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Biological activity of *Aronia melanocarpa* antioxidants pre-screening in an intervention study design

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Abstract: The beneficial effects of black chokeberry fruits and juices in health promotion and prevention of chronic diseases shown in both epidemiological and dietary intervention studies are often connected with their antioxidant activity. The aim of this study was to investigate the total phenolics and anthocyanins content, chemical antioxidant activity (DPPH-assay), antioxidant protection in erythrocytes and anti-platelet activity *in vitro* of three different chokeberry products: commercial and fresh pure chokeberry juice and a crude lyophilized water–ethanol extract of chokeberry fruits, as part of their pre-clinical evaluation. The obtained results indicated differences in chemical composition and antioxidant activity of the investigated products. Cellular effects, including both *in vitro* anti-platelet and antioxidant effects, were not directly correlated with the chemical antioxidant activity and the results obtained *in vitro* for anti-platelet effects were only partially consistent with the results obtained *in vivo*, in a pilot intervention trial. In conclusion, chemical analyses and *in vitro* experiments on foods and their bioactive substances are a valuable pre-screening tool for the evaluation of their biological activity. However, extrapolation of the obtained results to the *in vivo* settings is often limited and influenced by the bioavailability and metabolism of native dietary compounds or interactions with differrent molecules within the human body.

Keywords: chokeberry; erythrocytes; platelets; DPPH assay; flow cytometry.

INTRODUCTION

Black chokeberry (*Aronia melanocarpa*) belongs to the Rosaceae family. It is a native plant of North America and Canada, grown successfully in Europe since the beginning of the 20th century. The fruits and juices of black chokeberry (*A. melanocarpa*) are excellent sources of both nutritive and non-nutritive dietary

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compounds with numerous biological activities, including phenolics, vitamins and minerals. The phenolic compounds of chokeberries are procyanidins, anthocyanins, phenolic acids and tannins. Chokeberries are considered to be the best dietary sources of anthocyanins (25 % of total phenolics), one of the most powerful *in vitro* antioxidants. The fruits and juices of this plant have the highest antioxidant activity compared to other types of berries from the Rosaceae family.[1] A large number of dietary intervention studies showed the beneficial effects of the consumption of chokeberry juice and extracts on various risk factors for chronic diseases, including the parameters of oxidative stress,[2] total cholesterol, LDL, oxy-LDL, triglycerides, glucose, HbA1c, systolic and diastolic blood pressure,[3,4] platelet[5] and endothelial function.[6] The effects of black chokeberry consumption are often connected with their antioxidant activity. However, the very low bioavailability of anthocyanins observed after ingestion of anthocyanin-rich food questions the rationale for investigation of native compounds, instead of their metabolites, in *in vitro* models, in pre-screening for *in vivo* effects, including antioxidant activity. The aim of this study was to investigate the total contents of phenolics and anthocyanins, the chemical antioxidant activity (using the DPPH-assay), cellular antioxidant activity and anti-platelet activity *in vitro* and *ex vivo* of three different chokeberry products: commercial and fresh pure chokeberry juice and a crude lyophilized water–ethanol extract of chokeberry fruits.

EXPERIMENTAL

Chemicals

The following buffers and reagents were obtained from Sigma–Aldrich (Germany): phosphate-buffered saline (PBS), Folin–Ciocalteu's phenol reagent, gallic acid, potassium chloride, sodium acetate, 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ), ferrous sulphate heptahydrate, 2,2-diphenyl-1-picrylhydrazyl (DPPH), Dulbecco's modified Eagle's medium (DMEM), hydrogen peroxide 30 % solution (H_2O_2), 3,4-dihydroxybenzoic acid (protocatechuic acid), calcein acetoxymethyl ester (calcein AM), foetal bovine serum (FBS), bovine serum albumin (BSA), adenosine diphosphate (ADP) and 2′,7′-dichlorofluorescein diacetate (DCF-DA). Monoclonal antibodies: fluorescein isothiocyanate (FITC)-conjugated PAC1, phycoerythrine (PE)-conjugated CD62P, peridinin chlorophyll protein (PerCP)-conjugated CD61 and control immunoglobulin G-PE and immunoglobulin M-FITC were purchased from Becton Dickinson (USA).

Subjects

Whole blood was collected from subjects with metabolic syndrome for both *in vitro* experiments ($n = 3$) and *ex vivo* pilot study ($n = 6$; 3 males and 3 females). Metabolic syndrome was defined according to ATP III criteria.[7] All blood samples were taken by venipuncture according to the guidelines for blood sampling in platelet analysis. For the *ex vivo* pilot study participants were subjected to dietary intervention by acute intake of 200 mL of commercial pure chokeberry juice and blood samples were collected before and 2 h after the consumption. Whole blood was used for the isolation of plasma and platelets.

The study protocol was approved by the Ethical Committee of the Faculty of Pharmacy, University of Belgrade. The study was conducted in accordance with the revised Declaration of Helsinki. All participants provided written informed consent.

