Antioxidant Activity and Polyphenols of Aronia in Comparison to other Berry Species

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Summary

Total polyphenols, total anthocyanins and antioxidant activity of various berries: [aronia (*Aronia melanocarpa*), blackberry (*Rubus fruticosus*), red raspberry (*Rubus idaeus*), and strawberry (*Fragaria anannassa*)] have been evaluated in this study by using Folin-Ciocalteau, pH-differential, and DPPH method. Amount of anthocyanins ranged from 232 to 4341 mg kg^{-1} in strawberry and aronia, respectively. Total polyphenol content varied from 1005 mg kg^{-1} in strawberry to 10637 mg kg^{-1} in aronia. Aronia contains the highest amounts of polyphenols and anthocyanins among the berries studied. It shows the highest antioxidant activity, as well. Therefore, individual polyphenolic compounds of aronia were studied further in more details. The amount of flavonols (quercetin, myricetin, kaempferol) and anthocyanins in aronia was determined by using HPLC method. Anthocyanins found in aronia were derivatives of cyanidin of which the most abundant were cyanidin-3-galactoside (68.9%) and cyanidin-3-arabinoside (24.5%). Quercetin was the main flavonol, with 93.07 % in total flavonol amount. The level of kaempferol was low (6.93%) while myricetin was not identified.

Key words

berries, aronia, flavonols, anthocyanins and polyphenols

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Introduction

Berries are delicious, low energy fruit, rich in antioxidant vitamins, fibre and various polyphenolic compounds (Beattie et al., 2005). They are studied intensively, because of possible beneficial effects on human health (Beattie et al., 2005; Kris-Etherton et al., 2002). In Croatia, berries like red raspberries, blackberries and strawberries are commonly used in diet, but aronia is almost unknown fruit. *Aronia melanocarpa* (black chokeberry) is small, dark violet fruit, and belongs to Rosaceae family (Benvenuti et al., 2004). Chokeberries originate from North America and are of great interest for food-coloring purposes and functional food development (Benvenuti et al., 2004; Bermúdez-Soto et al., 2004).

Polyphenolic compounds (including anthocyanins and flavonols) are important components of berries (Määttä-Riihinen et al., 2004a; Määttä-Riihinen et al., 2004b). They are potent antioxidants *in vitro* (Kähkönen et al., 2003; Heinonen et al., 1998; Pekkarinen et al., 1999) and may be protective against many degenerative diseases (Joshipura et al., 2001; Knekt et al., 2002; Le Marchand, et al., 2000; Garcia-Closas et al., 1999). Numerous studies have shown that anthocyanins are absorbed in their original glycosylated forms in humans (Netzel et al., 2005). They appear in urine and in human plasma after supplementation with berries or berry extracts but in very low concentrations (Netzel et al., 2005). Glucosides of quercetin are more efficiently absorbed than quercetin itself, whereas rhamnoglucosides (rutin) are less efficiently and less rapidly absorbed (Manach et al., 2005).

There is still not enough knowledge about health effects of fruits, vegetables or antioxidant concentrates. Much more research is therefore needed on the composition of antioxidants in fruits and on antioxidant effects of these products (Heinonen et al., 2002). A need for such data still exists because of increasing popularity of fruit consumption lately and because of increasing consumer awareness concerning the nutritional value of all foods (including berries).

Therefore, the objective of this work was to evaluate total polyphenols, total anthocyanins and antioxidant activity of berries which are commonly consumed in diet (red raspberry, blackberry, strawberry) and to compare these berries with aronia, berry fruit which is not so common in Croatia. Furthermore, individual flavonols (quercetin, kaempferol and myricetin) and anthocyanins of aronia were determined using HPLC method.

Materials and methods

Chemicals

For this study, anthocyanin standard (cyanidin-3-*O*-glucoside chloride-kuromanin chloride 0915 S) was purchased from Extrasynthese (Genay, France). Methanol (HPLC grade) was obtained from Merck (Darmstadt, Germany) and ortho-phosphoric acid (85%, HPLC grade) was purchased from Fluka (Buchs, Switzerland). Hydrochloric acid (36.2 %), potassium chloride, sodium acetate trihydrate, Folin-Ciocalteau reagent were obtained from Kemika (Zagreb, Croatia). Myricetin (M6760), quercetin dihydrate (Q0125), kaempferol (K0133) and 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\cdot}$) (D9132) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Samples

Berries [red raspberry (*Rubus idaeus*), blackberry (*Rubus fruticosus*), strawberry (*Fragaria anannassa*) and chokeberry (*Aronia melanocarpa*)] were harvested in Slavonia region (Croatia) at the commercial maturity stage. Immediately after harvesting, fruits were frozen and stored at –20 °C until analysis.

