.

Table 2. Content of phenolic compounds (mg/g DBLF) in extracts: A-water; B-boiling water; C-ethanol/water (50:50); D-boiling ethanol/water (50:50); E-ethanol/water (80:20); F-boiling ethanol/water (80:20); values are means of three determinations \pm standard deviation. Values ^{a,b,c,d} in each row with the same superscript are not significantly different ($P < 0.05$)

Compound	Extract A	Extract B	Extract C	Extract D	Extract E	Extract F
Gallic acid	0.052 \pm 0.009 ^a	0.037 \pm 0.004 ^a	0.874 \pm 0.065 ^c	0.864 \pm 0.03 ^c	0.779 \pm 0.021 ^b	0.766 \pm 0.071 ^b
Protocatechuic acid	0.172 \pm 0.011 ^a	0.175 \pm 0.016 ^a	0.266 \pm 0.021 ^c	0.236 \pm 0.019 ^b	0.209 \pm 0.014 ^b	0.228 \pm 0.048 ^b
Caffeic acid	0.214 \pm 0.013 ^a	0.240 \pm 0.027 ^a	1.084 \pm 0.101 ^c	0.953 \pm 0.091 ^b	0.988 \pm 0.077 ^b	0.966 \pm 0.068 ^b
Catehin	0.093 \pm 0.007 ^b	0.062 \pm 0.007 ^a	0.088 \pm 0.005 ^b	0.087 \pm 0.005 ^b	0.090 \pm 0.003 ^b	0.092 \pm 0.008 ^b
Chlorogenic acid	1.652 \pm 0.075 ^d	1.621 \pm 0.111 ^d	0.855 \pm 0.042 ^b	0.950 \pm 0.036 ^c	0.772 \pm 0.052 ^a	0.743 \pm 0.026 ^a
Rutin	0.251 \pm 0.013 ^a	2.274 \pm 0.125 ^b	40.35 \pm 0.693 ^d	39.87 \pm 0.265 ^d	34.93 \pm 0.213 ^c	49.94 \pm 0.623 ^e
Quercetin	0.252 \pm 0.031 ^a	0.254 \pm 0.026 ^a	0.310 \pm 0.031 ^{bc}	0.306 \pm 0.034 ^{bc}	0.344 \pm 0.029 ^{bc}	0.260 \pm 0.022 ^{ab}

Antioxidant activity (AOA) of the extracts

Due to the complexity of oxidative processes in food matrices and cells and the versatile nature of plant extracts a single method for the screening of antioxidant potential is not recommended. Therefore, the antioxidant effects of plant products must be evaluated by combining at least two or more different *in vitro* assays to obtain relevant data. With respect to this, the antioxidant properties of the examined extracts were tested through their ability to donate electrons or H atoms, chelate/deactivate transition metal ions and to inhibit the process of lipid oxidation.

Free radical scavenging activity of the extracts was tested by applying the method towards long-lived DPPH^{*} while the electron-donating capacity was evaluated by measuring their reducing power. Referring to the results (Table 3) expressed as IC_{50} values, the extracts showed lower DPPH^{*} scavenging activity and reducing power than the reference compound BHT (Table 4). In comparison with other investigated samples, water extracts had significantly lower reducing power and DPPH^{*} scavenging activity than the ethanol/water extracts, which were not significantly different in both of the tests. Thermal treatment had a

significant positive influence only on water extraction. Also, the correlation between reducing activity and scavenging activity on DPPH^{*} (Table 4) was highly positive. Furthermore, reducing activity significantly correlated with rutin and total flavonoid contents (Table 4). Similarly, strong positive correlation was observed between scavenging activity on DPPH^{*} and rutin and total flavonoid contents (Table 4).

As the process of lipid oxidation occurs both in the high-fat food products causing rancidity and in living organisms resulting in cell damage, three different lipid model systems were used to measure the antioxidant activity of the extracts: β -carotene bleaching method, antihemolytic test and Schaal test.

