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Preliminary communication

ANTIOXIDATIVE ACTIVITY OF ANTHOCYANINS FROM SOUR CHERRIES

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The aim of this study was to examine antioxidant activities of the anthocyanins isolated from different sour cherry (*Prunus cerasus*) cultivars. DPPH radical method and photochemiluminescence detection method were employed. The effect of isolated anthocyanins on peroxidase activity was also investigated. In the DPPH method, methanol extract of isolated anthocyanins was employed and results showed the highest radical-scavenging activity of anthocyanins isolated from one genotype of Cigančica cherry (genotype VN 10-11), Petrovaradinska and Oblačinska cherry cultivar. Integral antioxidative capacity was determined by luminometry (Photochem), calculating the ascorbic acid equivalents. The best reducing power was shown by anthocyanins isolated from Maraska and Petrovaradinska cherry cultivars. Peroxidase activity of fresh plant material was determined and the influence of anthocyanins on peroxidase activity was investigated.

Keywords: anthocyanins, *Prunus cerasus*, antioxidative activity, DPPH, photochemiluminescence, peroxidase activity

Anthocyanins are members of the flavonoid group of phytochemicals, a group predominant in teas, honey, wines, fruits, vegetables, nuts, olive oil, cocoa, and cereals. Natural antioxidants and colourants present in foods have attracted interest because of their safety and potential nutritional and therapeutic effect. Anthocyanins are well-known alternatives to synthetic dyes (ESPÍN et al., 2000). Consumption of cherries and their products was reported to be health-promoting, particularly in alleviating arthritic pain and gout, and in reducing the incidence of cancer. Recently, reports have shown that the anthocyanins isolated from tart cherries exhibit in vitro antioxidative and anti-

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inflammatory activities (BURKHARDT et al., 2001). Antioxidant activity of our examined sour cherries has not been investigated.

The objectives of this study were to screen five different cherry cultivars: Petrovaradinska, Oblačinska, Erdy Jubileum, Maraska, and Cigančica (for Cigančica cultivar six different genotypes were investigated). The scavenging effects of different anthocyanin extracts were estimated in the presence of the DPPH radical, while antioxidative capacity was determined by luminometry, calculating the ascorbic acid equivalents. The influence of anthocyanins on peroxidase activity was also investigated to evaluate their possible inhibitory effect.

1. Materials and methods

1.1. Materials and reagents

Samples of sour cherry cultivars (Petrovaradinska, Cigančica (all genotypes), Erdy Jubileum and Oblačinska were harvested in 2003 in Slavonija and Baranja region, Croatia. Maraska cherry cultivar was harvested in 2005 in Dalmacija region. Within 1 h of harvest, all samples were packed in polyethylene bags and kept at –20 °C before analysis. XAD-2 Amberlit resin was purchased from Kemika (Zagreb, Croatia). Abs. ethanol and methanol for DPPH method were of analytical grade (99.9% purity) and was obtained from Kemika (Zagreb, Croatia), while (DPPH) 2,2-diphenyl-1-picrylhydrazil was purchased from Sigma Aldrich. The methanol for the PCL method was also of analytical grade (99.9% purity), obtained from Rohm and Haas. For peroxidase activity 0.1 M Tris/HCl buffer (pH 8.0), 25% glycerol, 10–15% polyvinylpirolidone, 5 mM guaiacol, 5 mM H_2O_2 in 0.2 M phosphate buffer (pH 5.8), liquid nitrogen were used.

1.2. Isolation of anthocyanins from sour cherries

The pitted cherries (400 g) were homogenised separately for 10 min and centrifuged at 3000 r.p.m. for 15 min at room temperature. The supernatant was then applied to an XAD-2 column which was prepared as described by CHANDRA and co-workers (1993). The column was washed with water until the colourless washings gave a pH of about 7. The adsorbed pigments were then eluted with methanol. The red methanolic solution was concentrated at 50 °C in vacuo to dryness.