Samples

Three different chokeberry products were used in the study: commercial pure chokeberry juice (CCJ) (Aronia Antioxi, Nutrika, Serbia), fresh pure chokeberry juice (FCJ) obtained in the laboratory by squeezing of fresh fruits (with a yield of 0.53 mL g^{-1} of fresh fruits) and crude lyophilized water–ethanol (40/60 % vol.) extract (WECE) of chokeberry fruits. All investigated chokeberry products were prepared from the fruits of *Aronia melanocarpa* var. *rubina*, grown in western Serbia and harvested during October/November 2012. For the experiments, the juices were diluted to the working concentrations in water or defined buffers. The crude water–ethanol extract was further extracted (50 mg mL^{-1}) with water or methanol and soluble fractions (WCE and MCE, respectively) obtained by centrifugation were used in further experiments. For the cell-based assay, the methanol soluble fraction of investigated extract was evaporated to dryness in a rotary evaporator and the residue was reconstructed with water immediately before addition to the cells. The cell-based antioxidant activity and anti-platelet activity were also evaluated for protocatechuic acid, one of the major metabolites of cyanidin-3-glucoside.[8]

Determination of the total phenolics

Content of total phenolics in investigated samples was analysed using a modified Folin––Ciocalteu method.[9,10]. Juices and extracts were diluted in distilled water to the working solutions that gave absorbances within the standard calibration curve (0–600 µg mL^{-1} of gallic acid). The results are expressed as milligrams of gallic acid equivalents (GAE) per mL of juices or of the investigated extracts. Data are presented as mean ±*SD* for three replications.

Determination of the total anthocyanins

The total anthocyanin content (*TAC*) was quantified using the pH differential method described by Lee *et al*.[11] Briefly, the investigated juices (CCJ and FCJ) and extract fractions (WCE and MCE) were dissolved in a potassium chloride buffer of pH 1.0 and sodium acetate buffer pH 4.5. The absorbance of both buffer solutions was measured at 520 and 700 nm. The results were expressed as milligrams of cyanidin-3-glucoside equivalents (CGE) per mL of juices or g of investigated extract. All experiments were performed in triplicate.

Determination of the antioxidant activity

Radical scavenging activity of the investigated samples was analysed using the DPPH assay.[12] The data are presented as the concentrations of the samples that inhibited 50 % of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical (0.05 mM) after 30 min of incubation, based on the decrease in absorbance measured at 517 nm.

The antioxidant activity of plasma obtained from whole blood before and 2 h after the dietary intervention with chokeberry juice was determined according to the method of Benzie and Strain,[13] as the ability of the plasma to reduce ferric ions. The data are presented as the Fe(III) concentration (mM) in the sample after reaction with the ferric tripyridyltriazine (Fe^{III}-TPTZ) complex, according to the calibration curve (0–2000 µM $FeSO_4$). All experiments were performed in triplicate.

Determination of the antioxidant protection of erythrocytes

The cellular antioxidant activity of the investigated samples was based on the antioxidant protection of erythrocytes exposed to reactive oxygen species (ROS). The cellular antioxidant protection assay was performed as previously described[14] with modifications regarding the exposure of erythrocytes to a lower level of extracellular ROS (1mM H_2O_2). Packed erythrocytes were isolated from the whole blood of the donors by three subsequent washings with

PBS. The obtained erythrocytes, re-suspended in PBS, were treated with serial dilutions of the investigated samples (1 h, 37 °C). After incubation, the cells were washed twice with PBS to remove extracellular antioxidants, and incubated with the intracellular dye 2′,7′-dichlorofluorescein diacetate (DCF-DA), washed again and treated with hydrogen peroxide (1 mM) for 30 min. The intracellular ROS levels were analysed by flow cytometry (FACSCalibur, BD, USA) based on the fluorescence of dichlorofluorescein (DCF), fluorescent product of DCF-DA in the reaction with intracellular H_2O_2. The results are expressed as the mean fluorescence intensity (*MFI*) of the total number of analysed erythrocytes (20000) and presented as mean ±*SD* of the data obtained in three subjects. All analyses were performed in duplicate.

Determination of platelet activation – in vitro

The platelet activation markers, P-selectin and GPIIb-IIIa, were measured by whole-blood flow cytometry according to a previously published protocol[15] with slight modifications for *in vitro* testing. In brief, after venipuncture, aliquots of dissolved (1:10 in Hepes-Tyrode Buffer, pH 7.4) anti-coagulated blood (3.2 % citrate) were incubated with serial (2×) dilutions of investigated samples (30 min, 37 °C) and subsequently incubated with CD61-PerCP (pan-platelet marker), CD62P-PE (anti-P-selectin) and PAC-1-FITC (antiGPIIb-IIIa) monoclonal antibodies with suboptimal concentration of platelet agonists (0.5 mM ADP) for 20 min in the dark, at room temperature. After the incubation with antibodies, the samples were fixed with paraformaldehyde solution (0.5 %) for 15 min and analysed. Sample analysis was performed using a FACSCalibur flow cytometer with CellQuest software (Becton Dickinson, USA). The results are presented as antigen positive platelets (%) in the platelet pool (20000 events).

