

Full Length Research Paper

Phenolic content, antioxidant capacity, radical oxygen species scavenging and lipid peroxidation inhibiting activities of extracts of five black chokeberry (*Aronia melanocarpa* (Michx.) Elliot) cultivars

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Black chokeberries (*Aronia melanocarpa* (Michx.) Elliot) are species, the fruit of which are considered to be one of the most valuable sources of phenolic substances and the research in the field of the fruit utilization as a food supplement is more than topical. Nevertheless, as far as this species is concerned, in particular cultivars there are differences which are caused by both their origin and genetic predispositions. Therefore, the aim of our measurement was to compare five widely grown cultivars of this plant, namely 'Aron' which is Danish in origin, 'Fertödi' which is Hungarian in origin, 'Hugin' which is of Swedish origin, 'Nero' which is of Czech origin and 'Viking' which is Finnish in origin. The highest contents of total phenolics (TPC) were observed in the cultivar 'Viking' with the value of 12.85 grams of gallic acid/kg FM and in the cultivar 'Nero' the value reached 11.12 grams of gallic acid/kg FM. Furthermore, in both mentioned cultivars the values of total antioxidant capacity (TAC) were the highest. The correlation coefficient between TPC and TAC was $r^2 = 0.943$. The content of chlorogenic and neochlorogenic acid showed an influence on TAC ($r^2 = 0.788$ and $r^2 = 0.940$). For comparison, scavenging activity of reactive oxygen species (superoxide anion, hydroxyl radical and nitric oxide) was determined by using 25% methanolic extracts of fruit of particular cultivars. In addition, antioxidant potential was assessed using the rat liver slice model. Similarly, the highest values of scavenging activity were found in the cultivars 'Viking' and 'Nero' which seem to be most promising for further utilization in alimentary and pharmaceutical practices.

Key words: Chokeberry, phenolics, antioxidant capacity, ROS, lipid peroxidation.

INTRODUCTION

Chokeberry (*Aronia melanocarpa* (Michx.) Elliot) is a member of the *Rosaceae* family and fruit are actually a pome (Walther and Schnell, 2009). Nowadays, the fruit are highlighted with respect to their potential as a food colorant (Bridle and Timberlake, 1997) and as a suitable

food supplement in relation to strengthening human immunity system (Jakobek et al., 2007). Perhaps the most important constituents found in chokeberries are the phenolic compounds which are responsible for many medicinal properties (Kulling and Rawel, 2008). In the

fruit, chlorogenic and neochlorogenic acids are dominant among the aromatic acids (Slimestad et al., 2005). High contents of cyanidin-3-arabinoside, cyanidin-3-galactoside and (-)-epicatechin are also typical of chokeberries (Skupien and Oszmianski, 2007). ROS are generated in many redox processes in human body and often induce oxidative damage to biomolecules. When the generation of ROS induced by various stimuli in the organism exceeds the antioxidant capacity of the organism, it will lead to a variety of pathophysiological processes (Yang et al., 2008). ROS can cause oxidation of biomolecules such as DNA, protein and enzymes (Wu and Ng, 2008). ROS can cause oxidation of biomolecules such as DNA, protein and enzymes. ROS also attack lipids to initiate free radical chain reactions and cause lipid peroxidation (Wu and Ng, 2008). Antioxidant capacity of phenolics against lipid peroxidation is very effective and was described by many authors e.g (Takao et al., 1994; Banerjee et al., 2005; Gracia-Alonso et al., 2004).

In comparison with other fruit species, relatively high values of antioxidant capacity were reported in chokeberry fruit (Kulling and Rawel, 2008). Antioxidant properties are ascribed to a high content of polyphenols (Walther and Schnell, 2009). The aim of our work was to measure TPC, TAC and the content of cyanidin-3-arabinoside, cyanidin-3-galactoside, (-)-epicatechin, chlorogenic and neochlorogenic acids as important phenolic constituents in black chokeberry fruit. To support the results, efficiency of particular chokeberry cultivar extracts on radical oxygen species (ROS) scavenging activity (hydroxyl radical, nitric oxide and superoxide anion) was measured. Antioxidant potential was also assessed using the rat liver slice model and lipid peroxidation scavenging activity was measured. The work is to provide with useful information when comparing the most widespread cultivars of this fruit species (particularly as far as TPC, TAC and ROS are concerned).

