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Kinetika inhibicije DPPH radikala i antiradikalna aktivnost polifenola iz plodova aronije i bazge

DPPH radical inhibition kinetic and antiradical activity of polyphenols from chokeberry and elderberry fruits

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SAŽETAK

Plodovi aronije i bazge bogat su izvor polifenola, spojeva sa značajnim antiradikalnim osobinama i osobinama stvaranja helata. Istražuju se intenzivno zbog pozitivnog utjecaja na ljudsko zdravlje. U ovom istraživanju izolirane su tri frakcije iz aronije i bazge obogaćene različitim skupinama polifenola da bi se istražila njihova antiradikalna aktivnost pomoću DPPH testa. Prva frakcija sadržavala je flavonole i fenolne kiseline, druga antocijanine, a treća proantocijanidine. Količina polifenola određena je reverzno-faznom tekućinskom kromatografijom visoke djelotvornosti. Pronađeno je da su antocijanini glavne polifenolne komponente ovog voća s najvećim udjelom u ukupnoj antiradikalnoj aktivnosti, a slijede ih proantocijanidini te fenolne kiseline i flavonoli. Pokazalo se također da su proantocijanidini efektivniji u hvatanju slobodnih DPPH$^{\bullet}$ radikala od flavonola, fenolnih kiselina i antocijanidina. Opažena je bifazna reakcija u reakciji između DPPH$^{\bullet}$ radikala i polifenola sa „brzim" i „sporim" razdobljem hvatanja. Sve skupine polifenola pokazale su snažnu antiradikalnu aktivnost za vrijeme prvog brzog perioda, dok je antiradikalna aktivnost opadala za vrijeme sporog razdoblja. Ovakva bifazna reakcija mogla bi biti važna u biološkom djelovanju aronije i bazge. Ovo istraživanje predstavlja doprinos boljem razumijevanju antiradikalne aktivnosti polifenola iz plodova aronije i bazge.

Ključne riječi: aronija, bazga, antiradikalna aktivnost, antocijanini, proantocijanidini, flavonoli, fenolne kiseline

ABSTRACT

Chokeberry and elderberry fruits are rich sources of polyphenols, the compounds with significant antiradical and chelating properties. They are studied intensively because of beneficial effects on human health. In this study, three fractions enriched with different classes of polyphenols were isolated from chokeberry and elderberry to study their antiradical activity by DPPH test. The first fraction contained flavonols and phenolic acids, the second anthocyanins, and third proanthocyanidins. The polyphenol

content was determined by reversed-phase high performance liquid chromatography. Anthocyanins were found to be the main polyphenolic components of these berries with the highest portion in total antiradical activity, followed by proanthocyanidins and phenolic acids and flavonols. Furthermore, proanthocyanidins were found to be more effective in free DPPH$^{\bullet}$ radical scavenging than flavonols, phenolic acids and anthocyanins. A biphasic reaction was observed in reaction between DPPH$^{\bullet}$ radicals and polyphenols, at "fast" and "slow" scavenging rate. All classes of polyphenols showed strong antiradical activity in the first fast period, while in the slow period antiradical activity of polyphenols decreased. This kind of biphasic reaction could be important in biological activity of chokeberry and elderberry. This study is a contribution to a better understanding of antiradical activity of polyphenols from chokeberry and elderberry.

Keywords: chokeberry, elderberry, antiradical activity, anthocyanins, proanthocyanidins, flavonols, phenolic acids

INTRODUCTION

Chokeberry and elderberry are dark coloured berries that belong to the *Rosaceae* and *Caprifoliaceae* family. Because of their intensive colour, extracts of these berries can be used as natural colorants in food industry. This application is important due to possible toxicity of some synthetic colorants. Extracts of these berries can also be used in production of dietary supplements rich in polyphenolic antioxidants (Netzel et al, 2005). Namely, chokeberries and elderberries are very rich sources of polyphenols. Chokeberries contain 6900 to 20100 mg kg^{-1} of total polyphenols, and in elderberries 19500 mg kg^{-1} of total polyphenols were found (Benvenuti et al, 2004; Zheng et al, 2003; Wu et al., 2004). Anthocyanins account for a major fraction of the total polyphenols in chokeberries (3166 to 14800 mg kg^{-1}) (Zheng et al, 2003; Wu et al., 2006) followed by proanthocyanidins (6637 mg kg^{-1}) (Wu et al., 2004), flavonols (99 to 575 mg kg^{-1}) (Jakobek et al, 2007a; Zheng et al, 2003) and phenolic acids (42 to 2617 mg kg^{-1}) (Jakobek et al, 2007a; Zheng et al, 2003). In elderberries, the dominant polyphenols are also anthocyanins (13744 mg kg^{-1}) (Wu et al, 2004). Besides anthocyanins elderberries contain proanthocyanidins (233 mg kg^{-1}) (Wu et al, 2004), flavonols (146 mg kg^{-1}) (Jakobek et al, 2007a) and phenolic acids (27 mg kg^{-1}) (Jakobek et al, 2007a).