1.3. Free radical scavenging activity on DPPH

Methanolic extracts of cherries' anthocyanins were analysed using HPLC-MS method as previously described (ŠIMUNIĆ et al., 2005). The free radical scavenging activities of anthocyanins isolated from different cherry cultivars were estimated according to the method of SANCHEZ-MORENO and co-workers (1998) with some modification. Dried anthocyanin extracts were resolubilised in methanol and different anthocyanins concentrations were prepared: 2.5, 5, 10, 15, 20, 25, 50, 75, 100, 150 μM. DPPH· was

dissolved in abs. ethanol to a concentration of 100 μM. The decrease in absorbance at 515 nm was determined continuously with data capturing at 60 s intervals with the UV-Vis spectrophotometer Specord 200, (Analytik Jena AG, Jena, Germany) until the reaction reached a plateau. Ethanol was used to zero the spectrophotometer. All determinations were performed in triplicate. The fraction of the residual DPPH radical (DPPH· res) was calculated as:

$$\% \text{ DPPH}\cdot_{res}=[\text{DPPH}\cdot]_t/[\text{DPPH}\cdot]_{t=0}\times 100$$

where $[\text{DPPH}\cdot]_t$ and $[\text{DPPH}\cdot]_{t=0}$ are concentrations of the DPPH· radical at t=0 and t=t, respectively. $[\text{DPPH}\cdot]_t$ was determined according to the linear regression equation below:

$$A_{515nm}=0.0097[\text{DPPH}\cdot]_T-0.0012$$

EC_{50} values were obtained from the inhibition curve. Considering EC_{50} and the time needed to reach the steady-state to EC_{50} concentration (T_{EC50}), which was calculated graphically, affect the antioxidative activity (AOA):

$$AOA=1/EC_{50}\times T_{EC50}$$

1.4. PCL method

The photochemiluminescence (PCL) assay, based on the methodology described by the manufacturer was used to measure the reducing power of anthocyanin extracts with Photochem instrument (Analytik Jena AG, Jena, Germany). The free radicals are visualised with a chemiluminescence detection reagent, luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), which acts as photosensitiser as well as oxygen radical detection reagent. Sample extracts were resolubilised in 100% methanol. In this study, measurements were conducted with the ACW kit (Analytik Jena). The calibration curve was constructed by measuring a series of standard solutions, namely 0.15, 0.3, 0.5, 1.2 nmol ascorbic acid. Lag time (L in s) for the ACW assay, obtained from the PCLsoft control and analysis software used as the radical-scavenging activity and the antioxidant capacity were estimated by comparison with an ascorbic acid standard curve. The antioxidant index was obtained by dividing the antioxidant capacity by lag time multiplied by 1000 (i.e., antioxidant activity/lag time × 1000). Antioxidant capacity was determined in duplicate.

1.5. Peroxidase activity

Peroxidases were isolated from fresh *Prunus cerasus* leaves. The tissue was smashed into powder by adding liquid nitrogen. Proteins were extracted with 0.1 M Tris-HCl buffer, pH 8.0, containing polyvinylpyrolidon – PVP (10 mg ml^{-1} buffer) and centrifuged for 10 min at 10 000 *g* at 4 °C. Total guaiacol peroxidase activity was determined spectrophotometrically (UV-Vis spectrophotometer Specord 200, Analytik

Jena AG, Jena, Germany) by measuring the absorbance increase at 470 nm. The reaction mixture contained 5 mM guaiacol and 5 mM H_2O_2 in 0.2 M phosphate buffer, pH 5.8 (SIEGEL & GALSTON, 1967). Two different anthocyanin solutions in the reaction mixture were prepared (0.01 mg ml^{-1} and 0.1 mg ml^{-1}). The reaction was started by adding 200 µl of protein extract into 800 µl of reaction mixture. The first measured sample was without added anthocyanins and after that samples with anthocyanins present in reaction mixture were measured. All measurements were done in duplicate and averaged.