MATERIALS AND METHODS

Description of locality

Fruit were harvested in experimental orchards of Tomas Bata University in Zlín within the period of 2008 – 2010. These orchards are situated in the south-western part of the White Carpathians

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Abbreviations: **AAE**, ascorbic acid equivalents; **DPPH**, 2,2-Diphenyl-1-picrylhydrazyl; **FM**, fresh mass; **GAE**, gallic acid; **ROS**, radical oxygen species; **TAC**, total antioxidant capacity; **TPC**, total phenolic content.

near Zlín, the Czech Republic. The average altitude is 340 m above sea level, and the mean annual temperature and precipitation are 7.9°C and 760 mm, respectively. The soil type was classified as the Mesotrophic Cambisol (Anonymous, 2007).

Collection and processing of samples for chemical analyses

Fruit were harvested in full ripeness (Hricovsky, 2002) from three plants of each cultivar under study in the course of August. 40 randomly chosen fruit from each plant were mixed together and used for analyses (that is, altogether 120 per each cultivar). Fruit of individual cultivars were processed immediately after the harvest (not later than within two days). Harvested fruit were puréed in a mixer and the average sample was obtained by dividing into quarters. Each parameter was measured in five replications. The results were expressed as average of a three-year experiment. The following cultivars of black chokeberries were analyzed – ‘Aron’ which is Danish in origin, a Hungarian cultivar ‘Fertödi’, ‘Hugin’ which is of Swedish origin, ‘Nero’ which is a Czech cultivar and ‘Viking’ which is Finnish in origin (Kulling and Rawel, 2008).

Sample preparation

Extraction was performed according to the method described by Kim et al. (2003) and modified according to Barros et al. (2007), using the following procedure: 10 g of a fresh sample were homogenized for 10 seconds in 100 mL of methanol. The resulting paste was placed into Erlenmeyer flasks (120 mL) and let to stand in a water bath with the temperature of +25°C for a period of 24 hours. The residue was then extracted with two additional portions of methanol. The combined methanolic extracts were evaporated at 40°C to dryness and redissolved in methanol at a concentration of 100 mg/mL, and stored at 4°C for further use.

Total phenolic content assay

To measure total contents of phenolic substances, 0.5 mL of the sample was taken and diluted with water in a 50 mL volumetric flask. Thereafter, 2.5 mL of Folin-Ciocalteu reagent and 7.5 mL of a 20% solution of sodium carbonate were added. The resulting absorbance was measured in the spectrophotometer LIBRA S6 at the wavelength of 765 nm against a blind sample, which was used as reference. The results were expressed as g of gallic acid/kg of fresh mass (FM) (Kim et al., 2003).

Antioxidant capacity by the DPPH test assay

The DPPH test (2,2-Diphenyl-1-picrylhydrazyl) was done according to the method of Brand-Williams et al. (1995) with some modifications (Thaipong et al., 2006). The stock solution was prepared by dissolving 24 mg of DPPH with 100 mL of methanol and then stored at -20°C until needed. The working solution was obtained by mixing 10 mL of the stock solution with 45 mL of methanol to obtain the absorbance of 1.1 ± 0.02 units at 515 nm using the spectrophotometer LIBRA S6. Fruit extracts (150 µL) were allowed to react with 2.850 µL of the DPPH solution for 1 h in the dark. Then, the absorbance was taken at 515 nm. The antioxidant capacity was calculated as a decrease in absorbance value using the formula:

$$(\%) = (A_0 - A_1/A_0) \times 100\%$$

Where, A_0 is the absorbance of the control (without the sample) and A_1 is the absorbance of the mixture containing the sample. The

results of absorbance were converted using a calibration curve of the standard and expressed in ascorbic acid equivalents (AAE)/kg FM (Rupasinghe et al., 2006).