Polyphenolic compounds represent a large group of secondary plant metabolites. Earlier studies have showed that polyphenols could be absorbed in human organism (Mülleder et al, 2002; Murkovic et al., 2001; Bitsch et al,

2004; Hollman et al, 1995; Suomela et al., 2006). Anthocyanins are absorbed in glycosidic forms after the intake of polyphenol-rich food and excreted from body after 4 h (Mülleder et al, 2002; Murkovic et al., 2001; Bitsch et al, 2004). Flavonols are absorbed as well, and absorption is enhanced if flavonol molecules are conjugated with glucose (Hollman et al, 1995; Suomela et al., 2006). Although the data on proanthocyanidin absorption are still largely unavailable, certain reports have indicated that at least monomers and smaller oligomeric procyanidins are absorbed (Nandakumar et al., 2008). After absorption, these antioxidative fitonutrients can exhibit a wide range of biological effects, including antioxidant and anticarcinogenic effects (Bermúdez-Soto et al., 2007a). Most of these beneficial health effects are attributed to their antiradical and chelating abilities (Heim et al. 2002).

Chokeberry and elderberry polyphenols exhibit stronger antiradical activity than polyphenols from some other berries like strawberries or raspberries (Jakobek et al., 2007a). Furthermore, earlier studies showed positive effects of chokeberry and elderberry polyphenols on human health. It was found that chokeberry flavonols can be used clinically for secondary prevention of ishaemic heart disease (Naruszewicz et al, 2007). Elderberry anthocyanins showed positive effects on vascular disease prevention (Youdim et al, 2000). Chokeberry juice inhibited the proliferation of colon carcinoma cells (Bermúdez-Soto et al, 2007a; Bermúdez-Soto et al, 2007b). Proanthocyanidins could serve as potential anti-carcinogenic agents (Nandakumar et al., 2008). Because of these positive effects on human health, chokeberry and elderberry are still under intensive investigation.

The aim of this study was to investigate antiradical activity of polyphenols from chokeberries and elderberries and kinetic of inhibition of free radicals. Three polyphenolic fractions enriched with various polyphenol classes were extracted from chokeberry and elderberry. The first fraction contained flavonols and phenolic acids, the second anthocyanins, and the third proanthocyanidins. Antiradical activity of polyphenol fractions as well as kinetic of inhibition of free radicals were studied by using DPPH test. Total amount of polyphenols in fractions was determined by using high-performance liquid chromatography (HPLC).

MATERIALS AND METHODS

Fruit samples

Chokeberries (*Aronia melanocarpa*) and elderberries (*Sambucus nigra*) were harvested at maturity in Slavonia (Croatia) in the year 2007. Immediately after harvesting, fruits were frozen and stored at –20º C until analysis.

Chemicals

Standards: gallic acid hydrate (398225), 4-hydroxybenzoic acid (H5376), (-)-epicatechin (E1753), caffeic acid (C0625), ferulic acid (F3500), *p*-coumaric acid (C9008), quercetin dihydrate (Q0125) and kaempferol (K0133)) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Standards were prepared in methanol in concentration range of 1-100 mg l^{-1}. Anthocyanin standard cyanidin-3-*O*-glucoside chloride (kuromanin chloride 0915 S) was purchased from Extrasynthese (Genay, France) and prepared in 0.1 % methanolic HCl in concentration range 1-100 mg l^{-1}. 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\cdot}$) (D9132) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Extraction of polyphenolic compounds from berries

Extraction of polyphenols was carried out by procedure already described in literature (Määttä-Riihinen et al. 2004a; Määttä-Riihinen et al. 2004b). The frozen fruits were homogenized, and the samples were weighed (3 g) into centrifuge tubes. The extractions were performed by repeated vortexing of samples with ethyl acetate (4 X 5 ml). Combined ethyl acetate extracts contained free and conjugated phenolic acids and flavonol glycosides. One portion of ethyl acetate extract (10 ml) was evaporated to dryness (35ºC) using a rotary evaporator and dissolved in 2 ml of methanol (fraction 1). The portion was subjected to direct antiradical activity determination by DPPH assay. Afterwards extract 1 was submitted to hydrolysis procedure in order to free aglycons from glycosides of polyphenolic compounds. The portion of fraction 1 was acidified to 0.6 M with concentrated HCl and heated for 5 min in a boiling water bath (70-80 °C). This hydrolysed fraction was used for analysis of flavonols and phenolic acids in the form of aglycons by HPLC.