Determination of platelet–endothelial cells adhesion – ex vivo

The effect of chokeberry juice consumption on platelet–endothelial adhesion was investigated *ex vivo* in a platelet–endothelial cell adhesion assay. EA.hy926, a continuously replicating cell line derived from primary human umbilical vein endothelial cells (HUVEC) was used as an endothelial cellular model.

EA.hy926 cells were cultured as a monolayer in DMEM supplemented with penicillin (192 U mL^{-1}), streptomycin (200 μg mL^{-1}) and 10 % heat-deactivated FBS. The cells were grown at 37 °C in 5 % CO_2 and humidified air atmosphere with twice-weekly subculture.

Platelet-coated surfaces were prepared as described previously[16] with modifications for *ex vivo* testing. Suspensions of platelets (0.1 mL containing 1×10^8 platelets in HEPES Tyrode's buffer), isolated from the whole blood before and after intervention, were added to plastic flat-bottomed micro-titre wells. The plates were incubated for 24 h at 4 °C. The day after, non-adherent platelets were removed by washing with PBS containing 1 % BSA. The same solution was used for the blocking of "free adherent" sites on the plastic (1 h at 37 °C). EA.hy926 were detached and re-suspended in PBS enriched with Ca^{2+} and Mg^{2+}. After staining with calcein-AM, 1×10^5 EA.hy926 cells were added to each platelet-coated well in the presence of thrombin (2 U mL^{-1}) and incubated for 1 h at 37 °C. The plates were then washed twice and the adherent cells were quantified in black 96-well plates with a fluorescence plate reader (Florosken Ascent FL, Thermo) with an excitation wavelength of 485 nm and an emission wavelength of 520 nm.

Statistical analysis

All results are presented as mean ± standard deviation (*SD*). The data were analysed by the one sample *t*-test and $p < 0.05$ was considered statistically significant. The SPSS program, version 19 (SPSS Inc., Chicago, IL), was used for the analysis.

RESULTS AND DISCUSSION

Total phenolics content

The results of the total phenolics and anthocyanin content analysis, as well as the radical (DPPH) scavenging activity of the investigated samples are summarized in Table I, showing values expressed per mL of juices and g of dry weight (DW) of the investigated juices, as well as per ml of the investigated extract fractions and g of investigated extract (WECE) used for the purpose of comparison. The total phenolics contents of CCJ and FCJ were similar ($p = 0.79$), showing that processes included in the production of the commercial juice (pectinase treatment, filtration) did not influence the phenolics of the juice. The obtained results were in accordance with the results of Mayer-Miebach *et al.*[17] The contents of total phenolics in WCE and MCE, expressed as mg GAE per g of WECE dry weight (DW), were 4 and 2.5 times lower, respectively, than phenollics content in the juices, calculated on DW. It could be concluded that the phenolics present in 100 mL of juice could be provided with at least 35 g of the investigated extract, indicating that the extraction process should be further optimized and/or prioritizing juice as the optimal source of chokeberry phenolics in future clinical studies. Results showing the significantly higher phenolic content in MCE compared to WCE ($p < 0.001$) also indicate that compounds with the phenolic structure present in WECE were extracted more efficiently with less polar solvents (methanol *vs*. water).

TABLE I. Total phenolics content, total anthocyanins content and radical scavenging activity of the analysed samples; CCJ – commercial chokeberry juice; FCJ – fresh chokeberry juice; WCE – water soluble fraction of the water–ethanol ($\varphi_{\text{water}} = 0.40$) extract of chokeberry fruits; MCE – methanol soluble fraction of the water–ethanol ($\varphi_{\text{water}} = 0.40$) extract of chokeberry fruits; GAE – gallic acid equivalents; DW – dry weight; CGE – cyanidin-3-glucoside equivalents

Samples	CCJ	FCJ	WCE	MCE
Total phenolics, mg GAE mL^{-1}	5.86±0.27	5.93±0.33	0.50±0.01	0.86±0.01
Total phenolics, mg GAE g^{-1} DW	41.2±1.9	43.3±2.4	10.15±0.22	17.12±0.18
Total anthocyanins, mg CGE mL^{-1}	0.15±0.02	2.18±0.09	0.0210±0.004	0.21±0.01
Total anthocyanins, mg CGE g^{-1} DW	1.07±0.14	15.91±0.65	0.42±0.02	4.12±0.18
IC_{50}[a] / μL mL^{-1}	0.44±0.03	0.52±0.03	7.41±0.46	4.12±0.38
IC_{50} / mg DW mL^{-1}	0.062±0.001	0.071±0.001	0.370±0.023	0.206±0.019

[a] Concentration of samples that inhibited 50 % of DPPH radical, based on absorbance measurements at 517 nm

Total anthocyanins content

Total anthocyanins (TA) content in FCJ was significantly higher than in CCJ ($p < 0.001$), supporting previously published data on the influence of storage and processing on the content of anthocyanins. Howard *et al.*[18] reported that both processing and storage of processed chokeberry products at ambient temperature induced significant losses of anthocyanins. Anthocyanins in juices were more susceptible to the degradation processes compared to other products, due to the removal of skin and seeds. Degradation of anthocyanins is accompanied with an increase in the products of their polymerization, designed as polymeric pigments, but the precise mechanism is still unknown.