Phenolic content assay

For the measurement of cyanidin-3-arabinoxide, cyanidin-3-galactoside, (-)-epicatechin, chlorogenic and neochlorogenic acids the stored methanolic extract was diluted a hundred times with ACS water, which is deionized water supplied by Sigma-Aldrich and filtrated through a membrane filter Nylon (0.45 µm Nylon filter disk). The instrument used for phenolic analysis consisted of a solvent delivery pump (ESA Inc., Model 582), guard cell (ESA Inc., Model 5010A, working electrode potential K1 = 600 mV, K2 = 650 mV), chromatographic column - Model Supelcosil LC8 (150.0 × 4.6 mm), 5 µm particle size and an electrochemical detector (Coulchem III). Chromatographic conditions were constant: 30°C, as a mobile phase methanol was used: H₂O:H₃PO₄ = 99:0.5:0.5, (filtrated through a filter Nylon, 0.2 µm), the type of elution was isocratic, the flow rate of the mobile phase was 1.1 mL/min (Miki, 1981). The content of phenols was calculated as mg/kg of fresh mass.

Reactive oxygen species scavenging activity assay

For the measurement of reactive oxygen species activity 25% extracts were prepared in phosphate buffer (20 mmol/L, pH 7.4). The hydroxyl radical scavenging activity was assayed according to the method of Ghiselli et al. (1998). 1 mL of the extract was mixed with 0.8 mL of a reaction buffer (phosphate buffer, 20 mmol/L, pH 7.4, deoxyribose, 1.75 µmol/L, iron ammonium sulphate, 0.1 µmol/L and EDTA, 0.1 µmol/L). 0.1 mL of H₂O₂ (0.01 mol/L) was then added to the reaction solution. The solution was incubated for 10 min at 37°C prior to the addition of 0.5 mL of 1% thiobarbituric acid and 1 mL of 2.8% trichloroacetic acid. The mixture was boiled for 10 min and cooled rapidly. The absorbance of the mixture was measured at 532 nm (apparatus LIBRA S6). The assay of nitric oxide scavenging activity was done according to the method described by Green et al. (1982). 1 mL of the extract was mixed with 1 mL of the reaction solution containing sodium nitroprusside (10 mmol/L) in phosphate buffer (20 mmol/L, pH 7.4). The incubation at 37°C for 1 h followed and 0.5 mL of aliquot was then mixed with 0.5 mL of Griess reagent. The absorbance was measured at 540 nm.

The superoxide anion scavenging activity was done according to the method described by Beissenhirtz et al. (2004) and it is based on the reduction of cytochrome c. 1 mL of the extract was mixed with 1 mL of the solution containing xanthine oxidase (0.07 U/mL), xanthine (100 µmol/L) and cytochrome c (50 µmol/L). After incubation at 20°C for 3 min, the absorbance at 550 nm was determined. All tests were performed in triplicate. The hydroxyl radical scavenging activity, nitric oxide scavenging activity and superoxide anion scavenging activity were calculated as follows:

$$\text{Scavenging activity (\%)} = (A_0 - A_1/A_0) \times 100\%$$

Where, A₀ is the absorbance of the control (without the sample) and A₁ is the absorbance of the mixture containing the sample.

Lipid peroxidation inhibition activity

The inhibition of lipid peroxidation was assayed by the method of Anup et al. (2006). 5 µg of rat liver were homogenized in 20 mL of Tris-HCl buffer (50 mmol/L, pH 7.6). 0.1 mL of the liver homogenate was incubated with the sample (0.2 mL of a 25% extract), 0.1 mL of KCl (30 mmol/L), 0.1 mL of FeSO₄ (0.16 mmol/L) and 0.1 mL of ascorbic acid (0.06 mmol/L) at 37°C for 1 h. Thereafter, 1 mL of 1% thiobarbituric acid (TBA) and 1 mL of 15% trichloroacetic acid