The berry residue remained after ethyl acetate extraction was acidified with HCl (2M, 1 ml) and extracted with 5 ml of methanol (4 to 8 times). Combined extracts contained anthocyanins in the form of flavylium cations. An aliquot of the methanol extract (10 ml) was evaporated to dryness, dissolved in 2 ml of methanol (fraction 2) and used for analysis of antiradical activity of anthocyanins by DPPH assay. The anthocyanins content was determined by HPLC analysis.

The berry residue that remained after anthocyanin extraction was suspended in 5 ml of methanol, acidified to 0.6 M with concentrated HCl, and refluxed for 2 h (60 to 70 ºC) (fraction 3). This extract contained proanthocyanidins. Antiradical activity was evaluated by DPPH test, and the amount of total proanthocyanidins by HPLC.

All extracts were prepared in duplicate and filtered through a 0.45 μm syringe filter before determination of polyphenol content.

Determination of polyphenols by HPLC methods

Quantification of polyphenolic compounds in extracts was performed by HPLC methods validated earlier in our laboratory (Jakobek et al., 2007a; Jakobek et al., 2007b). The chromatographic analyses were performed on a Varian HPLC system (USA) consisting of ProStar 230 solvent delivery module, and ProStar 330 PDA detector. Separation of phenols was done in an OmniSpher C18 column (250 mm x 4.6 mm inner diameter, 5 μm, Varian, USA) protected with guard column (ChromSep 1 cm x 3 mm, Varian, USA). Flavonols and phenolic acids in hydrolised fraction 1 and proanthocyanidins in fraction 3 were separated using 0.1 % phosphoric acid as solvent A and 100 % HPLC grade methanol as solvent B. The elution conditions were as follows: 0-30 min from 5 % B to 80 % B; 30-33 min 80 % B; 33-35 min from 80 % B to 5 % B; with flow rate=0.8 ml min^{-1}. UV-Vis spectra were recorded in wavelength range from 190-600 nm. Detection wavelength was 280 nm. The identification of flavonols and phenolic acids 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 berry extracts with phenolic standards. Identified flavonols and phenolic acids were quantified using calibration curves of authentic standards and expressed in mg kg^{-1} of fresh weight of berries. Proanthocyanidins were converted to anthocyanidins (cyanidin and delphinidin) by hydrolysis procedure. Total area at 280 nm was used for quantification of total proanthocyanidins by using calibration curve of (-)-epicatechin.

For separation of anthocyanins from fraction 2, 0.5 % phosphoric acid was used as solvent A and 100 % HPLC grade methanol as solvent B. The elution conditions were as follows: 0-38 min from 3 % B to 65 % B; from 38-45 min, 65 % B; with flow rate=1 ml min^{-1}. Anthocyanins were detected at 520 nm, tentatively identified by using available literature data (Jakobek et al., 2007a) and quantified by using calibration curve of cyanidin-3-glucoside. The results were expressed as total anthocyanins in mg cyanidin-3-glucoside equivalent / kg of fresh weight of berries.

Antiradical activity

The antiradical activity of fractions enriched with various classes of polyphenols was measured spectrophotometrically with a UV-Vis spectrophotometer (UV 2005, Barcelona, Spain) by using DPPH assay (Brand-Williams et al., 1995). DPPH solution was prepared by diluting 10 to 400 μl DPPH (1mmol dm^{-3}) in methanol to final volume of 3 ml and absorbance of these solutions was measured at 517 nm. DPPH calibration curve was constructed by plotting concentration of DPPH radical *vs* absorbance.

Afterwards, three dilutions of fractions 1, 2 and 3 were prepared. Each dilution contained increasing aliquots of fraction, 200 μl of methanolic DPPH$^{\bullet}$ solution (1mmol dm^{-3}) and methanol to final volume of 3 ml. The absorbance was read against the blank solution (prepared using 200 μl of methanol instead of DPPH$^{\bullet}$ solution) each minute in the period of 5 minutes, and then every 5-th minute, in the total period of 20 minutes. The amount of DPPH$^{\bullet}$ radicals in each moment of reaction was calculated according to DPPH calibration curve and the percentage of inhibition of DPPH$^{\bullet}$ radicals according to:

