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DPPH Radical Scavenging Activity and Phenolic Compound Content in Different Leaf Extracts from Selected Blackberry Species

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We used DPPH scavenging assays to study the antioxidant activity of three native Polish species of blackberry leaves (*Rubus kuleszae* Ziel., *R. fabrimontanus* (Sprib.) Sprib. and *R. capitulatus* Utsch.). All the studied extracts (methanolic, water, methanolic-water) showed high DPPH free radical scavenging activity (IC50 450.0–186.0 μg/ml). The most effective of the studied species was *Rubus kuleszae*. Total content of phenolic compounds (70.50–136.04 mg GAE/g) and phenolic acids (14.70–38.26 mg CAE/g) was determined spectrophotometrically. Antioxidant activity correlated positively with total content of phenolic compounds and phenolic acids.

Key words: *Rubus*, blackberry, leaf extract, antioxidant activity, DPPH, phenolic compounds, phenolic acids.

INTRODUCTION

The genus *Rubus* L. (blackberries/raspberries) comprises almost 700 species, making it the largest genus of the Rosaceae family and one of the most diverse of the plant kingdom (Alice and Campbell, 1999; Zieliński, 2004). Some species such as *R. idaeus* and *R. fruticosus* are valuable fruit plants and also used in medicine. The leaves of *Rubus* plants are known to contain astringent, antibacterial and antifungal agents, recommended internally in diarrhoea and inflammation of the gastrointestinal tract. Leaf infusions are also used externally for skin lesions and ulcers, and as a rinse in infections of the oral cavity and throat. This use of leaves of *Rubus* species relies on their high content of hydrolyzing (gallo- and ellagitannins) and condensed (catechins) tannins, and numerous other polyphenols including flavonoids (quercetin and kaempferol derivatives), anthocyanins and phenolic acids, particularly gallic and ellagic acid (Thiem, 2003; Benvenuti et al., 2004; Gudej and Tomczyk, 2004; Patel et al., 2004; Byamukama et al., 2005; Mertz et al., 2007; Ali et al., 2012).

Among many possible effects on biological processes in the human body, the antioxidant properties of phenolic compounds are the most important. Many data gathered in recent years indicate the participation of free radical processes in the emergence of such lifestyle diseases as atherosclerosis, heart attack, stroke, cancer, diabetes, senile cataracts and accelerated aging. The presence and distribution of numerous hydroxyl groups in the chemical structure of polyphenols make them excellent antioxidants. They are able to chelate transition metal ions, particularly those of iron and copper, which are involved in initiating free radical chain reactions. They also inhibit the activity of many enzymes participating in the formation of free radicals: for example, xanthine oxidase, responsible for the production of large amounts of reactive oxygen forms during reperfusion, and NADPH oxidase, responsible for the so-called respiratory burst (Rice-Evans et al., 1996; Pietta, 2000, Villańo et al., 2007; Li et al., 2008).

Phytochemical studies of phenolic compounds with a broad spectrum of biological activity are important to understanding the practical uses of blackberries. Correlations between the phenolic compound content and the antioxidant activity of plant extracts have been shown (Pietta, 2000; Benvenuti et al., 2004; Li et al., 2009). Our earlier

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phytochemical studies indicated high content of phenolic compounds in leaves of *R. fruticosus* L., including flavonoids, proanthocyanidins and phenolic acids, prompting us to investigate previously untested species of blackberry.

The purpose of this study was to make phytochemical analyses of leaf extracts of three native species of blackberry, including assessment of their antioxidant activity and determination of their content of phenolic acids and total phenolic compounds.

MATERIAL AND METHODS

PLANT MATERIAL

We studied three native species of blackberry from sect. *Corylifolii*: *Rubus kuleszae* Ziel., *R. fabrimontanus* (Sprib.) Sprib. and *R. capitulatus* Utsch. The plants grew in the Dendrological Garden of the University of Life Sciences in Poznań. We chose those species from the 80 blackberry and raspberry taxa growing there, on the basis of two years of phenological observations of the dynamics of vegetative and generative shoot growth, morphological studies, and species biomass determinations (Kluza-Wieloch and Maciejewska-Rutkowska, 2009), The plant material was air-dried for two weeks at room temperature. Voucher specimens are deposited in the herbarium of the Faculty of Pharmacognosy of the Poznan University of Medical Sciences.