were added. The final solution was heated at 100°C in a boiling water bath for 15 min, cooled with ice for 10 min, and then centrifuged at 5,000 rpm. for 10 min. The absorbance of the supernatant was measured at 532 nm, using the LIBRA S6 spectrophotometer. The blank was performed by substituting Tris-HCl buffer (50 mmol/L, pH 7.6) for the sample. The inhibition percentage of the formation of TBA-reactive substances was calculated as:

$$\text{Inhibition activity (\%)} = (A_0 - A_1/A_0) \times 100\%$$

Where, A₀ is the absorbance of the control (without the sample) and A₁ is the absorbance of the mixture containing the sample.

Statistical analysis

The data obtained were analyzed statistically by the analysis of variance (ANOVA) and Tukey's multiple range test for comparison of means (Snedecor and Cochran, 1967). Correlation functions were calculated using the statistical package Unistat, v. 5.1 and Office Excel® Microsoft.

RESULTS

From our results one can see high variability among the fruit of particular cultivars. In case of TPC the highest average values were obtained in the 'Viking' cultivar (12.85 g GAE/kg FM), followed by the 'Nero' (11.12 g GAE/kg FM) and the 'Hugin' (9.68 g GAE/kg FM) cultivars. On the contrary, in the 'Fertödi' and 'Aron' cultivars the values were only 7.78 g GAE/kg FM and 7.89 g GAE/kg FM, respectively. Similarly, the values of TAC corresponded to the measured contents. For example, in the 'Viking' cultivar, TAC was 15.96 g AAE/kg FM and in the 'Nero' cultivar the value was 15.96 g AAE/kg FM. On the other hand, the lowest amount of TAC was found in the fruit of the 'Fertödi' cultivar with the value being 8.89 g AAE/kg FM (Table 1). The correlation coefficient between TPC and TAC was $r^2 = 0.948$, $y = 1.5194x - 2.9191$.

In case of particular polyphenols statistical significant differences were observed among the cultivars. The values of their contents ranged from 941.82 mg/kg FM (the 'Aron' cultivar) to 1553.29 mg/kg FM (the 'Viking' cultivar) in cyanidin-3-arabinoxide; in cyanidin-3-galactoside from 1010.80 mg/kg FM (the 'Aron' cultivar) to 1203.56 mg/kg FM (the 'Viking' cultivar); in case of (-)-epicatechin the values were from 467.35 mg/kg FM (the 'Aron' cultivar) to 862.50 mg/kg FM (the 'Nero' cultivar). The lowest values of chlorogenic and neochlorogenic acids were measured in the 'Fertödi' cultivar (1131.15 mg/kg FM and 845.04 mg/kg FM).

The chromatogram representing the particular peaks is shown in Figure 1. The correlation coefficient between antioxidant capacity and chlorogenic acid was $r^2 = 0.788$, $y = 0.0075x - 0.1052$. On the contrary, in case of neochlorogenic acid the correlation coefficient was $r^2 = 0.940$, $y = 0.0258x - 1.3268$. For comparison, the correlation

Table 1. TPC and TAC contents – in case of TPC the results are expressed as grams of gallic acid/kg FM, in case of TAC the results are expressed as grams of AAE/kg FM.

Cultivar	TPC	TAC
Aron	7.89 ± 0.35 ^a	9.02 ± 0.56 ^a
Fertödi	7.78 ± 0.39 ^a	8.89 ± 0.71 ^a
Hugin	9.68 ± 0.44 ^b	11.15 ± 0.88 ^b
Nero	11.12 ± 0.57 ^c	15.32 ± 1.12 ^c
Viking	12.85 ± 0.56 ^c	15.96 ± 0.95 ^c

Different superscripts in each column indicate the significant differences in the mean at $p < 0.05$.

coefficients between TAC and cyanidin-3-arabioside, cyanidin-3-galactoside and (-)epicatechin were $r^2 = 0.958$, $y = 0.0116x - 1.6738$; $r^2 = 0.903$, $y = 0.0397x - 3.1910$; and $r^2 = 0.948$, $y = 0.0190x - 0.7533$, respectively.