2. Results and discussion

2.1. DPPH method

Our data showed that the DPPH· radical solution was bleached with all the samples tested. However, differences could be observed for the different cherry cultivars used and anthocyanin concentrations. The higher the concentration, the greater was the decrease in absorbance. Parameter EC_{50} "efficient concentration" is a direct quantitative measure for antioxidative activity. It is defined as the amount of antioxidant concentration needed to reduce the initial DPPH· concentration $C_{DPPH·}$ (t=0) by a factor of two (at steady-state). Highly effective (high antioxidative activity) antioxidant is characterised by a low EC_{50} value. "Efficient concentration" was calculated for each type of samples. Anthocyanin extract of one genotype (VN 10-11) of Cigančica cherry cultivar exhibited the lowest EC_{50} concentration 179.8 g ACN/kg DPPH·). Low EC_{50} concentration was observed for the anthocyanins from Petrovaradinska and Oblačinska cherry cultivars (186.4 and 230.3 g ACN/kg DPPH·).

Table 1. Fifty % radical scavenging activity concentration of antioxidants and their antioxidative activity

Anthocyanin extract from:	EC_{50} (g ACN/kg DPPH·)	T_{EC50} (min)	AOA ($\times10^{-3}$)
Petrovaradinska cherry	186.4	3.5	1.534
Oblačinska cherry	230.3	3.6	1.198
Erdy Jubileum cherry	254.6	3.8	1.043
Maraska cherry	735.7	5.1	0.266
Cigančica Genotype: VN 12-236	263.4	3.7	1.021
Cigančica Genotype: VN 2-26	820.8	5.4	0.227
Cigančica Genotype: VN 12-21	416.4	4.2	0.576
Cigančica Genotype: VS 10-28	598.2	4.7	0.355
Cigančica Genotype: VN 2-26-11	504.8	4.4	0.447
Cigančica Genotype: VN 10-11	179.8	3.5	1.600

The highest antioxidative activity was shown by anthocyanins isolated from one genotype of Cigančica cherry (VN 10-11), AOA=1.6×10^{-3}, but in spite of this, other genotypes of Cigančica cherry showed very weak antioxidative activity. Anthocyanins isolated from Maraska cherry cultivar exibited also very weak antioxidative activity,

AOA=0.266×10^{-3}. High antioxidant activity was exhibited by anthocyanins isolated from Petrovaradinska AOA=1.534×10^{-3} and Oblačinska cherry cultivars AOA=1.198×10^{-3}. Direct correlation between anthocyanin content and antioxidative activity could not be proven. It seems reasonable, while it is possible to ascertain direct correlation between the concentration of pure compounds and the antioxidant capability of their solutions, it is not so easy in the case of plant extracts (WANASUNDARA et al., 1996; RAKOTOARISON et al., 1997; MOURE et al., 2001; DAWIDOWICZ et al., 2006). The methods applied for the estimation of antioxidant effectiveness of the extracts do not eliminate the possibility of individual compound interactions, and synergistic and/or inhibiting effects with the system used for antioxidant testing. It will also be important to study the synergistic effect of different phenolic compounds contained in cherry extracts.

2.2. PCL method

PCL assay generated two indicators; lag time (L in s) – a parameter of delay in the photochemical generation of superoxide radical anion – and antioxidant capacity (AC, expressed as ascorbic acid equivalent) – a parameter of radical scavenging ability. Our results showed that anthocyanin compounds isolated from all cherry cultivars possess scavenging activity for the superoxide anion radical ($O_2^{-\cdot}$). There was a considerably high antioxidative capacity measured in two cherry cultivars; Maraska AC=1.819±1.0159 μm ascorbic acid with lag time of 5.61 s, and antioxidant index of 324.30 and Petrovaradinska AC=1.887±0.4575 μm ascorbic acid with lag time of 9.11 s and antioxidant index of 207.08. Thus, the high antioxidant index of the Maraska and Petrovaradinska cherry anthocyanins expresses both their relatively fast initiation of the luminol (photosensitiser) excitation and its antiradical capacity. Methanolic extract of anthocyanins isolated from different genotypes of Cigančica cherry exhibited weak antioxidant capacity (0.001±0.0027 to 0.30±0.0459 μm ascorbic acid), lag time (10.47 to 73.42 s) and antioxidant index (0.1 to 7.18) based on ACW measurements (Table 2). The lowest antioxidant capacity and index were expressed by anthocyanins isolated from one genotype (VN 2-26-11) of Cigančica cherry.