PLANT EXTRACT PREPARATION

We tested three different solvents to determine their extraction efficiency. Dried and powdered leaves of the studied blackberry species (0.5 g) were extracted twice with methanol (30.0 ml, 30 min for each extraction, in an ultrasound bath – extract I, MeOH), with hot water (30.0 ml, 30 min for each extraction, at boiling temperature in a water bath – extract II, H_2O), and with methanol-water (5.0 ml hot water for 30 min, then extracted twice with 30.0 ml methanol, 30 min for each extraction at boiling temperature in a water bath under reflux – extract III, MeOH-H_2O). After evaporation of the solvents, the dry extracts were diluted with water to 50.0 ml. Extracts I, II and III were used in the analyses.

DPPH FREE RADICAL SCAVENGING ASSAY

The DPPH method was used to determine the free radical scavenging activity of each sample (Brand-Williams et al., 1995; Molyneux, 2004; Assimopoulou et al., 2005; Li et al., 2009). We mixed 1.4 ml DPPH (2.2-diphenyl-1-picrylhydrazyl) solution (0.0062 g/100 ml MeOH) with 0.2 ml of the tested samples dissolved in water at different concentrations (sample concentrations corresponding to 0.5–50.0 mg of plant material per 1 ml final solution, prepared by diluting the original plant extracts with the extraction solvent). The reaction mixture was shaken and incubated in the dark at room temperature for 30 min. Absorbance (A) was measured at 536 nm against the blank (UV/VIS Perkin-Elmer Lambda 35 spectrophotometer). Controls were prepared as for the test group except that the antioxidant solution was replaced with the corresponding extraction solvent. Inhibition of the DPPH radical by the sample was calculated according to the following formula:

$$\text{DPPH scavenging activity (\%)} = [A_0 - A_1 / A_1] \times 100$$

where A_0 is the absorbance of the control and A_1 is the absorbance of the sample. The results are averages of five measurements. Free radical scavenging activity is expressed as the percentage of DPPH decrease. The IC_{50} value, the amount of antioxidant necessary to halve the initial DPPH concentration, was calculated from the results and used for comparing the quality of the antioxidant extracts. We also calculated the $1/IC_{50}$ coefficient in order to determine the correlation between antioxidant activity and the total phenolics content of the tested extracts. Vitamin C (5–100 μg/ml) and BHA (butylated hydroxyanisole) (5–5000 μg/ml) were used as standards.

DETERMINATION OF TOTAL PHENOLICS

Total phenolics content was determined by the Folin-Ciocalteu method (Djeridane et al., 2006). The extract (0.2 ml) was pipetted into a 10.0 ml volumetric flask containing 4.0 ml water; next, 0.5 ml Folin-Ciocalteu's reagent and, after 1 min, 2.0 ml 20% aqueous solution of sodium carbonate were added. The volume was made up to 10.0 ml with distilled water. After 30 min, absorbance was measured at 760 nm against the reference solution. The results are averages of five measurements.

The total phenolics concentration was calculated from a calibrated curve ($R^2 = 0.9954$), using gallic acid as standard (0.001–0.006 mg/ml). The results are expressed as gallic acid equivalent (mg GAE/g).

DETERMINATION OF TOTAL PHENOLIC ACIDS

Content of phenolic acids was determined by a spectrophotometric method described in *Polish Pharmacopeia VI* (2002) with Arnov's reagent (10.0 g sodium molybdate, 10.0 g sodium nitrite in 100.0 ml water). The sample (1.0 ml) was pipetted into a 10.0 ml volumetric flask containing 5.0 ml water; next, 1.0 ml HCl (18 g/l), 1.0 ml Arnov's reagent and 1.0 ml NaOH (40g/l) were added. The

volume was made up to 10.0 ml with distilled water. Total phenolic acids content was calculated according to the following formula:

$$(\%) = A \times 0.877/m,$$

where A is the absorbance of the examined solution at 490 nm and m is the mass of the sample in grams. The results are averages of five measurements, expressed as caffeic acid equivalent (mg CAE/g).