The TA contents of WCE and MCE were 0.42±0.02 and 4.12±0.18 mg CGE g^{-1} DW, respectively. The observed significant difference between the obtained values ($p < 0.001$), indicate that anthocyanins were more efficiently extracted with methanol and the content in MCE was more than 10 times higher than in WCE and almost 4 times higher than in CCJ calculated on DW, showing that 100 mL of CCJ is equivalent to approximately 3.7 g of extract.

Antioxidant activity (DPPH assay)

Antioxidant activity of juices and extracts was evaluated as the radical scavenging activity (RSA). After 30 min of incubation with 0.04 mM DPPH solution in methanol, 50% inhibition of absorbance measured at 517 nm (IC_{50} value) was obtained with 0.44±0.03 and 0.52±0.03 μL mL^{-1} of CCJ and FCJ, respectively, showing slight but significant difference between the obtained values ($p = 0.038$) and surprisingly higher antioxidant activity of CCJ. Regarding investigated extract MCE was more effective than WCE as DPPH radical scavenger, with the IC_{50} value of 0.206±0.019 and 0.370±0.023 mg of WECE used for extraction ($p = 0.007$). RSA did not correlate with the anthocyanin content in investigated juices and extract fractions, indicating the influence of other bioactive substances present in the samples. In strawberries, DPPH radical scavenging activity was not significantly influenced by processing and storage and did not reflect the decrease in anthocyanin content.[19] However, RSA of extract fractions, based on quantities relevant for consumption, is negligible compared to the investigated juices and consequently could not be taken as optimal intervention sample.

Antioxidant activity of plasma, measured using the ferric reducing antioxidant power (FRAP) assay within *in vivo* pilot study in six subjects significantly increased after single intervention with commercial chokeberry juice ($p = 0.001$), with the obtained values of 1.51±0.26 mM Fe^{2+} compared to the baseline values of 1.29±0.23 mM Fe^{2+}. The effect of acute intake of flavonoid-rich juice consumption on FRAP value of plasma was observed previously, but the authors suggested that the effect may be due to changes in uric acid concentration.[20] Data on acute intake of chokeberry juice on antioxidant capacity of plasma is lacking although Pilaczynska-Szczesniak *et al*. have shown that long term chokeberry juice consumption reduced parameters of lipid oxidation and increased the activities of antioxidative enzymes in erythrocytes.[2]

Antioxidant protection of erythrocytes

Erythrocytes were used as a simple cellular model for the evaluation of the bioactive antioxidant effects of chokeberry against moderate oxidative stress induced by hydrogen peroxide, influenced by the uptake through the biological membrane and overall bioavailability. Isolated red blood cells were pre-incubated with serial dilutions of investigated samples and exposed to H_2O_2 (1 mM). Intracellular H_2O_2 levels were determined according to the measured DCF fluorescence using flow cytometry. As shown in a representative histogram presenting the decrease in DCF fluorescence in erythrocytes pre-treated with MCE (5 mg mL^{-1}), compared to the non-treated cells, after the subsequent H_2O_2 exposure (Fig. 1), the mean DCF fluorescence (horizontal axis) of the analysed cells, corresponding with intracellular ROS levels, is shifted to the left to lower values compared to the fluorescence of non-pre-treated cells, indicating scavenging of H_2O_2. Figure 2 shows the decrease in intracellular ROS presented as the inhibition of DCF fluorescence in the DCF-DA-stained cells pre-treated with different concentrations (1.25, 2.5 and 5 mg mL^{-1}) of water soluble fraction (WCE) or methanol soluble fraction (MCE) of water–ethanol chokeberry fruit extract and subsequently exposed to H_2O_2 (1 mM), compared to the fluorescence in control cells (without pre-treatment). Based on the results obtained, both WCE and MCE showed antioxidant activity against H_2O_2-induced oxidative stress in erythrocytes, and the effect was more pronounced for MCE, with the inhibition levels (%)