Interesting results were given in case of scavenging effect on ROS of 25% black chokeberry fruit methanolic extracts. Comparing the particular results shown in Table 3, high variability between the cultivars is evident. Generally, the lowest values of scavenging activity were reached (the results are listed in order of hydroxyl radical, nitric oxide, superoxide anion and lipid peroxidation) in the 'Fertödi' cultivar (22.08, 27.59, 21.24, 12.05%) and that of the 'Aron' (25.01, 28.42, 22.22, 12.57%). Higher values were measured in the fruit of the 'Hugin' cultivar (31.12, 33.10, 30.48, 16.19%) and the highest scavenging activity was observed in the 'Nero' cultivar (33.51, 37.30, 35.96, 19.22%) and the 'Viking' cultivar (34.15, 41.46, 36.92, 19.81%).

DISCUSSION

Regarding to the fact that all cultivars were grown under identical conditions and in the same locality, it is possible to conclude that one can clearly see the cultivar variability, which is quite typical of fruit (Kopec, 1998). This variability became evident in case of TPC, TAC, ROS scavenging activity and also lipid peroxidation activity. In our measurement very high contents of polyphenolics were observed. The values of their contents ranged from 7.78 to 12.85 g of gallic acid/kg FM. These contents correspond to the values which were found out in black chokeberries by Zheng and Wang (2003). Moreover, some authors noticed high values of phenols in black chokeberry fruit, even higher in comparison with our results (Benvenuti et al., 2004; Oszmianski and Wojdylo, 2005). For most fruit species lower contents are typical. For example, in apples the common content is between 0.6 to 2.1 g of gallic acid/kg FM (Vrhovsek et al., 2004) or in plums it is 2.2 to 5.0 g of gallic acid/kg FM (Rop et al., 2009). Antioxidant capacity was determined by the DPPH test and it was the highest in the cultivar 'Viking' with the value of 15.96 g of AAE/kg

FM. In other fruit species, including apples (Huber and Rupasinghe, 2009), small berries and stone fruit (Chen et al., 2006), the values of antioxidant capacity are mostly much lower. For example, in cherries there are values of up to 0.9 g of AAE/kg FM (Usenik et al., 2008) and in plums the values can reach up to 6 g of AAE/kg FM (Rop et al., 2009). As far as statistical evaluation of the results is concerned, the highest values of correlation coefficient between antioxidant capacity and the total amount of phenolic substances were obtained (in case of the DPPH test $r^2 = 0.948$, $y = 1.5194x - 2.9191$). Many authors notice a high correlation between TPC and antioxidant capacity in fruit (Thompson and Chaovanalikit, 2003; Ercisli and Orhan, 2008). Interesting results were received in case of the contents of particular phenols. While in case of cyanidin-3-glucoside the differences in its content were slight among the cultivars (Table 2), in other phenols the differences were statistically significant. The highest contents were demonstrated in case of chlorogenic acid (from 1131.15 to 1960.72 mg/kg FM). This acid is an important phenol found in black chokeberry fruit (Skupien and Oszmianski, 2007). Zheng and Wang (2003) draw attention to the fact that chlorogenic acid is the most important antioxidant in black chokeberry, which was also confirmed in our measurement.

The methanolic extracts (25%) of black chokeberry fruit showed moderate inhibitory ability on hydroxyl radical (34.15%), nitric oxide (41.46%), superoxide anion (36.92%) and lipid peroxidation (19.81%) – the cultivar 'Viking'. ROS are known to cause aging, cancer and many other mal-effects on the human body and implicated in pathogenesis of several diseases (Sumanont et al., 2004). In this study, the black chokeberry fruit extract was evaluated for high ability to scavenge hydroxyl radical using the deoxyribose degradation assay. The cultivars 'Nero' and 'Viking' generally seemed to have scavenging activities of ROS including nitric oxide. Scavenging activity of superoxide anion in the extracts of particular cultivars was also demonstrated in the xanthine/xanthine oxidase system. Using extracts of black chokeberry fruit was more effective than in other fruit species, e.g. mulberry (Bae and Suh, 2007), apples (Maffei et al., 2007) or fruit of