$$\%inhibition = \frac{\gamma_0(DPPH) - \gamma_t(DPPH)}{\gamma_0(DPPH)/100}$$

$\gamma_0(DPPH)$ = mass concentration of DPPH radicals (g/ml) in t=o

$\gamma_t(DPPH)$ = mass concentration of DPPH radicals (g/ml) in the

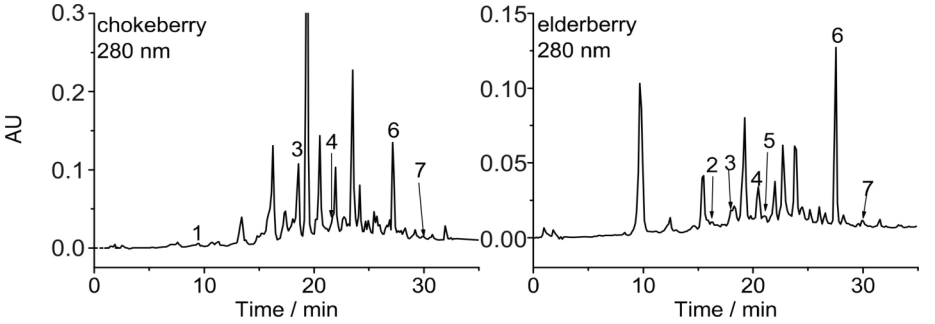
percentage of inhibition of DPPH$^{\bullet}$ radicals was plotted against the amount of berry after 20 minutes (g of berry / g of DPPH). This curve was used to calculate EC_{50} value. This is the value which represents the amount of berries needed to inhibit 50% of DPPH$^{\bullet}$ radicals in 20 minutes. Lower EC_{50} value represents higher antiradical activity. Due to easier interpretation of results, antiradical power (ARP) was calculated as well.

$ARP = 1/EC_{50}$

Lower ARP value represents lower antiradical activity.

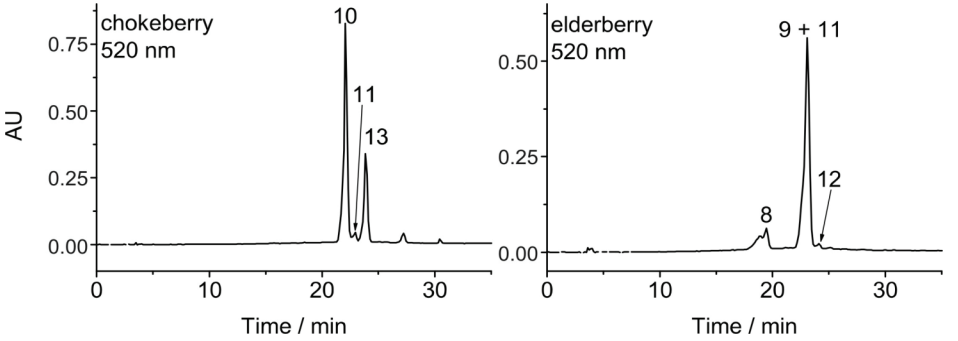
RESULTS AND DISCUSSION

Figure 1 and 2 show HPLC chromatograms of chokeberry and elderberry fractions enriched with polyphenols with identified polyphenolic compounds. The polyphenols content is shown in Table 1. Phenolic acids and flavonols were detected in chokeberry and elderberry fractions extracted with ethyl acetate (fraction 1): derivatives of gallic acid, caffeic acid, *p*-coumaric acid, *p*-hydroxybenzoic acid, quercetin and kaempferol. In chokeberry, the phenolic acids content was significant (340.1 mg/kg). Caffeic acid derivatives were the dominant phenolic acids because they were found in considerable amount (301 mg/kg). The dominant flavonols in chokeberries were quercetin derivatives (394 mg/kg). Elderberries contained lower amount of phenolic acids (11.8 mg/kg) than chokeberries, and the dominant flavonols were quercetin derivatives (148 mg/kg). Anthocyanins were detected in fractions extracted with acidified methanol (fraction 2). The anthocyanins content in both, chokeberry and



Slika 1. HPLC kromatogrami hidroliziranih polifenolnih frakcija ekstrahiranih s etil acetatom iz aronije i bazge snimljenih na 280 nm. Identifikacija pikova: 1=galna kiselina, 2=*p*-hidroksibenzojeva kiselina, 3=kafeinska kiselina, 4=*p*-kumarinska kiselina, 5=ferulična kiselina, 6=kvercetin, 7=kemferol

Figure 1. HPLC chromatograms of hydrolised polyphenolic fractions extracted with ethyl acetate from chokeberry and elderberry recorded at 280 nm. Peak identification: 1=gallic acid, 2=*p*-hydroxybenzoic acid, 3=caffeic acid, 4=*p*-coumaric acid, 5=ferulic acid, 6=quercetin, 7=kaempferol.



