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Phenolic acids, flavonols, anthocyanins and antiradical activity of "Nero", "Viking", "Galicianka" and wild chokeberries

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ABSTRACT

Chokeberries are a subject of numerous studies due to a high phenolic compound content, antioxidant properties and potential positive influence on the health. In this work, phenolic compounds and the antiradical activity of three chokeberry cultivars ("Nero", "Viking", "Galicianka") and wild chokeberries were compared during two consecutive year. The phenolic compound profile determined by high performance liquid chromatography (HPLC) was the same in all chokeberries. But some differences were found in the content of phenolic compounds. The total phenol and total anthocyanin content was higher in cultivars "Viking" and "Nero". Cultivar "Galicianka" had the lowest phenolic compound content. The flavonol content was similar in all chokeberries. Phenolic acid content was high in wild chokeberries and in cultivar "Viking". The antiradical activity was the strongest in wild chokeberries and in cultivar "Viking". Wild chokeberries differed from other investigated chokeberries by somewhat higher amount of phenolic acids which might affect their antiradical activity.

1. Introduction

Polyphenols or plant phenols are compounds that have shown potential positive influence on the health through numerous studies. They showed anti-inflammatory (Larrosa et al., 2010), anti-tumor activity (Bermúdez-Soto et al., 2007b), and might have a significant role in eye health (Kalt et al., 2010). Because of these effects, they are still intensively studied.

Large amounts of polyphenols can be found in fruits. Chokeberry fruits are a type of fruit that have one of the greatest quantities of polyphenol, higher that in blueberries, raspberries, blackberries, *etc.* (Jakobek et al., 2007b). The most numerous are anthocyanins, proanthocyanidins, flavonols, phenolic acids (Kulling and Rawel, 2008; Slimestad et al., 2005). Phenolic compounds from chokeberries were reported to have antimutagenic activity (Gąsiorowski et al., 1997), blood pressure lowering properties (Hellström et al., 2010), they can inhibit the proliferation of some cancer cells (Bermúdez-Soto et al., 2007a). Chokeberries also showed gastroprotective effect (Matsumoto et al., 2004). In the bioavailability studies, after the consumption of chokeberry juice, chokeberry anthocyanins or their metabolites were identified in the human plasma and urine although in a relatively low amount (Wiczkowski et al., 2010). Chokeberry phenolics and phenolics in general are intensively studied to clarify their bioavailability, metabolism and real clinical effectiveness that they or their metabolites might have in the human body (Chrubasik et al., 2010; Larrosa et al., 2006; Lattanzio et al., 2008).

In the fruits of various cultivars, differences in the polyphenol amount may occur, like in different apple cultivars (Khanizadeh et al., 2008), almond cultivars (Bolling et al., 2010) or apricot cultivars (Dragovic-Uzelac et al., 2007). Seasonal differences and geographic origin can also affect the amount of polyphenols in some fruits. The same kind of fruit grown in different regions may show differences in the amount of polyphenols due to different amounts of rainfall, number of sunny days, *etc.* But according to some studies, the influence of cultivars on the polyphenols is probably more significant than the influence of seasonal differences (Bolling et al., 2010). The differences in the polyphenol content in the various cultivars can mean that there can be some differences in the quality of fruits.

Since chokeberries are rich in phenolic compounds which might have potential positive effects, and the phenolic composition may differ in different cultivars, the aim of this study was to study differences in the quantity and distribution of polyphenols in different chokeberry cultivars. Anthocyanins, phenolic acids, and flavonols were investigated in three different cultivars and wild chokeberries. Polyphenol composition was determined by using HPLC with PDA detection. In addition, total phenol content was determined

Abbreviations: HPLC, high performance liquid chromatography; PDA, photo diode array detector; DPPH, 2,2-diphenyl-1-picrylhydrazyl radical; ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt.

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by Folin-Ciocalteu method. The antiradical activity of chokeberries was determined by using two methods – DPPH and ABTS methods.

2. Materials and methods

2.1. Chemicals

Chemicals used in this study were purchased from several firms: the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•) (D9132), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt (ABTS) (A1888), gallic acid monohydrate (398225), chlorogenic acid (3-o-caffeoylquinic acid) (C3878), quercetin dihydrate (Q0125), rutin hydrate (quercetin-3-rutinoside hydrate) (R5143) were purchased from Sigma–Aldrich (St. Louis, MO, USA); cyanidin-3-*O*-glucoside chloride (kuromanin chloride) (0915 S) from Extrasynthese (Genay, France); ammonium peroxodisulfate (09915) from Fluka (Buchs, Switzerland), methanol from Merck (Darmstadt, Germany); hydrochloric acid (36.2%), sodium carbonate and Folin-Ciocalteu reagent from Kemika (Zagreb, Croatia).

2.2. Chokeberry samples

Three chokeberry (*Aronia melanocarpa*) cultivars ("Viking", "Nero", "Galicianka") and wild chokeberries were harvested in Croatia, region Slavonia during two consecutive years (2010 and 2011). Immediately after harvesting, fruits were frozen at −20 °C.

2.3. Polyphenol extraction

Frozen berries were homogenized, weighed (∼15 g), and extracted (45 ml) in the ethanolic solution acidified with 1% hydrochloric acid. Extracts were shaken (30 min), left to stand 24 h and evaporated (35 °C) using a rotary evaporator. Two extracts were made for each chokeberry sample.