Sample preparation

For total anthocyanin, total polyphenol, and antioxidant activity determination, three replicates of berry extracts were prepared and analyzed the day of preparation. Berry extracts for anthocyanin analysis (Kalt et al., 1999) were obtained by grinding berries (~5 g) in methanol (20 mL) acidified with HCl (0.1 %, v/v) for 2 min. The mixture was stored at room temperature in the dark for 16 h and then filtered. Berry extracts for polyphenolic analysis (Kalt et al., 1999) were obtained by grinding berries (~5 g) in hot methanol (10 mL) for 2 min. The solution was filtered. The extraction of the residue was repeated twice following above mentioned procedure. Three extracts were combined. Berry extracts for antioxidant activity determination were obtained by grinding berries (~20 g) in methanol (20mL) acidified with HCl (0.1%). After 60 min the solution was filtered. The residue was extracted again. The extracts were combined and diluted to volume of 50 mL with methanol acidified with HCl (0.1%).

For flavonol analysis by HPLC method, three replicates of aronia extract were prepared and analyzed the same day. Method for extraction and hydrolysis of flavonols was developed by Hertog et al., (1992), optimized by Häkkinen et al., (1998) and applied in this work with further modifications. Ascorbic acid (80 mg) was dissolved in distilled water (5 mL) and 5 g of homogenized fruit sample, 25 mL of methanol and 10 mL of 6 M HCl were added to ascorbic acid solution. Water was added to this mixture to obtain a final volume of 50 mL and final concentration of HCl 1.2 M. This solution was refluxed on water bath for 2 h at 85 °C. After cooling, extracts were filtered. A 20 mL portion of the filtrate was evaporated to dryness on a rotary evaporator using water bath and temperature of 35°C. The residue was dissolved in 2 mL of methanol and filtered

through a 0.45 µm syringe filter (VariSep PTFE, 0.45 µm, 25 mm-Varian) prior to injection into the HPLC.

For anthocyanin analysis by HPLC method, three replicates of aronia extract were prepared and analyzed the same day. Aronia extract was obtained by grinding berries (~20 g) in methanol (20 mL) acidified with HCl (0.1 %). After 60 min the solution was filtered. The residue was extracted again in the same way. The extracts were combined and diluted to volume of 50 mL with methanol acidified with HCl (0.1%). An aliquot of extract (2 mL) was filtered again through a 0.45 µm syringe filter (VariSep PTFE, 0.45 µm, 25 mm-Varian) prior to injection into the HPLC.

Determination of total polyphenols

Total polyphenols were determined by Folin-Ciocalteau micro method (Waterhouse, 2006). An aliquot (20 µL) of extract was mixed with 1580 µL of distilled water and 100 µL of Folin-Ciocalteau reagent. 300 µL of sodium carbonate solution (200 g L^{-1}) was added to the mixture. After incubation at 40 °C for 30 min in water bath, absorbance of the mixture was read against the prepared blank at 765 nm. Total polyphenolics were expressed as mg of gallic acid equivalents (GAE) per kg of berry. Data presented are mean ± standard deviation (SD).

Determination of total anthocyanins

Total anthocyanins were estimated by a pH-differential method (Giusti et al., 2001). Two dilutions of berry extracts were prepared, one with potassium chloride buffer (pH 1.0) (1.86 g KCl in 1 L of distilled water, pH value adjusted to 1.0 with concentrated HCl), and the other with sodium acetate buffer (pH 4.5) (54.43 g $CH_3CO_2Na{\cdot}3H_2O$ in 1 L of distilled water, pH value adjusted to 4.5 with concentrated HCl), diluting each by the previously determined dilution factor [strawberry and red raspberry 1:5 (v/v), blackberry 1:20 (v/v), aronia 1:100 (v/v)]. Absorbance was measured simultaneously at 510 and 700 nm after 15 min incubation at room temperature. The content of total anthocyanins was expressed in mg of cyanidin-3-glucoside equivalents (CGE) per kg of berries using a molar extinction coefficient (ε) of cyanidin-3-glucoside of 26 900 L mol^{-1} cm^{-1} and molar weight (MW) (449.2 g mol^{-1}). Data presented are mean ± standard deviation (SD).