STATISTICAL ANALYSES

The data obtained in this study are means ±confidence interval of five replicate determinations. Statistical comparisons employed Student's test, with $P<0.05$ considered statistically significant. Concentrations yielding 50% inhibition (IC_{50}) were found by interpolation from linear regression analysis. All statistical analyses used Microsoft Excel 2007 software.

RESULTS AND DISCUSSION

Some plants of the genus *Rubus* have been tested for antioxidant activity previously. There are many studies of the most common *Rubus* species, such as *Rubus ideaus* and *Rubus fruticosus* (Wang and Lin, 2000; Benvenuti et al., 2004; Jiao et al., 2005; Reyes-Carmona et al., 2005; Venskutonis et al., 2007; Dall'Acqua et al., 2008; Witkowska and Zujko, 2009), but there are no published data on the species we tested: *R. kuleszae*, *R. fabrimontanus* and R. *capitulates*. We analyzed three different extracts (methanol, water, methanol-water) from leaves of these three native species of blackberry, measuring the antioxidant activity of the extracts and correlating those data with total phenolic compounds and phenolic acids content.

The antioxidant activity of the leaf extracts was assessed by spectrophotometry of the presence of the DPPH radical, which is often used to compare the activity of plant extracts. DPPH is a stable free radical which dissolves in methanol and shows characteristic absorption at 536 nm. When an antioxidant scavenges free radicals by hydrogen donation, the DPPH assay solution becomes lighter in color (Molyneux, 2004; Villaňo et al., 2007). Samples with a raw material concentration range of 10.0–1000.0 μg/ml were analyzed. For comparison we also measured the radical scavenging activity of vitamin C and BHA in the same conditions. Table 1 shows the antioxidant activity of the tested extracts and the positive control (BHA, vitamin C), expressed as the percentage of deactivation of the DPPH free radicals.

The quality of the antioxidants in the extracts was determined by the IC_{50} values, denoting the concentration of the sample required to scavenge 50% of the DPPH free radicals. The $1/IC_{50}$ coefficient was calculated to compare the antioxidant activity of individual extracts (Tab. 2).

The antioxidant activity of the tested extracts increased with the quantity of raw material in the extract. For all the tested *Rubus* species, activity at a given concentration was highest for the water extracts (IC_{50} = 232.0–186.0 μg/ml; $1/IC_{50}$ = 0.0043–0.0054), and was similar to the activity of the positive control, BHA (IC_{50} = 200.05 μg/ml; $1/IC_{50}$ = 0.0050), but almost seven times less than that of vitamin C (IC_{50} = 30.60 μg/ml, $1/IC_{50}$ = 0.0327). The methanol-water extracts (IC_{50} = 414.0–284.0 μg/ml; $1/IC_{50}$ = 0.0024–0.0035) and methanol extracts (IC_{50} = 450.0–392.0 μg/ml; $1/IC_{50}$ = 0.0022–0.0026) were about half as active as the water extracts of a given *Rubus* species.

We used colorimetric methods to determine the content of total phenolic compounds and phenolic acids. Total phenolics were assayed by Folin-Ciocalteau's method, which can estimate all flavonoids, anthocyanins and nonflavonoid phenolic compounds, that is, all the phenolic compounds present in the extracts. This method is based on an oxidation-reduction reaction in which phenolic compounds are oxidized with simultaneous reduction of a phosphotungsten-phosphomolybdate complex in an alkaline medium, reacting blue. Table 2 gives the results for total phenolics, expressed as gallic acid equivalent (mg GAE/g).

The investigated blackberry species were rich in phenolic compounds, regardless of the method of extract preparation. Their content ranged from 70.50 to 136.04 mg GAE/g, highest in water extracts of *R. kuleszae* (136.04 mg GAE/g) and slightly lower in *R. fabrimontanus* (120.77 GAE/g) and *R. capitulatus* (111.73 mg GAE/g). The methanol and methanol-water extracts were similar in total phenolics content (70.50–89.21 mg GAE/g). Other authors have reported high content of polyphenols, especially tannins and ellagic acid, in leaves and fruits of *Rubus* species (Benvenuti et al., 2004; Gudej and Tomczyk, 2004; Reyes-Carmona et al., 2005; Witkowska and Zujko, 2009; Komes et al., 2010).