2.4. Total polyphenol determination

Total polyphenols were determined by Folin-Ciocalteu micro method (Waterhouse). A 0.1 ml of chokeberry extract was diluted with 4.9 ml of distilled H_2O (dilution factor 50). An aliquot of diluted chokeberry extract (20 μl) was mixed with 1580 μl of distilled water and 100 μl of Folin-Ciocalteu reagent. 300 μl of sodium carbonate solution (200 g/l) was added to the mixture and shaken. After the incubation at 40 °C for 30 min in the water bath, the absorbance was read against the blank at 765 nm. Total polyphenols were expressed as mg of gallic acid equivalents (GAE) per kg of fruit's fresh weight (FW). Data are mean ± standard deviation of two parallels each measured two times.

2.5. HPLC determination of phenolic compounds

Phenolic compounds were identified and quantified using Varian HPLC system (USA) consisting of ProStar 230 solvent delivery module, ProStar 330 PDA detector (photodiode array) and OmniSpher C18 column (250 mm × 4.6 mm inner diameter, 5 μm, Varian, USA) (guard column ChromSep 1 cm × 3 mm, Varian, USA). The flavonols and phenolic acids were separated using 0.1% water solution of phosphoric acid as a solvent A and 100% HPLC grade methanol as a solvent B (elution conditions: 0–30 min from 5 to 80% B; 30–33 min 80% B; 33–35 min from 80 to 5% B; flow rate = 0.8 ml min^{-1}; injection volumes 20 μl). For the anthocyanin separation 0.5% water solution of phosphoric acid was used as a solvent A and 100% HPLC grade methanol as a solvent B (elution conditions: 0–38 min from 3 to 65% B; from 38–45 min, 65% B; flow rate = 1 ml min^{-1}, injection volumes were 20 μl) (Jakobek et al., 2007a, 2007b). Prior to injection into the HPLC system, extracts were diluted with methanol 1:6, 1:5 or 1:4 and filtrated. Identification of flavonols and phenolic acids was conducted by comparing retention times and spectra with those of standard compounds. Quantification was made by using calibration curves of standards. Anthocyanin identification was done by comparing UV/vis spectra and retention time with those of standards and by using literature data (Slimestad et al., 2005; Määttä-Riihinen et al., 2004). Anthocyanins were quantified by using cyanidin-3-glucoside calibration curve.

2.6. Antiradical activity

The antiradical activity was measured with a UV-Vis spectrophotometer (UV 2005, Barcelona, Spain) by using the DPPH (Brand Williams et al., 1995) and ABTS test (Ivekovic et al., 2005).

DPPH assay: Reaction solutions contained increasing aliquots of chokeberry extract, 200 μl of methanolic DPPH• solution (1 mmol dm^{-3}) and methanol to the final volume of 3 ml. The absorbance was read at 517 nm against the blank solution every 5 or 10 min until the steady state. The extract concentration expressed as g fruit/g DPPH was plotted against % inhibition of DPPH radicals, in the steady state. From that curve, the EC_{50} (efficient concentration) value was calculated which represents the amount of phenols (expressed as g fruit/g DPPH) necessary to reduce the initial DPPH• concentration by 50%. Lower EC_{50} represents higher antiradical activity.

ABTS assay: $ABTS^{\bullet+}$ radical cation was chemically generated with the ammonium peroxodisulfate solution by mixing 0.2 ml of ammonium peroxodisulfate (65 mmol dm^{-3}) with 50 ml of the ABTS solution (1 mmol dm^{-3}, prepared in 0.1 mol dm^{-3} phosphate buffer pH 7.4) and the mixture was left to stand overnight. A 0.5 ml of the $ABTS^{\bullet+}$ solution was mixed with 2 ml of phosphate buffer (pH 7.4) in the cuvette and the absorbance was read at 734 nm. Subsequently, increasing aliquots of diluted chokeberry extract (dilution 1:70) was added into the cuvette and the absorbance was read at 734 nm every five minute until the steady state, around 30 min. The extract concentration expressed as g fruit/g DPPH was plotted against % inhibition of ABTS radicals, in the steady state. From that curve, the EC_{50} (efficient concentration) value was calculated. It represents the amount of phenols (expressed as g fruit/g ABTS) necessary to reduce the initial ABTS• concentration by 50%. Lower EC_{50} represents higher antiradical activity.

2.7. Statistical analysis

MS Excel (Microsoft Corporation, USA) was used for the data analyzing and the results are presented as mean values ± standard deviation. One way analysis of variance (ANOVA) was used to study the differences between chokeberries, and if justified by statistical probability ($p < 0.05$) the means were compared using Turkey test ($p < 0.05$) (OriginLab Corporation, Northampton, MA, USA). The correlation between polyphenol content and the antiradical activity was expressed by r (correlation coefficient) (OriginLab Corporation, Northampton, MA, USA).

3. Results

The results for the total phenolic compound amount determined by Folin-Ciocalteu methods are shown in Table 1. Cultivar "Viking", wild chokeberries and cultivar "Nero" had similar total phenol content (9012–10,804 mg kg^{-1} in the first year, 9361–12,055 mg kg^{-1} FW in the second year). Only cultivar "Galicianka" had somewhat lower total phenol content (8564 mg kg^{-1} FW first year, 8600 mg kg^{-1} FW second year). Statistically significant difference