Slika 2. HPLC kromatogrami polifenolnih frakcija ekstrahiranih sa zakiseljenim metanolom iz aronije i bazge snimljenih na 520 nm. Djelomična identifikacija pikova: 8=cijanidin-3-sambubiozid-5-glukozid, 9=cijanidin-3-sambubiozid, 10=cijanidin-3-galaktozid, 11=cijanidin-3-glukozid, 12= cijanidin-3-rutinozid, 13=cijanidin-3-arabinozid

Figure 2. HPLC chromatograms of polyphenolic fractions extracted with acidified methanol from chokeberry and elderberry recorded at 520 nm. Tentative peak identification: 8=cyanidin-3-sambubioside-5-glucoside, 9=cyanidin-3-sambubioside, 10=cyanidin-3-galactoside, 11=cyanidin-3-glucoside, 12=cyanidin-3-rutinoside, 13=cyanidin-3-arabinoside.

Tablica 1. Sadržaj polifenolnih spojeva u polifenolnim frakcijama iz aronije i bazge izražen u mg/kg svježe težine ploda

Table 1. Polyphenolic compounds content in polyphenolic fractions from chokeberry and elderberry fruits expressed in mg/kg of fresh weight of fruits

Flavonols and phenolic acids	Chokeberry	%	Elderberry	%
Gallic acid	24.9±0.5	0.18		
p-hydroxybenzoic acid			3.8±0.1	0.04
Caffeic acid	301.4±1.5	2.17	3.2±0.2	0.03
p-coumaric acid	13.8±0.5	0.10	3.7±0.3	0.04
Ferulic acid			1.1±0.1	0.01
Quercetin	393.7±2.2	2.83	148.1±0.5	1.52
Kaempferol	4.0±0.3	0.03	1.6±0.1	0.02
Anthocyanins				
Total anthocyanins	12527.8±10.2	90.14	9539.8±8.9	98.10
Proanthocyanins				
Total proanthocyanins	633.3±5.2	4.56	22.9±1.3	0.23

elderberry fractions, was high (12528 and 9540 mg/kg respectively). Furthermore, anthocyanins contributed a high portion to total polyphenol content of chokeberry and elderberry (90 and 98 %, respectively). Proanthocyanidins were detected in the third fraction from chokeberry and elderberry and their content was significant (633 and 23 mg/kg, respectively). The amount of polyphenols found in elderberry and chokeberry is in accordance with literature data (Jakobek et al., 2007a; Jakobek et al., 2007b; Wu et al., 2004; Zheng et al., 2003.; Wu et al., 2006.; Määttä-Riihinen et al. 2004b).

Antiradical activity of polyphenolic fractions was determined by DPPH test which is considered as easy and valid assay to evaluate antiradical activity of various antioxidants. Polyphenols and $DPPH^{\bullet}$ radicals were dissolved in methanol in the reaction solution. Free $DPPH^{\bullet}$ radicals which show absorption at 517 nm are reduced to the corresponding hydrazine when they react with hydrogen donors such as polyphenol molecules. The decrease in $DPPH^{\bullet}$ radical concentration is accompanied by the decrease in absorbance at 517 nm. In this study antiradical activity was expressed as ARP value (Table 2). Higher value represents higher antiradical activity. Anthocyanin fractions from chokeberry

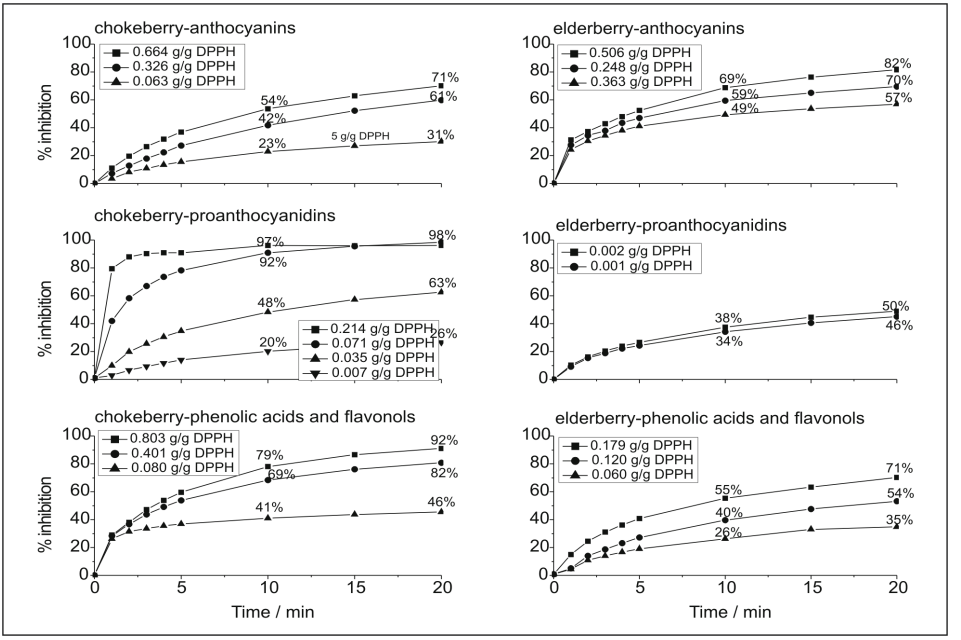
Tablica 2. Antiradikalna aktivnost polifenola iz aronije i bazge