β -Carotene bleaching test measures the loss of the yellow colour of β -carotene due to its reaction with radicals that are formed by linoleic acid oxidation in an emulsion. Antioxidant activity of an extract in this case refers to its ability to protect β -carotene from oxidative damage. The test results (Table 3) indicated the following order in antioxidant activities: boiling ethanol/water (50:50) = ethanol/water (50:50) = boiling ethanol/water (80:20) > ethanol/water (80:20) > water extracts. The extracts with the highest activity

Table 3. Antioxidant activity and total flavonoid content of dried buckwheat leaf and flower (DBLF) extracts: A-water; B-boiling water; C-ethanol/water (50:50); D-boiling ethanol/water (50:50); E-ethanol/water (80:20); F-boiling ethanol/water (80:20) and the control substances; values are means of three determinations \pm standard deviation. Values in each column with the same superscript ^{a,b,c,d} are not significantly different ($P < 0.05$)

Extract/control substance	IC_{50} in mg plant material/mL or mg control substance/mL					Total flavonoid content g/100g plant material
	DPPH [*] scavenging activity	Chelating activity on Fe ²⁺	Reducing power	β -Carotene bleaching method	Antihemolytic activity	
A	3.79 \pm 0.177 ^c	272.8 \pm 31.1 ^d	15.10 \pm 2.43 ^d	5.04 \pm 0.083 ^c	42.1 \pm 7.27 ^d	0.22 \pm 0.026 ^a
B	2.78 \pm 0.044 ^b	64.8 \pm 0.961 ^c	12.46 \pm 1.89 ^c	4.76 \pm 0.400 ^c	29.4 \pm 9.04 ^c	0.72 \pm 0.035 ^b
C	0.800 \pm 0.074 ^a	34.4 \pm 0.559 ^b	3.19 \pm 0.135 ^b	1.88 \pm 0.142 ^a	3.43 \pm 0.207 ^b	2.57 \pm 0.031 ^d
D	0.679 \pm 0.018 ^a	25.7 \pm 1.01 ^b	2.81 \pm 0.093 ^b	1.87 \pm 0.054 ^a	3.12 \pm 0.370 ^b	4.16 \pm 0.032 ^e
E	0.816 \pm 0.025 ^a	39.8 \pm 0.240 ^b	3.40 \pm 0.342 ^b	3.82 \pm 0.197 ^b	5.53 \pm 0.339 ^b	2.42 \pm 0.021 ^c
F	0.672 \pm 0.009 ^a	30.6 \pm 0.580 ^b	2.66 \pm 0.040 ^b	1.78 \pm 0.003 ^a	2.30 \pm 0.377 ^b	4.92 \pm 0.026 ^f
BHT	0.560 \pm 0.001 ^a	-	0.360 \pm 0.010 ^a	1.78 \pm 0.060 ^a	-	-
Ascorbic acid	-	-	-	-	0.18 \pm 0.010 ^a	-
EDTA	-	0.039 \pm 0.001 ^a	-	-	-	-

Table 4. Correlation coefficients between IC_{50} values of antioxidant activity and phenolic compounds (total flavonoid and rutin) content ($P < 0.05$)

Parameter	Chelating activity on Fe ²⁺	Antihemolytic activity	β -Carotene bleaching method	Reducing power	DPPH [*] scavenging activity
Rutin content	Not significant	- 0.963	- 0.924	-0.9587	-0.974
Total flavonoid content	Not significant	- 0.874	- 0.905	-0.871	-0.879
DPPH [*] scavenging activity	0.871	0.999	0.867	0.996	-
Reducing power	0.823	0.995	0.880	-	-
β -carotene bleaching method	Not significant	0.881	-	-	-
Antihemolytic activity	0.871	-	-	-	-

were as efficient as BHT. Significant correlation was found between these results and rutin content and total flavonoid content (Table 4). Also, these results significantly correlated with scavenging activity on DPPH^{*} and the results of reducing power (Table 4).