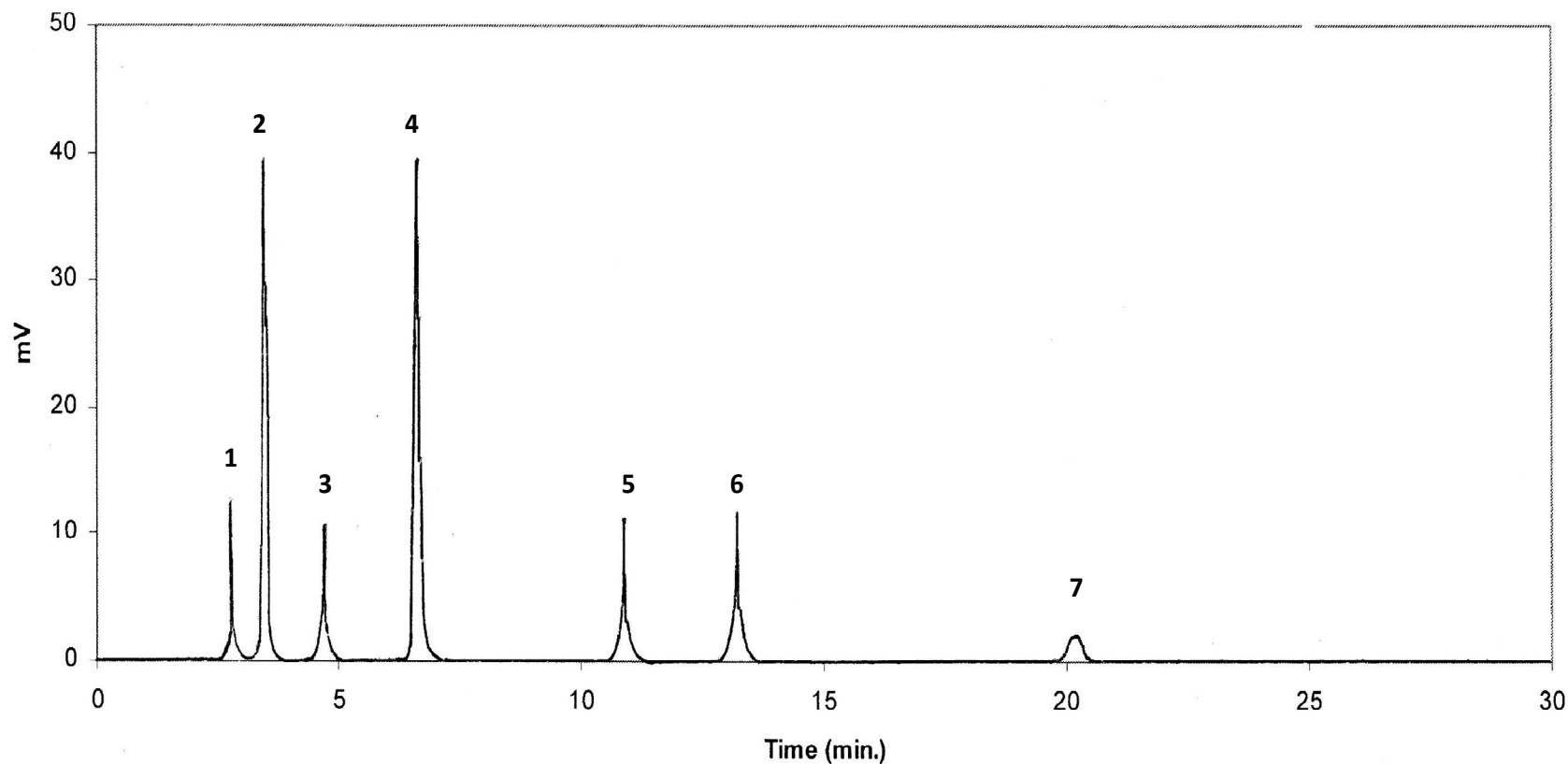


Figure 1. Chromatogram representing the peaks of phenolics – the ‘Viking’ cultivar (the peak 2 = cyanidin-3-arabinoside, the peak 4 = cyanidin-3-galactoside, the peak 5 = chlorogenic acid, the peak 6 = neochlorogenic acid; the peak 7 = (-) epicatechin.