Table 2. Antiradical activity of polyphenols from chokeberry and elderberry

Berry	EC_{50} (g of fruit /g DPPH)	ARP	%
	Anthocyanins		
Chokeberry	16.2	0.0617	66.7
Elderberry	18.3	0.0546	82.6
	Proanthocyanidins		
Chokeberry	43.1	0.0232	25.1
Elderberry	98.6	0.0101	15.3
	Phenolic acids and flavonols		
Chokeberry	131.8	0.0076	8.2
Elderberry	691.1	0.0014	2.1

EC_{50} = g voća potrebni da reduciraju g DPPH$^{\bullet}$ radikala za 50 %.
ARP = antiradical power, izračunat kao $1/EC_{50}$

EC_{50} = g of fruit needed to reduce g of DPPH$^{\bullet}$ radicals by 50 %.
ARP = antiradical power, expressed as $1/EC_{50}$



Slika 3. Kinetičko ponašanje antocijanina, proantocijanidina, flavonola i fenolnih kiselina iz aronije i bazge u reakciji s DPPH$^{\bullet}$ radikalima

Figure 3. Kinetic behaviour of anthocyanins, proanthocyanidins, flavonols and phenolic acids from chokeberry and elderberry in reaction with DPPH$^{\bullet}$ radicals

and elderberry showed the highest antiradical activity (ARP = 0.0617 and 0.0546 respectively) followed by proanthocyanidin fractions (ARP = 0.0232 and 0.0101 respectively) and phenolic acid and flavonol fractions (ARP = 0.0076 and 0.0014 respectively). Furthermore, anthocyanins comprised 66.7 % of total antiradical activity of chokeberry and 82.6 % of total antiradical activity of elderberry. These results indicate that antiradical activity of chokeberry and elderberry was mostly affected by anthocyanins, especially in elderberries. But, proanthocyanidins had a significant portion in antiradical activity as well (25.1 % in chokeberry, 15.3 % in elderberry), especially in chokeberry in which these compounds were found in high amount. In earlier study conducted by Zheng et al., 2003, the portion of anthocyanins in total antiradical activity of chokeberry determined by ORAC assay, was 53 % which is similar to the results in this study. Besides polyphenols, berries contain some other antioxidants like vitamins which may have a certain portion in total antiradical activity of berries as well. It was found that chokeberries contain 131 mg / kg of vitamin C (Benvenuti et al., 2004.) and elderberry 60 to 250 mg/kg (Kaack et al., 1998). Vitamin C has an antiradical activity similar to Trolox, and somewhat lower than polyphenols. It was reported that antioxidant activity of vitamin C was 2.5-fold lower than that of gallic acid and (-)-epicatechin, 2.2-fold than that of (+)-catechin, 5.3-fold that of procyanidin B2 and 2.6-fold that of quercetin (Soobrattee et al., 2005). But vitamin C can still have a smaller portion in total antiradical activity of chokeberry and elderberry fruits.

It is well known that intensity and effectiveness of antiradical activity of polyphenolic compounds differ due to differences in their chemical structure. One of the most effective and the strongest scavengers of free radicals are proanthocyanidins, followed by flavan-3-ols, flavonols, hydroxycinnamic acids and simple phenolic acids (Soobrattee et al., 2005). Anthocyanidins show similar or somewhat lower antiradical activity than hydroxycinnamic acids or flavonols in DPPH test (Kähkönen et al., 2003). The differences in effectiveness of antioxidants can be seen from the amount of antioxidants which are needed to inhibit the same quantity of free radicals. More effective antioxidants are needed in a lower amount than less effective antioxidants, to accomplish the same inhibition effect. In order to see which polyphenols from chokeberry and elderberry fruits are the most effective and the strongest scavengers of free radicals, the amount of polyphenols which caused 50 % of inhibition of DPPH radicals was calculated. The results are shown in Table 3. In chokeberry and elderberry, the most effective and strongest scavengers of free radicals were proanthocyanidins because the lowest amounts of proanthocyanidins inhibited 50% of DPPH radicals (0.027 and 0.002 g proanthocyanidins / g DPPH).

