The influence of interactions among phenolic compounds from chokeberry on the antiradical activity of chokeberry

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The influence of interactions among phenolic compounds from chokeberry on the antiradical activity of chokeberry

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Summary

In this work, interactions between chokeberry (*Aronia melanocarpa*) phenols and their influence on the antiradical activity were studied. Three fractions were extracted from chokeberries. Every fraction was enriched with different classes of phenolic compounds, first with flavonols and hydroxycinnamic acids, the second with anthocyanins and the third with insoluble phenols and proanthocyanidins. Antiradical activities of phenolic fractions and their mixtures were determined by using the DPPH test. Results showed that the reaction between phenols and $DPPH^{\bullet}$ radicals was a biphasic reaction with "fast" and "slow" scavenging rates. Phenolic mixtures showed lower antiradical activity in comparison to the antiradical activity of individual fractions which can be the result of various interactions between phenolic compounds. This suggests that interactions among phenols promoted a negative synergistic effect on the antiradical activity of chokeberries.

Keywords: phenolic compounds, antiradical activity, chokeberry, interactions, synergism

Introduction

Aronia berries (*Aronia melanocarpa*), commonly known as black chokeberries, are a member of Rosaceae family (Kulling and Rawel, 2008). Many studies have demonstrated the potential positive effects of chokeberries or their phenolic components on the human health. These include antioxidant effect (Kulling and Rawel, 2008; Wu et al., 2004), the inhibition of cancer cell proliferation, antimutagenic effects, cardioprotective effects, antidiabetes effects (Kulling and Rawel, 2008, Bermudez-Soto et al., 2007a; Bermudez-Soto et al., 2007b; Jurgonski et al., 2008). The antiradical activity or ability to scavenge free radicals has an important role in beneficial effects of chokeberry phenols on the human health and is an important characteristic of phenolic compounds. Chokeberry phenols showed stronger antiradical activity than other fruits and berries (Wu et al., 2004) which makes these berries interesting for various investigations.
A series of papers investigated the antiradical activity of fruits (Kulling and Rawel, 2008; Wu et al., 2004.; Zheng et al., 2003; Määttä-Riihinen et al., 2005).

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But very little is known about interactions that can occur between phenolic compounds or between other fruit components, and the effects of these interactions on the antiradical activity of fruits. Some studies have shown that interactions among different compounds and among phenols itself can promote changes in the antiradical capacity of fruits (Pinelo et al., 2004). Interactions can lead to synergistic effects, negative synergism and additive effect (Pinelo et al., 2004).
The aim of this work was to study the antiradical activity of phenolic compounds from chokeberries, interactions among them and their influence on the total antiradical activity of chokeberries. Phenols were separated into three fractions. The first fraction was enriched with hydroxycinnamic acids, flavonol glycosides, the second with anthocyanins, and the third with insoluble phenols and high molecular weight (HMW) proanthocyanidins. The antiradical activity of fractions was studied by using the DPPH test. Fractions were then mixed and the antiradical activity of mixtures was evaluated and compared to the trend followed by each phenol fraction.

Materials and methods

Chokeberry samples

Black chokeberries (*Aronia melanocarpa*) were harvested at maturity in Slavonia (Croatia) in 2009. Immediately after harvesting, fruits were frozen and stored at –20 ºC until analysis.

Extraction of phenolic fractions

The extraction was carried out by procedure already described in the literature (Määttä-Riihinen et al. 2004a; Määttä-Riihinen et al. 2004b). The frozen berries were homogenized, weighed (~ 10 g), and extracted with ethyl acetate (4 x 20 ml). The combined ethyl acetate extracts contained free and conjugated hydroxycinnamic acids, flavonol glycosides. An aliquot of ethyl acetate extract (50 ml) was evaporated to dryness (35 ºC) using a rotary evaporator and dissolved in 4 ml of methanol (fraction 1).
The remaining berry residue (after ethyl acetate extraction) was acidified with HCl (2 mol dm^{-3}, 4 ml) and extracted with methanol (4 x 25 ml). The combined extracts contained anthocyanins in the form of flavylium cations. An aliquot of the methanol extract (40 ml) was evaporated to dryness, dissolved in 5 ml of methanol (fraction 2).
The berry residue remaining after anthocyanin extraction was suspended in 20 ml of methanol, acidified to 0.6 mol dm^{-3} with concentrated HCl, and refluxed for 2 h (60 to 70 ºC) (fraction 3). This extract contained insoluble glycosidic forms of hydroxycinnamic acids and flavonols and HMW proanthocyanidins.

Proanthocyanidins were converted to anthocyanidins under the conditions in reflux procedure.
Fractions were directly used in the HPLC analysis and in the antiradical activity determinations.