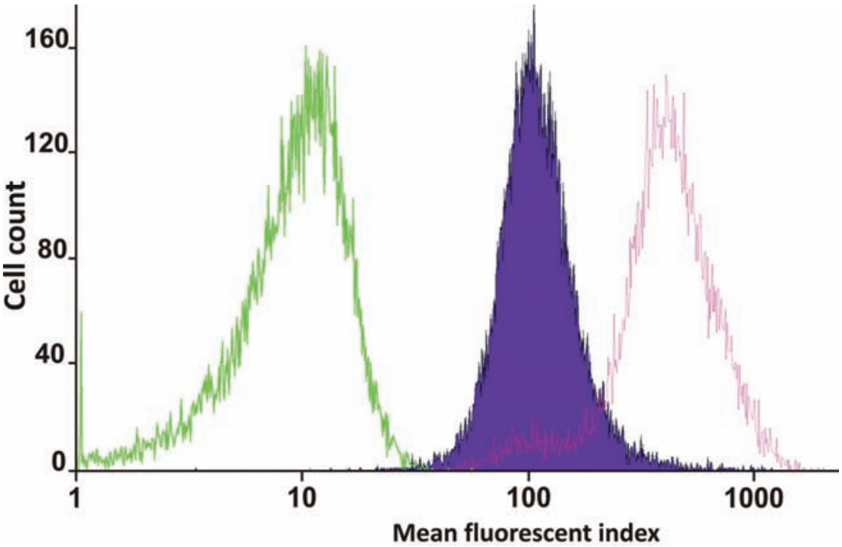

Fig. 1. Representative histogram showing DCF fluorescence decrease in erythrocytes pre-treated with the methanol soluble fraction (MCE) of chokeberry extract and subsequently exposed to H_2O_2 (full area), compared to the fluorescence of non-pre-treated cells, exposed to H_2O_2 (purple borderline) and cells without H_2O_2 exposure (green borderline).

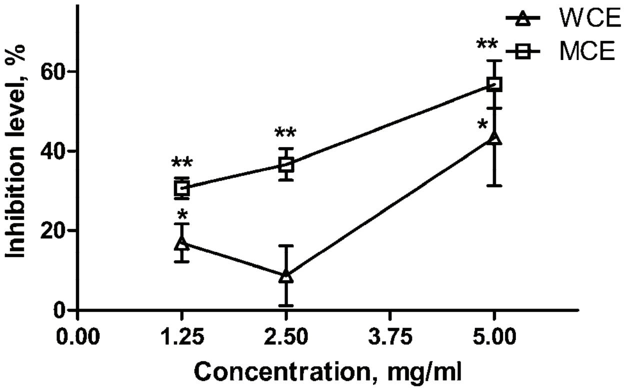

Fig. 2. Intracellular ROS decrease in erythrocytes pre-treated with different concentrations of the water soluble fraction (WCE) and methanol soluble fraction (MCE) of chokeberry extract and subsequently exposed to extracellular oxidative stress, compared to the control, non-pre-treated cells (significantly different from control cells: **p*<0.05 and ***p*<0.01).

of measured fluorescence of 30.65±2.61 ($p < 0.01$), 36.67±3.98 ($p < 0.01$) and 56.83±5.96 ($p < 0.01$), for the investigated concentrations of 1.25, 2.5 and 5 mg mL^{-1}, respectively, compared to the values obtained for WCE of 16.89±4.78 ($p <$ < 0.05), 8.69±7.45 ($p = 0.18$) and 43.42±12.08 ($p < 0.05$), for the same concentrations, respectively. The obtained results could be hypothetically explained with the higher amount of anthocyanins in the total content of phenolics of MCE. The main difference between the effects observed for MCE and WCE was the dose response, which was shown to be non-linear for WCE and linear for MCE, suggesting that the bioactive substances extracted with water or methanol could have antioxidant effects with different modes and activity. The inhibition level (%) was higher for MCE than for WCE at all the investigated concentrations, but was statistically significant only for 1.25 and 2.5 mg mL^{-1} ($p < 0.05$ and $p < 0.01$, respectively), showing that in higher concentrations, the cellular antioxidant activity could be influenced by the uptake of potential antioxidants into the living cell. The effects of investigated juices were evaluated in the concentration range of 0.25–75 μL mL^{-1}, with the maximum concentration hypothetically correlated with the concentration in plasma after the consumption of 200 mL of juice and the pre-assumption of complete availability of the bioactives present in the juices. Both juices showed antioxidant potential in concentrations corresponding to their IC_{50} values determined using DPPH assay, but the effect of FCJ was more pronounced ($p = 0.049$) with an inhibition level (%) of 63.7±8.4 at the IC_{50} concentration (0.44 μL mL^{-1}) compared to 47.3±5.7 obtained with CCJ at the IC_{50} concentration of 0.52 μL mL^{-1}. Interestingly, in the low concentration range (0.5–4 μL mL^{-1}), the observed antioxidant activity was inversely correlated with concentration, while at higher concentrations, a direct correlation could be observed.