Tablica 3. Antiradikalna efikasnost polifenola iz aronije i bazge izražena kao koncentracija polifenola potrebna da inhibira 50 % DPPH radikala

Tablica 3. Antiradical effectiveness of polyphenols from chokeberry and elderberry expressed as polyphenol concentration needed to inhibit 50 % of DPPH radicals

Berry	g of polyphenols/g DPPH
	Anthocyanins
Chokeberry	0.197
Elderberry	0.475
	Proanthocyanidins
Chokeberry	0.027
Elderberry	0.0022
	Phenolic acids and flavonols
Chokeberry	0.097
Elderberry	0.112

Proanthocyanidins were followed by phenolic acids and flavonols (0.097 and 0.112 g phenolic acids and flavonols / g DPPH). Chokeberry and elderberry anthocyanins showed somewhat lower effectiveness in scavenging free radicals (0.197 and 0.475 g anthocyanins / g DPPH). These results are consisted with the results of some previous studies in which proanthocyanidins were found to be stronger scavengers of free radicals than flavonols, anthocyanins or phenolic acids (Soobrattee et al., 2005). Proanthocyanidins are a class of polyphenols that take the form of oligomers or polymers of flavan-3-ol units such as (+)-catechin and (-)-epicatechin (Nandakumar et al., 2008). These compounds exhibit very high antiradical activity due to hydroxyl groups that are potential hydrogen donors. Also, increasing degree of polymerization enhances the effectiveness of proanthocyanidins against a variety of radical species (Heim et al., 2002). This specific polymer structure is probably responsible for stronger antiradical activity of proanthocyanidins in comparison to other polyphenolic compounds tested.

Overall results on antiradical activity of polyphenols from chokeberry and elderberry fruits suggest that anthocyanins are somewhat less effective in scavenging free radicals than proanthocyanidins or flavonols. But anthocyanins were found in these berries in a considerably higher amount than other polyphenol groups and, because of that, have the highest portion in total antiradical activity of chokeberry and elderberry. Antiradical activity of

polyphenols in berries is dependent on both, effectiveness in scavenging free radicals and content the polyphenols. These results are in accordance with some previous studies which found high correlations between the amount of anthocyanins, flavonols, hydroxycinnamic acids and antioxidant activity of fruits (Jakobek et al., 2007b). High correlations suggest that increased polyphenol concentration in fruits increases antioxidant activity of fruits which agrees with the results of this study.

Figure 3 represents the kinetic of inhibition of DPPH radicals by polyphenols from chokeberry and elderberry in total time of 20 minutes. It can be seen that reaction between DPPH$^{\bullet}$ radicals and polyphenols can be divided into two periods, the period of "fast" scavenging rate which lasts for the first 10 minutes, and the period of "slow" scavenging rate which lasts from 10-th to 20-th minute. In investigated concentration range chokeberry anthocyanins inhibited the highest amount of DPPH$^{\bullet}$ radicals during the fast period (23, 42 and 54 %). In the slower period which lasts from 10-th to 20-th minute, chokeberry anthocyanins inhibited a lower amount of DPPH$^{\bullet}$ radicals (8, 19 and 17 %) and the amount of total inhibition was 31, 61 and 71 %. Similar fast and slow scavenging rates were observed in reaction between proanthocyanidins, phenolic acids or flavonols and DPPH$^{\bullet}$ radicals. These polyphenolic groups inhibited the highest amount of DPPH$^{\bullet}$ radicals during the fast period (proanthocyanidins 20, 48, 92 and 97 %; phenolic acids and flavonols 41, 69, 79 %) while inhibition rate decreased during the slower reaction period (proanthocyanidins 6, 15, 7and 2 %; phenolic acids and flavonols 5, 13, 13 %). Elderberry polyphenols followed similar free radicals scavenging kinetic. In investigated concentration range, elderberry anthocyanins inhibited the highest amount of DPPH$^{\bullet}$ radicals (49, 59 and 69 %) during the fast period, while in the slower period the inhibition rate slowed down (8, 11 and 13 %). The total inhibition after 20 minutes was 57, 70 and 82 %. Proanthocyanidins and phenolic acids and flavonols from elderberry inhibited the highest amount of DPPH$^{\bullet}$ radicals during the fast period as well (proanthocyanidins 34 and 38 %, phenolic acids and flavonols 26, 40 and 55 %). The inhibition rate decreased from 10-th to 20-th minute (proanthocyanidins 12 and 12 %, phenolic acids and flavonols 9, 14, 16 %).