Table 3. Scavenging effect of 25% black chokeberry fruit methanol extracts (percentage of inhibition).

Cultivar	Hydroxyl radical	Nitric oxide	Superoxide anion	Lipid peroxidation
Aron	25.01 ± 0.89 ^a	28.42 ± 0.25 ^a	22.22 ± 0.29 ^a	12.57 ± 0.30 ^a
Fertödi	22.08 ± 0.58 ^b	27.59 ± 0.29 ^b	21.24 ± 0.37 ^b	12.05 ± 0.24 ^a
Hugin	31.12 ± 0.68 ^c	33.10 ± 0.31 ^c	30.48 ± 0.40 ^c	16.19 ± 0.21 ^b
Nero	33.51 ± 0.67 ^d	37.30 ± 0.28 ^d	35.96 ± 0.36 ^d	19.22 ± 0.20 ^c
Viking	34.15 ± 0.51 ^d	41.46 ± 0.31 ^e	36.92 ± 0.38 ^e	19.81 ± 0.26 ^c

Different superscripts in each column indicate the significant differences in the mean at $p < 0.05$.

Table 2. The content of phenolics (mg/kg FM).

Cultivar	cyanidin-3 arabinoside	cyanidin-3-galactoside	(-)-epicatechin	chlorogenic acid	neochlorogenic acid
Aron	941.82 ± 18.25 ^a	1010.80 ± 34.25 ^a	467.35 ± 4.56 ^a	1254.20 ± 51.18 ^a	883.31 ± 26.11 ^a
Fertödi	972.90 ± 24.56 ^a	1074.41 ± 31.50 ^a	560.22 ± 9.74 ^b	1131.15 ± 24.36 ^b	845.04 ± 19.52 ^a
Hugin	1015.04 ± 29.01 ^b	1071.91 ± 39.83 ^a	647.08 ± 6.09 ^c	1845.19 ± 21.12 ^c	965.81 ± 27.41 ^b
Nero	1420.17 ± 24.12 ^c	1181.15 ± 28.20 ^b	862.50 ± 8.70 ^d	1931.45 ± 40.01 ^d	1057.00 ± 25.30 ^c
Viking	1553.29 ± 35.44 ^d	1203.56 ± 31.31 ^b	844.44 ± 10.10 ^d	1960.72 ± 37.11 ^d	1156.59 ± 28.02 ^d

Different superscripts in each column indicate the significant differences in the mean at $p < 0.05$.

Prunus species (Jung et al., 2002). Lipid peroxidation is often caused by ROS as an oxidative alteration of polyunsaturated fatty acids (Wang et al., 2009). In a biological system, lipid peroxidation generates a number of degradation products and it is found to be an important cause of cell membrane destruction and cell damage (Wang et al., 2008). The highest values of lipid peroxidation activities were observed in the cultivars 'Nero' and 'Viking', which confirms biological efficiency of these cultivars in comparison with other cultivars evaluated in this study.

Conclusion

In our measurement, very high contents of polyphenolic substances and total antioxidant capacity were observed in the black chokeberry fruit. Furthermore, there was a high correlation between polyphenolic substances and antioxidant capacity. In addition, high efficiency became evident in reactive oxygen species and lipid peroxidation scavenging activities. From this point of view, the fruit of black chokeberry have unique properties in comparison with other fruit species. They can become a valuable source of nutritionally important substances in human nutrition. Moreover, they can have a significant influence on strengthening human immunity and the prevention

of many diseases.

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