Slatnar *et al*. investigated the cellular antioxidant effects of different berry juices using *Saccharomyces cerevisiae* as an *in vitro* cellular model, DCF-DA staining but without ROS exposure. They showed that the results obtained in the yeast cells were markedly different from the data obtained by the DPPH assay and concluded that the major factors found to influence *in vivo* antioxidant activity were not only the cellular availability of the polyphenols present in juices but also the ratio of specific polyphenol present in the juices and consumed by the cell, with the favourable effects of the high anthocyanin content and low content of hydroxycinnamic acids. They also found that when the uptake of polyphenols by the yeast cells was low, the antioxidant activity increased. High hydroxycinnamic acid uptake with low anthocyanin intake induced higher intracellular oxidation.[21] The cellular antioxidant activity of protocatechuic acid (PCA), a simple phenolic acid with *in vitro* antioxidant properties, was also investigated and it was found to be one of the major metabolites of cyanidin-glucosides.[8,22] The decrease in intracellular ROS, based on the inhibition of DCF fluorescence in DCF-DA stained cells pre-treated with different concentrations (10, 100 and 1000 µM) of PCA and subsequently exposed to H_2O_2 (1 mM), compared to the fluorescence in control cells (without pre-treatment) is shown in Fig. 3. Antioxidant protection of erythrocytes by PCA showed a statistically significant inverse dose response correlation (p = 0.0065), with the lowest dose (10 µM) that induced the highest reduction in intracellular ROS (52.09±7.29), suggesting that antioxidant activity of PCA is not mediated by its direct antioxidant action. The published data on the antioxidant properties of PCA within cellular systems and in animal models are controversial. Nakamura *et al*. showed dose and time dependant effects of PCA in animal models of skin tumours, with beneficial ef-

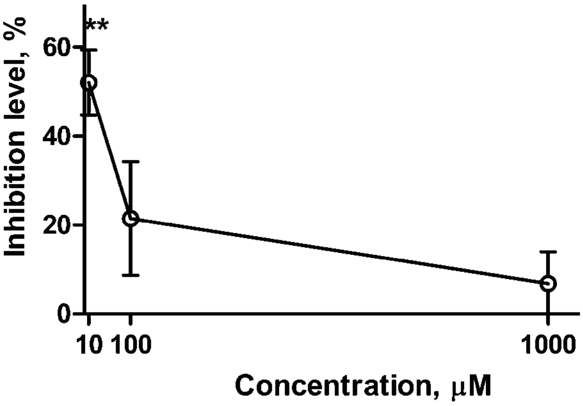

Fig. 3. Intracellular ROS decrease in erythrocytes pre-treated with different concentrations of protocatechuic acid (PCA) and subsequently exposed to extracellular oxidative stress, compared to the control, non-pre-treated, cells (significantly different from control cells: $^{**}p < 0.01$).

fects of lower doses and pro-oxidant effects of higher doses and longer exposure.[23] At doses higher than 10 mM, PCA was also found to induce oxidative stress in both transformed and malignant cells from oral tissue, but in lower non-toxic doses (2.5 mM), it sensitized cells to the pro-oxidant stimuli.[24] Opposing results showing protective effects of PCA isolated from natural source against H_2O_2 induced oxidative stress both *in vitro*, in PC12 cells, as well as *in vivo* in animal models were reported by Shi *et al.*[25] These discrepancies could be partly explained by the reported induction of cellular antioxidant enzymes by the lower concentrations of PCA.[26]

Inhibition of platelet activation – in vitro

The influence of the analysed samples on the expression of two activation markers, P-selectin and GPIIb-IIIa, on the platelet surface after *ex vivo* action of a suboptimal concentration of ADP was investigated. The effects were focused on the activities of WECE regarding its further use in clinical trials. As shown in Fig. 4, P-selectin expression in the whole blood platelets incubated with different concentrations (1.25, 2.5 and 5 mg mL^{-1}) of the water soluble fraction (WCE) and the methanol soluble fraction (MCE) of the water–ethanol chokeberry fruit extract and subsequently treated with ADP (0.5 µM) was not significantly different from the expression of this activation marker in activated non-pre-treated (control) cells. Inhibition levels (%) of P-selectin expression determined after incubation with 1.25, 2.5 and 5 mg mL^{-1} of WCE were –12.64±5.52, –4.84±13.09 and 1.51±5.52, respectively, while with MCE, the obtained values were –2.86±5.99, 1.37±3.72 and 7.50±5.73, respectively, and none of the observed changes were statistically significant. Linearity of the dose-response curves for the investigated concentrations and P-selectin expression were not observed either. Inhibition of

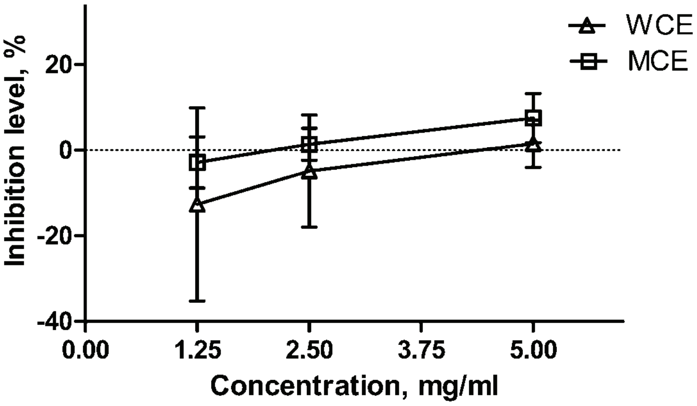

Fig. 4. Inhibition of P-selectin expression in ADP-stimulated platelets pre-treated with different concentrations of the water soluble fraction (WCE) and methanol soluble fraction (MCE) of chokeberry extract compared to control, non-pre-treated cells.