High performance liquid chromatography

Main phenolic compounds in fractions were identified by using the high performance liquid chromatography (HPLC) with photo diode array detection (PDA). The instrument was Varian HPLC system (USA) consisting of ProStar 230 solvent delivery module, ProStar 330 PDA detector and OmniSpher C18 column (250 x 4.6 mm inner diameter, 5 μm, Varian, USA).
The flavonols and phenolic acids were separated using 0.1 % phosphoric acid as solvent A and 100 % HPLC grade methanol as solvent B (elution conditions: 0-30 min from 5 % B to 80 % B; 30-33 min 80 % B; 33-35 min from 80 % B to 5 % B; flow rate=0.8 ml min^{-1}; injection volumes 20 μl). 0.5 % phosphoric acid was used as solvent A and 100 % HPLC grade methanol as solvent B for separation of anthocyanins (elution conditions: 0-38 min from 3 % B to 65 % B; from 38-45 min, 65 % B; flow rate=1 ml min^{-1}, injection volumes were 20 μl) (Jakobek et al., 2007a, Jakobek et al., 2007b). UV-Vis spectra were recorded in a wavelength range from 190-600 nm (the detection wavelength was; 320 nm for chlorogenic, neochlorogenic acid; 360 nm for quercetin-3-rutinsoide; 520 nm for anthocyanins). The total peak area at 320 nm was used for the quantification of total phenolic compounds in fraction 1 by using the chlorogenic acid calibration curve. The total peak area at 520 nm was used for the quantification of total phenolic compounds in fraction 2 and 3 by using the cyanidin-3-glucoside calibration curve.

Antiradical activity

The antiradical activity was measured spectrophotometrically with a UV-Vis spectrophotometer (UV 2005, Barcelona, Spain) by using the DPPH test (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 was measured at 517 nm. The DPPH calibration curve was constructed by plotting the $DPPH^{\bullet}$ radical amount (μmol) *vs* absorbance (Eq. 1):

$$y = 0.2594x + 0.0042 \quad (1)$$

where:

y = amount of $DPPH^{\bullet}$ radicals (μmol)
x = absorbance

Three dilutions of each phenol fractions (1, 2 and 3) were prepared. Each dilution contained an aliquot of individual 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) in period of 300 minutes. The amount of $DPPH^{\bullet}$ radicals were calculated in each moment of reaction according to the DPPH calibration curve and % of remaining $DPPH^{\bullet}$ radicals according to Eq. 2:

$$\% \text{ of remaining DPPH} = \frac{n(DPPH)_t}{n(DPPH)_0 / 100} \qquad (2)$$

where:

$n(DPPH)_t$ = amount of $DPPH^{\bullet}$ radicals (µmol) in time t

$n(DPPH)_0$ = amount of $DPPH^{\bullet}$ radicals (µmol) in t=0

% inhibition of $DPPH^{\bullet}$ radicals were calculated according to Eq. 3:

$$\% \text{ inhibition} = 100 - \% \text{ of remaining DPPH} \qquad (3)$$

Afterwards, the same volumes of phenolic fractions were mixed at various combinations. The antiradical activity of mixtures was determined by the same procedure as in individual phenol fractions. The antiradical activity of individual fractions was summed up and compared to the antiradical activity of mixtures.

Results and discussion

Phenolic fractions were extracted from chokeberries by procedure already described in the literature (Määttä-Riihinen et al., 2004a; Määttä-Riihinen et al., 2004b). The major phenolic compounds were identified by HPLC. Fig. 1, 2 and 3 show HPLC chromatograms of phenolic fractions with identified phenolic compounds.

In the first fraction, the major phenolic compounds extracted with ethyl acetate (Fig. 1) were neochlorogenic acid, chlorogenic acid and quercetin-3-rutinoside (rutin), in the total amount of 1472 mg/kg of fresh weight (F.W.). This agrees with literature data (Määttä-Riihinen et al., 2004a, Bermúdez-Soto and Tomás-Barberán, 2004; Slimestad et al., 2005).

The second fraction contained four major anthocyanins (Fig. 2), in the total amount of 8503 mg kg^{-1} of FW. All of them were cyanidin derivatives. This agrees with our previous investigations and with literature data (Määttä-Riihinen et al., 2004a; Jakobek et al., 2007a; Bermúdez-Soto and Tomás-Barberán, 2004; Slimestad et al., 2005).

After the extraction of major parts of free and conjugated forms of hydroxycinnamic acids, flavonols (Fraction 1) and anthocyanins (Fraction 2), the extraction residue was acid hydrolyzed to liberate insoluble high molecular weight (HMW) proanthocyanidins as anthocyanidins. Fig. 3 shows the chromatogram of the third fraction with the total amount of phenols 235 mg kg^{-1} F.W.