Earlier studies on antiradical activity of individual antioxidants found that some antioxidants inhibited DPPH$^{\bullet}$ radicals rapidly and such reaction reached steady state quickly. Contrary to that, some antioxidants inhibit DPPH$^{\bullet}$ radicals during a longer period of time, reaction is slower and such antioxidants retain their antiradical activity longer (Brand-Williams et al., 1995). The results of this study suggest that chokeberry and elderberry polyphenols belong to the polyphenols that retain their antiradical activity during a longer period of time

which might be important in biological activity of chokeberry and elderberry. Namely, Russo 2007 pointed out that low concentrations of antioxidants acting in a long time were probably sufficient to prevent the occurrence of some diseases. Furthermore, biphasic reaction was observed between chokeberry and elderberry polyphenols and DPPH$^{\bullet}$ radicals, at a fast and slow scavenging rate. Similar biphasic reaction was found in earlier investigation of inhibition of DPPH$^{\bullet}$ radicals by some dietary polyphenols (Goupy et al., 2003) which agrees with the results of this study. Biphasic reaction was found in earlier investigation of antiradical activity of individual polyphenols by ABTS assay as well (van den Berg et al., 1999) which is consistent with our results. It is not yet known whether fast and slow radical scavenging mechanisms between polyphenols and free radicals occur *in vivo*. But since free radicals have short half-lives *in vivo*, fast period might be important in human organism (van den Berg et al., 1999).

According to the earlier studies, the most accepted reaction for scavenging free DPPH$^{\bullet}$ radicals is reaction:

Fl-OH + DPPH$^{\bullet}$ $\rightarrow$ Fl-O$^{\bullet}$ + $DPPH_2$ (Seyoum et al., 2006)

Aroxyl radicals follow one or more termination mechanisms

Fl-O$^{\bullet}$ + Fl-O$^{\bullet}$ $\rightarrow$ Fl-O-O-Fl

Fl-O$^{\bullet}$ + DPPH$^{\bullet}$ $\rightarrow$ Fl-O + DPPH

Fl-O$^{\bullet}$ (semiquinone) $\xrightarrow{-H^{\bullet}}$ Fl=O (quinone) (Seyoum et al., 2006)

Termination reactions do not necessarily have to lead to termination of antiradical activity. Oxidation products and their degradation products may further be reactive towards DPPH$^{\bullet}$ radicals. Slower reaction period in reaction between chokeberry and elderberry polyphenols and DPPH$^{\bullet}$ radicals can, therefore, be explained by formation of reaction products which react slower with DPPH$^{\bullet}$ radicals. Furthermore, intramolecular rearrangement in the molecule of antioxidants can cause slower period (Seyoum et al., 2006). Since, the determination of antiradical activity of polyphenol mixtures is far more complicated than determination of antiradical activity of individual polyphenols, it is difficult to explain the mechanism of antiradical activity, but it is possible that similar reactions can affect antiradical activity of chokeberry and elderberry polyphenols which might cause slower scavenging rates.

CONCLUSIONS

Chokeberry and elderberry have extremely high amount of polyphenols which give them high antiradical activity. This feature is important in possible positive effects of chokeberry and elderberry on human health. That is why antiradical activity and mechanism of scavenging of free radicals by chokeberry

and elderberry polyphenols are studied intensively. Anthocyanins were found to be the predominant polyphenols of chokeberry and elderberry. Furthermore, these polyphenols were the most dominant contributors to total antiradical activity of chokeberry and elderberry. They comprised 66.7 % of total antiradical activity of chokeberry and 82.6 % of total antiradical activity of elderberry. The portion of other polyphenols was lower. Proanthocyanidins were found to be the most effective scavengers of free radicals because low amount of proanthocyanidins caused inhibition of $DPPH^{\bullet}$ radicals. Proanthocyanidins were followed by flavonols, phenolic acids and anthocyanins. All chokeberry and elderberry polyphenols showed the strongest antiradical activity in the first fast reaction period, followed by the slower reaction period during which antiradical activity of polyphenols decreased. This biphasic reaction could be important in reactions of chokeberry and elderberry polyphenols in *vivo*. This study gives further insight into antiradical activity of chokeberry and elderberry polyphenols.

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