the GPIIb-IIIa activation marker was influenced by the pre-treatment under the same experimental conditions with inhibition levels (%) of –2.96±1.83, 4.25±3.95 and 18.83±8.36 for WCE, and –4.27±2.46, 4.28±1.49 and 21.18±8.79 for MCE, for concentrations of 1.25, 2.5 and 5 mg mL^{-1}, respectively. Although the statistical significance was compromised by the high inter-individual variations, dose-response linearity could be observed (Fig. 5). CCJ and FCJ did not influence the expression of either of measured antigens in analysed concentration range (0.25––75 µL mL^{-1}). An effect of PCA in investigated concentration range (10–1000 µM) was also not observed (data not shown).

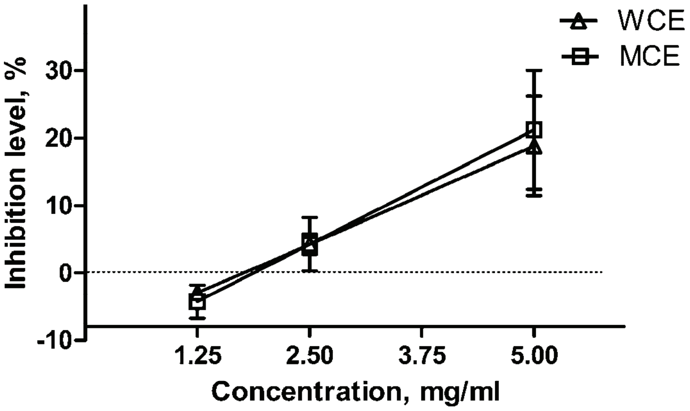

Fig. 5. Inhibition of GPIIb-IIIa expression in ADP-stimulated platelets pre-treated with different concentrations of the water soluble fraction (WCE) and methanol soluble fraction (MCE) of chokeberry extract compared to control, non-pre-treated cells.

Expressions of the platelet surface antigens, P-selectin and GPIIb–IIIa, are sensitive indicators of platelet activation.[15] P-selectin is a protein constitutively expressed in the α-granules of platelets and following platelet stimulation, it becomes expressed on the platelet surface. The active role of P-selectin in thrombosis, coagulation and its crucial role in the pathogenesis of atherosclerosis implies mostly its role in leukocyte recruitment (notably monocytes) as an inflammation process, mediated *via* its interaction with PSGL-1 (P-selectin glycoprotein ligand) constitutively expressed in almost all leucocytes.[27] GPIIb-IIIa is an activation-dependent receptor for fibrinogen and it mediates homotypic platelet aggregation with a crucial role in thrombosis.[28] Based on the results of previous studies, dietary bioactive compounds, including polyphenols, can modulate platelet activation in response to agonist, both *in vitro* and *ex vivo*.[29] Numerous studies reported beneficial effects of chokeberry products or extracts on platelet function *in vitro*, including reduction of nitrative and oxidative stress in platelets[30–32] and platelet aggregation,[33,34] but anti-platelet effects regarding expression of GPIIb-IIIa in response to suboptimal ADP were not investigated previously. Influence of chokeberry fruit extract on P-selectin expression after ADP action was previously investigated.[35] Regarding the anti-platelet activity of PCA, previous studies showed that PCA selectively inhibited shear stress-induced platelet activation and aggregation but did not inhibit platelet aggregation induced by ADP and other endogenous agonists (collagen, thrombin). The proposed mechanism of the observed effects of PCA is the blockage of the von Willebrand factor binding to the activated glycoprotein Ib.[36]

Inhibition of platelet–endothelial cells adhesion – ex vivo *pilot study*

The *ex vivo* effects of CCJ consumption were assessed by employment of the platelet–endothelial cells adhesion assay, according to a previously described procedure. Adhesion of platelets, isolated 2 h after the consumption of CCJ, to endothelial cells (EA.hy926) in culture in the presence of thrombin was inhibited in all subjects compared to the adhesion of the platelets isolated before the consumption. The mean value of inhibition (%) obtained in six subjects was 37.83±22.64, showing significant inhibition of platelet adhesion after chokeberry juice consumption ($p = 0.009$) with a marked inter-individual variation of the obtained values. The results obtained in this pilot study indicate a rationale for future investigation of the observed effects in a larger population and within a controlled study.