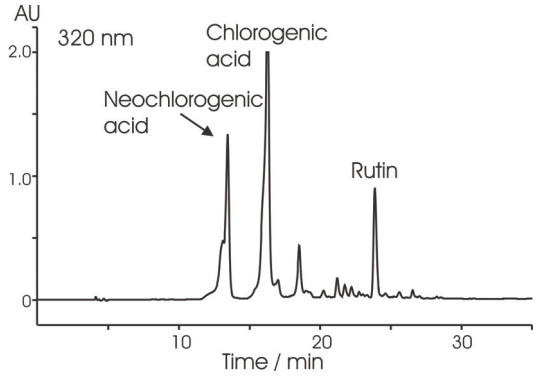


Fig. 1. The HPLC chromatogram of the first fraction extracted with ethyl acetate, recorded at 320 nm, with identified major phenolic acids and flavonols

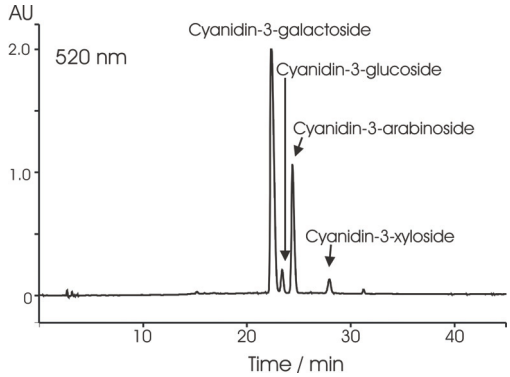


Fig. 2. The HPLC chromatogram of the second fraction extracted with acidified methanol, recorded at 520 nm, with identified major anthocyanins

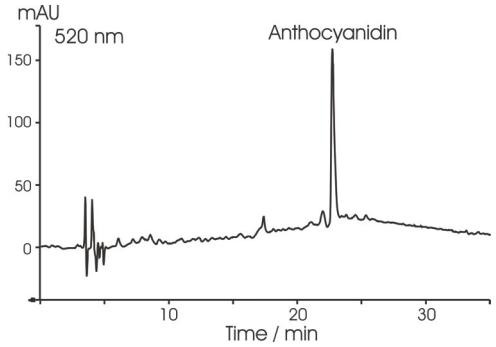
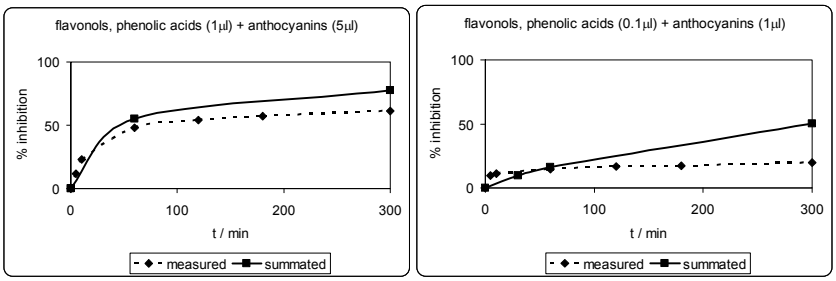


Fig. 3. The HPLC chromatogram of the third fraction extracted with acidified methanol and reflux, recorded at 520 nm

The ability of chokeberry phenols to scavenge free DPPH• radicals was studied by using the DPPH method. The reaction was monitored over a period of 300 minutes. The antiradical activity was monitored in individual phenolic fractions and expressed as % inhibition of DPPH• radicals. After that, the same concentrations of phenolic fractions were mixed and their antiradical activity was determined and expressed as % inhibition of DPPH• radicals. The measured antiradical activity caused by phenolic mixtures was compared to the summated values which were obtained by summing up the antiradical activity of individual phenolic fractions. In that way it could be seen if the antiradical activity of phenolic mixtures was lower or higher than predicted antiradical activity (summated values).