We also used Arnov's reagent to determine the content of phenolic acids, calculated as caffeic acid equivalent (mg CAE/g). This method is recommended by *Polish Pharmacopoeia VI* for standardization of plant materials. Phenolic acids constituted only 20–30% of total phenolics in the tested extracts. Water extracts were richest in these compounds: 38.26 mg CAE/g for *R. kuleszae*, 33.90 mg CAE/g for *R. fabrimontanus*, and 26.84 mg CAE/g for *R. capitulatus* (Tab. 2).

Previous analyses of blackberry extracts have found phenolic compounds to be the main antioxi-

TABLE 1. DPPH radical scavenging activity (%) of various extracts (I – MeOH; II – H_2O, III – MeOH-H_2O) from *Rubus* species, vitamin C and BHA

	Concentration (μg/ml)	DPPH radical scavenging activity (%)		
		R. kuleszae	*R. fabrimontanus*	*R. capitulatus*
Extract I	10.0	1.38±1.20	5.43±1.85	2.32±0.64
	50.0	4.14±2.80	7.77±0.68	8.08±1.23
	100.0	7.54±1.27	13.70±1.21	13.31±1.31
	200.0	14.40±2.42	26.69±3.76	27.34±0.49
	300.0	21.84±1.21	34.05±0.86	38.42±1.33
	500.0	40.71±1.89	55.51±2.02	63.34±1.06
	700.0	54.71±1.03	76.24±1.03	79.25±1.42
	1000.0	82.71±0.35	86.93±0.82	90.25±1.01
Extract II	10.0	1.52±0.10	2.26±1.64	1.30±0.41
	50.0	12.17±1.20	12.66±1.00	12.28±6.49
	100.0	28.48±1.59	26.11±1.91	23.30±0.91
	200.0	53.72±0.83	50.22±1.68	44.23±1.40
	300.0	73.44±1.30	71.75±6.04	62.63±0.97
	500.0	89.16±0.58	87.10±1.17	77.29±0.50
	700.0	90.62±0.24	90.72±0.66	87.63±0.27
	1000.0	89.64±0.47	90.99±0.62	88.56±0.33
Extract III	10.0	1.83±0.87	3.13±0.63	3.90±2.09
	50.0	6.93±2.84	9.64±1.52	6.09±0.62
	100.0	16.16±1.92	15.59±1.36	12.85±0.57
	200.0	32.21±1.27	35.33±4.34	25.98±1.98
	300.0	45.15±1.34	52.48±0.45	37.63±1.01
	500.0	66.74±3.08	75.87±2.80	59.67±1.37
	700.0	83.49±0.71	89.10±0.43	76.04±2.22
	1000.0	89.96±0.48	89.83±0.72	89.50±0.86
BHA	10	3.26±0.31		
	25	9.38±1.54		
	50	21.42±0.30		
	125	33.97±1.87		
	250	55.70±0.42		
	500	60.60±0.27		
	1000	65.23±0.56		
Vitamin C	5.0	9.42±1.65		
	12.5	19.31±0.28		
	25.0	39.15±1.76		
	50.0	78.84±0.91		
	75.0	85.90±1.74		
	100.0	86.11±0.35		

TABLE 2. IC_{50} and $1/IC_{50}$ values and total content of phenolic compounds and phenolic acids in various extracts (I – MeOH; II – H_2O, III – MeOH-H_2O) from *Rubus* species, and IC_{50} and $1/IC_{50}$ for standard controls (vitamin C, BHA)

	Extract	IC_{50} (μg/ml)	$1/IC_{50}$	Total content of phenolic compounds (mg GAE/g)*	Content of phenolic acids (mg CAE/g)
R. kuleszae	I	392.00	0.0026	73.32±1.46	15.46±0.52
	II	186.00	0.0054	136.04±0.67	38.26±0.28
	III	348.00	0.0029	70.50±0.92	14.70±0.43
R. fabrimontanus	I	450.00	0.0022	80.16±1.71	17.28±0.21
	II	200.00	0.0050	120.77±0.92	33.90±0.31
	III	284.00	0.0035	80.62±1.18	17.85±1.28
R. capitulatus	I	392.00	0.0026	89.21±0.45	24.01±0.95
	II	232.00	0.0043	111.73±0.70	26.84±0.25
	III	414.00	0.0024	87.24±1.76	21.36±1.67
BHA		200.05	0.0050		
Vitamin C		30.60	0.0327		