Determination of antioxidant activity

Antioxidant activity was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. By the DPPH method (Benvenuti et al., 2004), five dilutions of each berry extract were analyzed. Reaction solution was prepared by mixing 50 µL of diluted berry extract with 300 µL of methanolic DPPH· solution (1mM) and brought to 3 mL with methanol. The solution was kept in dark at room temperature for 15 minutes. The absorbance ($A_{extract}$) was read against the prepared blank (50 µL diluted fruit extract, 2950 µL methanol) at 517 nm. A DPPH· blank solution was prepared (300 µL of 1mM DPPH· solution, 2.7 mL of methanol) and measured daily. Percent inhibition of DPPH radical was calculated for each dilution of berry extract according to formula: % inhibition=$[(A_{DPPH}-A_{extract})/A_{DPPH})]$x100, where A_{DPPH} is the absorbance value of the DPPH· blank solution and $A_{extract}$ is absorbance value of the sample solution.

HPLC analysis of anthocyanins and flavonols

The analytical HPLC system employed consisted of a Varian LC system (USA) equipped with a ProStar 230 solvent delivery module, ProStar 310 UV-Vis Detector, and ProStar 330 PDA Detector. Anthocyanin and flavonol separation was done in an OmniSpher C18 column (250 x 4.6 mm inner diameter, 5 µm, Varian, USA) with a ChromSep C18 guard column (1 cm x 3 mm, Varian, USA). The data were collected and analyzed on IBM computing system equipped with Star Chromatography Workstation software (version 5.52).

For anthocyanin analysis, mobile phase A was 0.5 % water solution of phosphoric acid and mobile phase B was 100 % HPLC grade methanol. The elution conditions were as follows: 3-65 % B 0-38 min; 65 % B 38-45 min; with flow rate 1 mL min^{-1}. Operating conditions were: column temperature 20°C and 10 µL injection volumes of the standard and samples. A 10-minute re-equilibration period was used between individual runs. UV-Vis spectra were recorded in wavelength range from 190-600 nm (detection wavelength was 520 nm).

For flavonol analysis, mobile phase A was 0.1 % water solution of phosphoric acid and mobile phase B was 100 % HPLC grade methanol. The elution conditions were as follows: 5-80 % B, 0-30 min; 80 % B 30-33 min; 80-5 % B, 33-35 min; with flow rate 0.8 mL min^{-1}. Operating conditions were: column temperature 20°C and 10 µL injection volumes of the standards and samples. A 10-minute re-equilibration period was used between individual runs. UV-Vis spectra were recorded in wavelength range from 190-600 nm (detection wavelength was 360 nm).

Identification and peak assignment of anthocyanins and flavonols was based on comparison of their retention times and spectral data (190-600 nm) with those of authentic standards. Additional identification was carried out by spiking the extracts with available standards. Anthocyanin profiles of berries were compared with those found in literature (Määttä-Riihinen et al., 2004a) which gave additional information on anthocyanin identification. Calibration curves of the standards were made by diluting stock solutions in methanol to yield 1-80 mg L^{-1} (myricetin and kaempferol) and 1-250 mg L^{-1} (quercetin), or in 0.1 % methanolic HCl solution to yield 1-100 mg L^{-1} (cyanidin-3-glucoside). Identified anthocyanins

were quantified using calibration curve of cyanidin-3-glucoside and the concentrations of anthocyanins were expressed in mg of cyanidin-3-glucoside equivalent per kg of berries. Flavonols were quantified using calibration curves of quercetin, myricetin and kaempferol. Data presented are mean ± standard deviation (SD).