Antihemolytic activity assay shows the ability of the tested extracts to inhibit hydrogen-peroxide induced oxidation of the lipids in the phospholipid bilayer of erythrocyte membranes and has the advantage of using biological system instead of simpler membrane models like phosphatidylcholine liposomes [26]. Although much higher than the IC_{50} of ascorbic acid, calculated IC_{50} values of the investigated extracts (Table 3) significantly correlated with the total flavonoid content and even better with the rutin content (Table 4) of the samples, which indicates that flavonoids play a crucial role in preventing oxidative damage to erythrocyte cell membranes *in vitro*. Also, the highly significant correlation of antihemolytic activity with scavenging activity on DPPH^{*} and reducing power (Table 4) of the extracts implies the major mechanisms of antioxidant protection under the given experimental conditions.

In the third model system, which could be relevant to lipid oxidation processes in high-fat food products, sunflower oil was used as a lipid substrate. The courses of oxidation were followed gravimetrically and demonstrated by using derivative plots (Figure 1). The inflection points obtained in this manner corresponded to the maximum change in weight gain over time and are convenient for the induction period determination. As it can be seen from the inflection points in Figure 1, the oxidation process of the sunflower oil (control) began on the fifth day. With the addition of DBLF ethanolic extracts, the induction period in vegetable oil was prolonged. After BHT,

which showed the highest efficiency in delaying oxidative changes (induction period of 8 days), boiling ethanol/water (80:20) extract showed the highest activity prolonging the induction period of sunflower oil for 7.5 days. The ethanol/water (80:20) extract was capable of prolonging the beginning of the oxidation process until 7th day. However, boiling ethanol/water (50:50) and ethanol/water (50:50) extracts were able to postpone the oxidation process only for 5 and 5.5 days, respectively. Water extracts were not tested as they had been previously demonstrated to be less efficient by other tests (Table 3).

The ability of an antioxidant to chelate/deactivate transition metal ions, which can catalyze hydroperoxide decomposition and Fenton-type reactions, is considered an important mechanism of AOA, and therefore the chelating activity test was used to evaluate the chelating efficiency of investigated extracts. Obtained IC_{50} values indicate that investigated extracts possess significant chelating activities and may be able to play a protective role against oxidative damage by sequestering Fe²⁺, but they showed much lower chelating activity than the reference compound EDTA. The water extracts were shown to be less efficient than the ethanol/water extracts, which were not statistically different (Table 3). The correlation between Fe²⁺ chelating activity rutin and total flavonoid content was positive, but not statistically significant, which indicates that the other compounds present in the extracts, along with flavonoids contribute to the chelating activity. Chlorogenic acid was reported to be a powerful chelator of iron ions, capable of forming iron/chlorogenic acid complex, which was not capable of generating the hydroxyl radicals in Fenton model system [27]. However, the correlation between chlorogenic acid content and chelating activity of the

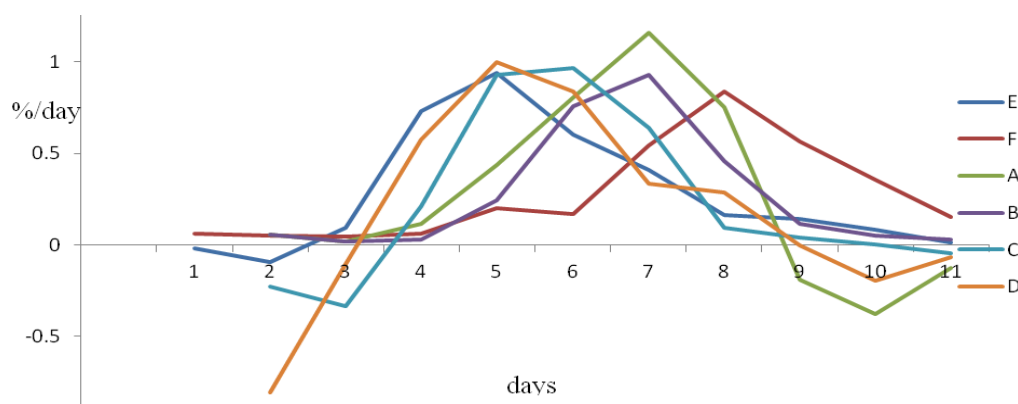


Figure 1. Antioxidant effectiveness of dried buckwheat leaf and flower (DBLF) ethanolic extracts tested by Schaal oven test. Changes in the mass per day (%/day) of sunflower oil samples supplemented with: A-boiling ethanol/water (80:20) DBLF extract; B-ethanol/water (80:20) DBLF extract; C-boiling ethanol/water (50:50) DBLF extract; D-ethanol/water (50:50) DBLF extract; E-control; F-0.01% BHT during accelerated oxidation.