*The measurements (λ = 536 nm) were made after 30 min incubation.

dant components, their total content being proportional to antioxidant activity. Wang and Lin (2000) reported a linear correlation between total phenolics and antioxidant activity for fruits and leaves of different thornless blackberry cultivars. High antioxidant activity and phenolic compound content were observed in leaf extracts of *Rubus ulmifolius* Schott. and *R. fruticosus* L. (Dall'Acqua et al., 2008; Comes et al., 2010). Other authors have noted a similar relationship for fruit extracts of the blackberry species *R. fruticosus* L. and *R. caesius* L. (Benvenuti et al., 2004; Jabłońska-Ryś et al., 2009).

Our quantitative analyses examined the correlation between antioxidant activity and phenolic compound content in different extracts of *Rubus* leaves (Tab. 2), with $1/IC_{50}$ values taken to indicate antioxidant activity; correlation coefficients were calculated (R^2 = 0.811, y = 0,5 × 10^{-5} x – 0.001), and the correlations ($1/IC_{50}$ vs. total phenolics) are shown in Figure 1. High antioxidant activity corresponded to high total phenolics in the tested extracts. This result suggests that 81.1% of the antioxidant activity in extracts from these *Rubus* species is due to the contribution of phenolic compounds, and that the antioxidant activity of these plant extracts is not limited to phenolic compounds but may also be related to the presence of other antioxidant secondary metabolites such as carotenoids and vitamins, which in this case contributed to 18.9% of the antioxidant capacity. The antioxidant activity of polyphenols is due mainly to their redox properties, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers. They may also have metal-chelating potential (Rice-Evans et al., 1996).

The solvents we used for extraction differed in the content of phenolic compounds they yielded in the *Rubus* species leaf extracts, and consequently the antioxidant activity of the extracts differed as well. Hot water extraction (extract II) gave the highest content of phenolic compounds and phenolic acids, and the highest antiradical activity.

Here we showed that the *Rubus* species we studied represent important dietary sources of phenolic antioxidants. Using water as an extraction solvent, with methods not very different from the way herbal infusions and teas are prepared at home, we can obtain a product with high antioxidant activity.

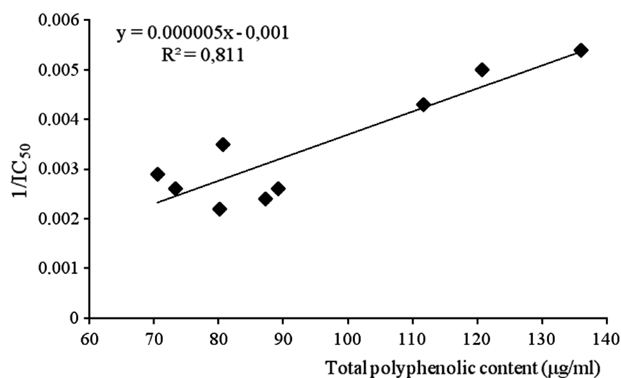

Fig. 1. Correlations of $1/IC_{50}$ values with total phenolics in different extracts from *Rubus* species.

CONCLUSIONS

The tested leaf extracts of *Rubus* species were similar in their content of total phenolic compounds and phenolic acids, and their antioxidant activity, but *R. kuleszae* measured highest.

The quality of the obtained extracts was affected mainly by the solvent used for extraction. Water was the best solvent for preparing herbal infusions, yielding the strongest antioxidant activity in the extract.

The study confirmed a strong correlation between antioxidant activity and the content of phenolic compounds (including phenolic acids) in the extracts.

Extracts from leaves of these *Rubus* species are good sources of antioxidants (similar to the synthetic antioxidant BHA, and about seven times less active than vitamin C), and their antioxidant properties are comparable to those reported in other research (Wang and Lin, 2000; Benvenuti et al., 2004; Dall'Acqua et al., 2008; Jabłońska-Ryś et al., 2009; Komes et al., 2010).

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