Results and discussion

Berry extracts were analyzed using a pH-differential and Folin-Ciocalteau method in order to examine their total anthocyanin (TA) and total polyphenol (TP) content. The portion of anthocyanins in total polyphenol concentration was evaluated by calculating TA/TP ratio. The concentrations of total anthocyanins, total polyphenols, and TA/TP ratios are shown in Table 1. Anthocyanins were found in the highest concentrations in aronia (4341 mg kg^{-1}) whereas the concentrations of anthocyanins in blackberry, red raspberry and strawberry were considerably lower (1109 mg kg^{-1}, 243 mg kg^{-1}, 232 mg kg^{-1}, respectively). Data presented by other authors also confirmed that the amount of total anthocyanins were higher in aronia than in other red fruits like blackberry, raspberry or red currant (Benvenuti et al., 2004).

Polyphenols were found in the highest concentrations in aronia (10637 mg kg^{-1}). High concentrations of polyphenols were found in blackberry as well (2485 mg kg^{-1}), while red raspberry and strawberry had relatively lower concentrations of polyphenols (1256 mg kg^{-1}, 1005 mg kg^{-1}, respectively). According to data presented by other authors, aronia contained higher concentrations of polyphenols than berries like red currant, blackberry and raspberry (Benvenuti et al., 2004) which agree with our results.

Anthocyanins represented significant portion in total polyphenol concentration of blackberry (45 %) or aronia (41 %). The portion of anthocyanins in strawberry (23 %) and red raspberry (19%) was considerably lower (Table 1).

In order to evaluate antioxidant activity of chosen berries, DPPH· assay was applied and the results are presented in Figure 1. All investigated berry extracts exhibited potent radical scavenging activities. The strongest radical scavenging activity showed aronia, while activities of other berries were considerably lower. There are already a number of reports on the antioxidant activity of berry extracts by several methods such as oxygen radical absorbance capacity (Wu et al., 2004) or DPPH radical scavenging capacity (Benvenuti et al., 2004) indicating that berries like aronia, elderberry and black currant possess strong antiradical activities. Antioxidant activity of aronia juice concentrate against DPPH radical was stronger than antioxidant activity of strawberry and red raspberry concentrate (Bermúdez-Soto et al., 2004), which agrees with our results.

Table 1. Concentrations of total anthocyanins (TA) (mg CGE kg^{-1})[a], total polyphenols (TP) (mg GAE kg^{-1})[a] and TA/TP ratio

Berries	Total anthocyanins (mg kg^{-1})	Total polyphenols (mg kg^{-1})	TA/TP
Red raspberry	242.90±3	1256.16±133	0.19
Blackberry	1108.87±6	2484.87±234	0.45
Strawberry	232.16±10	1005.17±56	0.23
Aronia	4341.06±22	10637.20±571	0.41

* values are means ± SD (n=3)

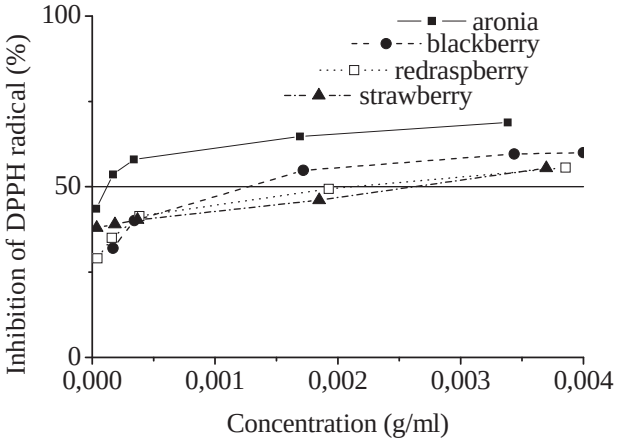

Figure 1. Radical scavenging activities of berry extracts. The berry extracts were incubated with DPPH for 15 minutes

In order to separate and determine individual polyphenolic compounds present in aronia, HPLC method was applied. The HPLC chromatogram of aronia recorded at 520 nm (representing anthocyanins) is shown in Figure 2. Chromatogram recorded at 360 nm represents flavonols and is shown in Figure 3. The concentrations and percent distribution of individual anthocyanins and flavonols in aronia are shown in Table 2.