Table 2. Water soluble antioxidative capacity (ACW) of anthocyanin extracts isolated from different sour cherry cultivars and genotypes, Antioxidative index = ACW/lag time×1000

Anthocyanin extract from:	Ascorbic acid equivalent (ACW kit) (μm/g)±SD	Lag time (L) (s)	Antioxidant index
Petrovaradinska cherry	1.887±0.4575	9.11	207.08
Oblačinska cherry	0.256±0.1419	48.13	5.31
Erdy Jubileum cherry	0.033±0.0049	24.94	1.30
Maraska cherry	1.819±1.0159	5.61	324.30
Cigančica Genotype: VN 12-236	0.037±0.0557	16.38	2.26
Cigančica Genotype: VN 2-26	0.035±0.0018	17.05	2.05
Cigančica Genotype: VN 12-21	0.061±0.0028	23.25	2.62
Cigančica Genotype: VS 10-28	0.023±0.0012	73.42	0.31
Cigančica Genotype: VN 2-26-11	0.001±0.0027	10.47	0.10
Cigančica Genotype: VN 10-11	0.305±0.0459	42.43	7.18

2.3. Influence of anthocyanins on peroxidase activity

Total peroxidase activity was measured in fresh cherry leaves. In non-affected cherry leaves samples total peroxidase activity was 7.98 min^{-1} ml^{-1}. All samples with added anthocyanins showed a decrease in absorbance and peroxidase activity (Table 3). There might be two possible reasons for that. First, anthocyanins are probably favourable peroxidase substrates, since they have a great propensity for accommodation in the catalytic center of the enzyme. They may act therefore as a competitive inhibitor of this hemeprotein, so catalytic activity of enzyme can be changed. It is revealed by means of peroxidase activities decrease. The second possible pathway is oxidation of anthocyanins which had corresponding inhibitory effect on guaiacol oxidation. The highest inhibition of peroxidase activity was manifested by anthocyanins isolated from the Petrovaradinska and Oblačinska cherry cultivars, while the lowest inhibition was observed for anthocyanins isolated from the Cigančica cultivar. Possible explanation of inhibitory effect was based on anthocyanin content, which we investigated in our previous work (ŠIMUNIĆ et al., 2005) where the highest total anthocyanin content was observed in Petrovaradinska and Oblačinska cultivars and the lowest in Cigančica cultivar.

Table 3. Effects of anthocyanins on peroxidase activity

Cherry cultivars	Anthocyanin concentration (0.01 mg ml^{-1})	Anthocyanin concentration (0.1 mg ml^{-1})
	Activity of guaiacol peroxidase (min^{-1} ml^{-1})	Activity of guaiacol peroxidase (min^{-1} ml^{-1})
Petrovaradinska	6.642±0.498	2.376±0.012
Oblačinska	6.000±0.084	1.623±0.441
Erdy Jubileum	7.053±0.669	4.884±0.132
Maraska	6.732±0.384	2.796±0.222
Cigančica Genotype: VN 12-236	7.728±0.060	4.350±0.402
Cigančica Genotype: VN 2-26	6.312±0.192	4.806±0.450
Cigančica Genotype: VN 12-21	7.662±0.198	5.346±0.066
CigančicaGenotype: VS 10-28	6.786±0.438	4.878±1.026
Cigančica Genotype: VN 2-26-11	7.278±0.150	5.064±0.132
Cigančica Genotype: VS 10-11	7.008±0.240	4.092±0.528

Total peroxidase activity was 7.98 min^{-1} ml^{-1} with no-added anthocyanins

3. Conclusions

Results in this study clearly indicate that anthocyanins isolated from cherry cultivars Oblačinska and Petrovaradinska possess strong antioxidative activity measured with DPPH method, but the strongest antioxidative activity was measured in one genotype of Cigančica cherry (VN 10-11). In PCL method, the strongest antioxidative activity was shown by Petrovaradinska and Maraska cultivars. In peroxydase assay, anthocyanins

isolated from Oblačinska and Petrovaradinska cultivars showed the strongest inhibitory effect on peroxidase activity. It is concluded that the best antioxidant capacity and hence the best health benefit may be derived from those cherry cultivars.

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