As platelet–endothelial adhesion is considered to be a GPIIb-IIIa dependant process,[37] the results obtained *in vivo* are consistent with the results obtained for GPIIb-IIIa inhibition *in vitro*. It is noteworthy that the results of the present pilot study investigating the effects of CCJ consumption on both P-selectin and GPIIb-IIIa expression after *ex vivo* suboptimal ADP action, evaluated by flow cytometry, were also observed (data not shown). The *in vitro* effects of chokeberry extract on platelet–endothelial cells adhesion was previously investigated by Luzak *et al*., who reported that incubation with a low concentration (5 μg mL^{-1}) of chokeberry extract increased the efficacy of human umbilical endothelial cells in culture to inhibit ADP activated platelet adhesion *in vitro*.[35] In the performed study, the effects of chokeberry consumption targeted on the function of platelets were investigated. The assay itself has numerous advantages in the screening of different agents for *in vitro* anti-platelet effects targeted at the inhibition of GPIIb-IIIa or endothelial cell-mediated inhibition of platelet aggregation or effect of the consumption food and food bioactives on *ex vivo* agonist-induced platelet activation and the consequential platelet adherence to endothelial cells in culture.

The only reliable option to confirm the beneficial effects of anthocyanin-rich food in health promotion and the prevention of chronic diseases and to prove the hypothesis that the effects mostly rely on anthocyanins is to conduct a human intervention study designed as a randomized placebo-controlled trial. Numerous constrains on intervention trials with anthocyanins within food matrix include the design of the placebo, the rapid degradation of the bioactive substances, the relevancy of their antioxidant activity in pre-screening for relevant effects of cardiovascular diseases (CVD) and surrogate outcomes.

Biological relevance of direct antioxidant effects of polyphenols in the prevention of chronic diseases, including cardiovascular disease, has recently been re-evaluated. More often experts in the field accept the opinion that the direct antioxidant effect could not explain the numerous health effects observed in both intervention and epidemiological studies.[38] This opinion is based on their poor bioavailability, low concentrations in blood compared with other antioxidants and the decline in their antioxidant activity following ingestion. Based on the results obtained in this study, the *in vitro* antioxidant potential is not reliable for the prediction of numerous effects relevant in CVD prevention.

The degradation of anthocyanins within berry products (especially juices) could be an issue in long term trials and additional strategies for mitigating anthocyanins losses during the intervention period are required. The extract of chokeberries evaluated in this study did not provide satisfactory results compared to the juice and cellular *in vitro* effects were obtained in high non-relevant doses regarding formulation in dosage forms or juice supplementation, suggesting that further optimization in the extraction process is needed. Two cell-based assays, investigating the antioxidant potential and anti-platelet effects were also not correlated for the evaluated fractions of extracts, showing that antioxidant activity is not always a prerequisite for other cellular effects as previously suggested.[38] The *in vitro* anti-platelet effects, although evaluated at high concentrations of extract, are supported by the pilot *ex vivo* trial, but the final conclusions could be made only in an intervention trial. Subjected to intense metabolic transformation, the bioactives could also provide metabolites with opposing effects, compared to the native forms. The effects of long-term consumption of chokeberry extract on platelet aggregation induced by ADP *ex vivo* were recently reported in patients with metabolic syndrome,[5] but the effects on specific activation markers and both homotypic and heterotypic platelet aggregation, with optimal and suboptimal agonist action should be further investigated.

In conclusion, a multifaceted approach in preclinical investigation is the optimal strategy in the screening for potential candidates for health promotion and prevention of chronic diseases, but it still could not guarantee the effects *in vivo*.

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ИЗВОД

ИСПИТИВАЊЕ БИОЛОШКЕ АКТИВНОСТИ АНТИОКСИДАНАСА АРОНИЈЕ (*Aronia melanocarpa*) У ДИЗАЈНИРАЊУ ИНТЕРВЕНТНИХ СТУДИЈА

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Повољни ефекти плодова и сокова аронијe у промоцији здравља и превенцији болести показани су у многим епидемиолошким и интервентним студијама и веома често се повезују са антиоксидативним деловањем њихових састојака. Циљ ове студије је био да се испита антиоксидативно деловање на еритроците и анти-тромбоцитно деловање три различита производа добијена од плода аронијe: комерцијалног сока, свеже цеђеног сока и лиофилизованог водено–етанолног екстракта плода аронијe, у *in vitro* експерименталним условима. Добијени резултати поређени су у односу на садржај укупних полифенола и антоцијана, као и на антиоксидативну активност одређену DPPH тестом. Резултати су указали на значајне разлике у хемијском саставу и антиоксидативној активности испитиваних производа, али директна веза са ефектима на ћелијама, укључујући и антиоксидативно деловање на еритроците и анти-тромбоцитно деловање *in vitro*, није показана. Резултати добијени *ex vivo* у оквиру пилот студије, са једним од испитиваних узорака, једним делом су потврдили резултате добијене *in vitro*. На основу резултата добијених испитивањем антиоксиданаса аронијe показано је да хемијска анализа и *in vitro* експерименти на ћелијским моделима имају велики значај у процени њихове биолошке вредности. Екстраполација добијених резултата у ситуацију *in vitro* је, међутим, често отежана и под утицајем биорасположивости и метаболизма дијетарних биоактивних супстанци.

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