Aronia contained a mixture of four different cyanidin-glycosides: 3-galactoside, 3-arabinoside, 3-glucoside, and 3-xyloside of cyanidin. The most abundant were cyanidin-3-galactoside and cyanidin-3-arabinoside (2795 mg kg^{-1}, 994 mg kg^{-1}, respectively, >93 % of total anthocyanin content) whereas the level of cyanidin-3-xyloside (146 mg kg^{-1}, 3.6 %) and cyanidin-3-glucoside (122 mg kg^{-1}, 3%) were relatively low. The data presented by other authors are showing that the main anthocyanins in aronia are cyanidin-3-galactoside and cyanidin-3-arabinoside; the following are cyanidin-3-xyloside and cyanidin-3-glucoside (Bermúdez-Soto et al. 2004; Määttä-Riihinen et al., 2004a; Wu et al., 2004), which is consistent with our results.

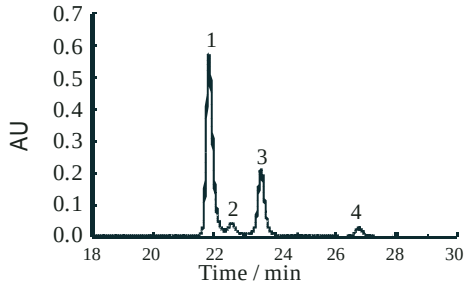

Figure 2. Chromatogram of aronia extract recorded at 520 nm. Peak identification: (1) cyanidin-3-galactoside, (2) cyanidin-3-glucoside, (3) cyanidin-3-arabinoside, (4) cyanidin-3-xyloside

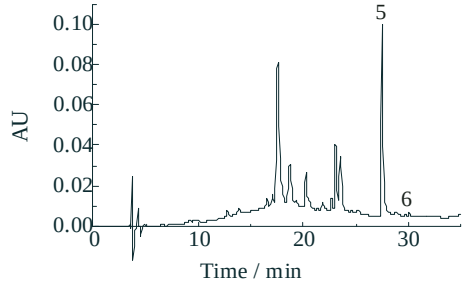

Figure 3. Chromatogram of aronia extract recorded at 360 nm. Peak identification: (5) quercetin, (6) kaempferol

Two flavonols were identified in aronia: quercetin and kaempferol. The main flavonol was quercetin (71.13 mg kg^{-1}; 93.07 %) while the concentration of kaempferol was low (5.3 mg kg^{-1}; 6.9 %) (Table 2). These results are in accordance with those reported by Määttä-Riihinen et al. (2004a). Häkkinen et al. (1999b) found only quercetin while kaempferol was not identified. According to Häkkinen et al., (1999a) aronia contains quercetin as the main flavonol and the relative content of myricetin and kaempferol was low.

Conclusion

Blackberry, red raspberry and strawberry have considerably lower amount of polyphenols and anthocyanins in comparison to aronia which is very rich in these phytochemicals. Aronia possesses the highest antioxidant activity against DPPH radical, as well. The main anthocyanins of aronia are derivatives of cyanidin among which cyanidin-3-galactoside and cyanidin-3-arabinoside were the most abundant. The main flavonol in aronia is quercetin. Although all berries studied can serve as a good source of bioactive phytochemicals in the human diet, aronia stands out in high polyphenol and anthocyanin content and high antioxidant activity. From the view of the phenolic content and antioxidant activity aronia can be regarded as good candidate for raw materials in production of health beneficial functional foods.

Table 2. Concentrations of anthocyanins and flavonols in aronia (mg kg^{-1})[a] determined by HPLC method and percentage distribution of anthocyanins and flavonols.

Berry	Anthocyanins (mg kg^{-1})	Percentage of total anthocyanins
Aronia		
cy-3-gal	2794.74±4.0	68.9
cy-3-glu	121.69±0.1	3.0
cy-3-ara	993.77±1.1	24.5
cy-3-xyl	146.02±0.3	3.6
Total	4056.22±5.5	100.0
	Flavonols (mg kg^{-1})	Percentage of total flavonols
Quercetin	71.13±1.5	93.07
Kaempferol	5.30±0.5	6.93
Total	76.43±2.0	100.00

* values are means ± SD (n=3); cy, cyanidin; glu, glucoside; gal, galactoside; ara, arabinoside; xyl, xyloside.

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