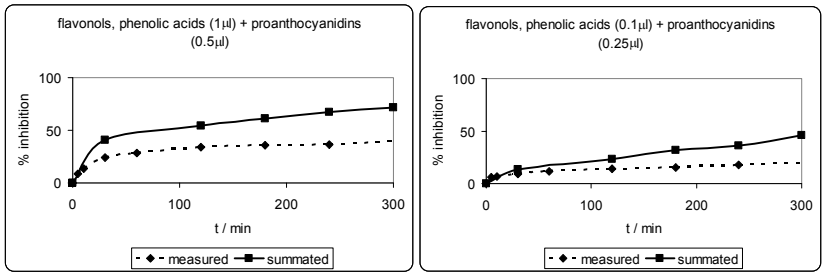
Fig. 4, 5, and 6 show the antiradical activity of individual phenol fractions (summated values) and phenolic mixtures (measured values). The results show that all phenols needed 4 or 5 hours to reach the steady state. Similar type of behavior was reported earlier in the study of Brand-Williams et al. (1995). They reported three types of kinetic behavior in the reaction between antioxidant compounds and DPPH• radicals, rapid, intermediate and very slow kinetic behavior (Brand-Williams et al., 1995). In this study, in the reaction between chokeberry phenols and free radicals, slow kinetic behavior was observed. Furthermore, all chokeberry phenols showed a biphasic reaction with fast and slow scavenging rates. Mixtures followed the reaction kinetic similar to that of individual fractions. Kinetic behavior was slow and mixed phenols needed 4 to 5 hour to reach the steady state. Moreover, the reaction between mixtures and DPPH• radicals was biphasic with fast and slow scavenging rates.

Furthermore, the results show that all phenolic mixtures (measured values) had lower antiradical activity than it was predicted by summing up antiradical activities of individual fractions (Fig. 4, 5 and 6). The mixing of various phenolic fractions caused the decrease of the antiradical activity. This indicated that phenolic combinations showed a tendency toward decreased antiradical activity. In other words, mixtures of various chokeberry phenols showed negative synergism. Negative synergism was observed earlier as well (Pinelo et al., 2004). In the study of Pinelo et al., (2004), the antiradical activity was determined by using the DPPH test, and the mixture of catechin, resveratrol and quercetin showed lower antioxidant activity in comparison to the trend followed by each single phenol. Negative synergism can be the result of various interactions between phenolic compounds. Phenolic compounds can be involved in various polymerization reactions which can increase their molecular complexity and affect their ability to react with free radicals. This leads to the decrease of the antiradical activity. Interactions between phenolic compounds from chokeberries could affect the total antiradical activity of chokeberries. The results also suggest that more complex systems could have lower antiradical activity in comparison to the simple ones.



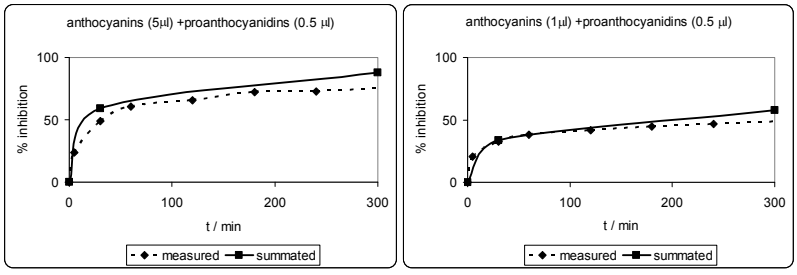
a) b)

Fig. 4. The antiradical activity of mixtures of fraction 1 (flavonols, phenolic acids) and fraction 2 (anthocyanins) and comparison with the trend followed by individual fractions. The antiradical activity was expressed as % inhibition of DPPH• radicals, and figures a) and b) represent different concentrations of mixtures. Summated antiradical activity denotes the sum of antiradical activities of individual phenol fractions. Measured antiradical activity represents total antiradical activity of the phenol mixture.



a) b)

Fig. 5. The antiradical activity of mixtures of fraction 1 (flavonols, phenolic acids) and fraction 3 (proanthocyanidins as anthocyanidins) and comparison with trend followed by individual fractions. The antiradical activity was expressed as % inhibition of DPPH• radicals, and figures a) and b) represent different concentrations of mixtures. Summated antiradical activity denotes the sum of antiradical activities of individual phenol fractions. Measured antiradical activity represents total antiradical activity of the phenol mixture.



a) b)

Fig. 6. The antiradical activity of mixtures of fraction 2 (anthocyanins) and fraction 3 (proanthocyanidins as anthocyanidins) and comparison with trend followed by individual fractions. The antiradical activity was expressed as % inhibition of DPPH• radicals, and figures a) and b) represent different concentrations of mixtures. Summated antiradical activity denotes the sum of antiradical activities of individual phenol fractions. Measured antiradical activity represents total antiradical activity of the phenol mixture.

Conclusions

Results obtained in this study showed that the mixing of phenolic compounds from chokeberries created more complex phenolic system, and promoted changes in the antiradical activity of chokeberries. Phenolic mixtures showed the tendency toward the decrease of the antiradical activity of chokeberries. Lower antiradical activity of phenolic mixtures can be the result of various interactions occurring between phenolic compounds in more complex systems. When producing food enriched with phenolic compounds, all of these effects should be considered.

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