extracts was not established. On the contrary, the results of chelating activity significantly correlated with those of scavenging activity on DPPH[•] and reducing power (Table 4). Unlike the poor correlation with β -carotene bleaching test, the results of anti-hemolytic activity assay significantly correlated with those of chelating activity on Fe²⁺ (Table 4). Contrary to the β -carotene bleaching test, the antihemolytic activity assay represents an iron ion-dependent lipid peroxidation system, where a potential of an antioxidant to act as a chelator becomes important [28].

CONCLUSION

DBLF was shown to be a rich source of antioxidants. Extraction with ethanol/water mixture (80:20 *V/V*) for 24 h at room temperature, after the mixture was just brought to boil was demonstrated to be an efficient and cheap way for the extraction of antioxidants.

Flavonoids, primarily rutin, were shown to be the most responsible for the antioxidant activity in all investigated lipid model systems, acting as free radical scavengers, electron-donating substances and iron ion chelators. In the β -carotene bleaching test, the extracts with the highest activity were as efficient as BHT. Furthermore, the extracts were able to protect erythrocyte cell membranes from oxidative damage and to prolong the induction period of sunflower oil for 3.5 days under the accelerated oxidation conditions.

Finally, the DBLF extracts obtained by this relatively cheap and environmentally friendly extraction procedure show a very good potential for incorporation in certain fat-containing food products as a rich source of natural antioxidants, which, beside their already proven health benefits, would provide a substantial protection against oxidative-induced rancidity. However, in order to fully optimize the extraction method, further research, which would include testing of ratio of liquid to raw material, extraction time, the ultrasonic power and radiation time on the extraction efficiency needs to be done.

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NAUČNI RAD

FENOLNI PROFIL I ANTIOKSIDANTNA SVOJSTVA EKSTRAKATA OSUŠENIH LISTOVA I CVETOVA HELJDE

Formulacije na bazi osušenih listova i cvetova heljde (DBLF) su dokazano efikasne u tretiranju bolesti krvnih sudova, a što se dovodi u vezu sa visokim sadržajem rutina (2-10%). U cilju pronalazaženja ekonomičnog načina ekstrakcije antioksidanata testirani su uticaj odnosa etanola i vode, kao i temperature na efikasnost ekstrakcije fenolnih jedinjenja i mehanizme antioksidantnog delovanja ekstrakata. Ekstrakcija smešom etanol/voda (zapreminski odnos 80:20) tokom 24 h na sobnoj temperaturi, nakon što je smeša zagrejana do ključanja, pokazala se kao efikasan i ekonomičan način za dobijanje visokog sadržaja rutina ($49,94 \pm 0,623$ mg/g DBLF). Najzastupljenija fenolna jedinjenja u DBLF ekstraktima bila su rutin i hlorogenska kiselina. Pokazano je da su flavonoidi, a posebno rutin, najodgovorniji za antioksidantnu aktivnost ekstrakata u lipidnim model sistemima, delujući kao "hvatači" slobodnih radikala, elektron-donorske supstance i helatori jona gvožđa. U testu ispitivanja antioksidantne aktivnosti β -karoten metodom najpotentniji ekstrakti bili su jednako efikasni kao i BHT (butilovani hidroksitoluen). Rezultati antihemolitičkog i Schaal testa ukazali su na izuzetnu sposobnost ekstrakata da inhibiraju oksidativnu razgradnju eritrocita i produže period do početka oksidativnih promena u suncokretovom ulju.

Ključne reči: osušeni listovi i cvetovi heljde; rutin; antioksidantna aktivnost; oksidacija lipida; ekstrakcija.