Eur J Nutr (2013) 52:1049–1057
DOI 10.1007/s00394-012-0411-8

ORIGINAL CONTRIBUTION

The polyphenol-rich extracts from black chokeberry and grape seeds impair changes in the platelet adhesion and aggregation induced by a model of hyperhomocysteinemia

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Received: 4 April 2012 / Accepted: 22 June 2012 / Published online: 19 July 2012

Abstract
Objective The mechanism action of the polyphenol-rich extracts from berries of *Aronia melanocarpa* (black chokeberry) and from grape seeds in the defence against homocysteine (Hcy) and its derivatives action in blood platelets is still unknown. In this study, the influence of the aronia extract and grape seeds extract (GSE) on the platelet adhesion to collagen and fibrinogen and the platelet aggregation during a model of hyperhomocysteinemia was investigated. The aim of our study in vitro was also to investigate superoxide anion radicals ($O_2^{-\bullet}$) production after incubation of platelets with Hcy, HTL and the aronia extract and GSE during a model of hyperhomocysteinemia (induced by reduced form of homocysteine at final dose of 100 μM) and the most reactive form of Hcy—its cyclic thioester, homocysteine thiolactone (HTL, 1 μM). Moreover, the additional aim of our study was also to establish and compare the influence of the aronia extract, GSE and resveratrol (3,4′,5-trihydroxystilben), a phenolic compound, which has been supposed to be beneficial for the prevention of cardiovascular events, on selected steps of platelet activation.
Methods The effects of tested extracts on adhesion of blood platelets to collagen and fibrinogen were determined according to Tuszynski and Murphy. The platelet aggregation was determined by turbidimetry method using a Chrono-log Lumi-aggregometer.
Results We have observed that HTL, like its precursor—Hcy stimulated the generation of $O_2^{-\bullet}$ (measured by the superoxide dismutase—inhibitable reduction of cytochrome c) in platelets and caused an augmentation of the platelet adhesion and aggregation induced by the strong physiological agonist—thrombin. Our present results in vitro also demonstrated that the aronia extract and grape seeds extract reduced the toxicity action of Hcy and HTL on blood platelet adhesion to collagen and fibrinogen, the platelet aggregation and superoxide anion radicals production in platelets, suggesting its potential protective effects on hemostasis during hyperhomocysteinemia.
Conclusion In the comparative studies, the aronia extract was found to be more effective antiplatelet factors, than GSE or resveratrol during a model of hyperhomocysteinemia. It gives hopes for development of diet supplements, which may be important during hyperhomocysteinemia.

Keywords Blood platelet · Homocysteine thiolactone · Homocysteine · Aronia · Grape seeds

Introduction

The black chokeberry *Aronia melanocarpa* (*A. melanocarpa*; Family Rosaceae) is native to eastern North America and Canada, and became popular in Eastern Europe and Russia about a century ago. *A. melanocarpa* fruits are one of the richest plant sources of phenolic substances, mainly in flavonoids from the anthocyanin subclass. The anthocyanins are water-soluble plant pigments with antioxidant, anti-inflammatory, antimicrobial, hepatoprotective, gastroprotective and other activities

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[1–4]. Most of the effects of *A. melanocarpa* anthocyanins are due to their antioxidative activity. Our earlier experiments also show that the extract from berries of *A. melanocarpa* reduces in vitro different steps of the platelet activation (the platelet adhesion to collagen and the platelet aggregation) and the production of reactive oxygen species in resting blood platelets and platelets activated by the strong physiological agonist—thrombin [5, 6]. Products derived from the black chokeberry are claimed to be beneficial in disorders or diseases associated with the oxidative stress [7]. Our previous studies demonstrate that a polyphenol-rich commercial extract of *A. melanocarpa* (Aronox) has a protective action on the oxidative/nitrative stress in plasma and blood platelets isolated from breast cancer patients before and after the surgery or various phases of the chemotherapy [8–10]. Moreover, our preliminary results indicate that this extract significantly reduces the oxidative stress in human plasma during the model of hyperhomocysteinemia [11]. Other our experiments showed that the grape seeds extract (rich in phenolic compounds, a mixture of about 95 % oligomeric phenols; GSE) has also antiplatelet properties [12, 13] and suppresses toxicity of hyperhomocysteinemia on the fibrinolytic system [14]. The seeds and skins of grapes contain 50–100 μg of resveratrol/mg of dry weight [15], which inhibits the platelet activation [16–18].

Blood platelet activation, including the platelet adhesion and aggregation under physiological conditions, is an important process to stop bleeding, but it is considered that excessive platelet adhesion and aggregation causes thrombosis and atherosclerosis [19]. A large number of physiological and pharmacological compounds have been found to inhibit the platelet activation [16, 17]. The list includes plant extracts—the aronia extract and GSE [12, 13]. Therefore, the aim of our study was to establish the influence of a natural extract from the berries of *A. melanocarpa* (black chokeberry) and from grape seeds, which have not only antiplatelet, but also antioxidative properties [5, 6] on the selected hemostatic properties of blood platelets (the platelet adhesion and aggregation induced by thrombin) during hyperhomocysteinemia-related blood platelet activation [20] and cardiovascular diseases. We induced hyperhomocysteinemia using the reduced form of Hcy in the concentration 100 μM and HTL in the concentration 1 μM, which correspond to levels found in human plasma during hyperhomocysteinemia in vivo.

Since reactive oxygen/nitrogen species, including superoxide anion radicals ($O_2^{-\bullet}$), play an important role (e.g., as second messengers) in blood platelet activation induced by various physiological agonists [21, 22], we measured the production of $O_2^{-\bullet}$ by the method of reduction of cytochrome c in blood platelets treated with the aronia extract and GSE during a model of hyperhomocysteinemia. In these studies, we also compared the action of tested extracts with effects of well-known commercial monomeric polyphenol—resveratrol (3,4′,5-trihydroxystilben) during a model of hyperhomocysteinemia.

Materials and methods

Materials

Reduced form of D, L-homocysteine, D, L-homocysteine thiolactone, resveratrol, collagen type I and thrombin was purchased from Sigma (St Louis, MO, USA). Fibrinogen was prepared from human blood according to Doolittle et al.' [23]. All other reagents were of analytical grade and were provided by commercial suppliers.

Plant material, extraction and isolation

The *Aronia melanocarpa* has been grown in Poland at large plantations to be used to produce phenolic-rich juice, jams and phenolic-rich extracts. The material used for phenolic-rich extract production came from commercial production of aronia berries.

The HPLC separation of the phenolic-rich extract from berries of aronia (the commercial product—Aronox by Agropharm Ltd, Poland; batch no. 020/2007 k) was described earlier [6, 24]. The total concentration of phenolics in the phenolic-rich powder used in this study was 309.6 mg/g of extract including phenolic acids (isomers of chlorogenic acid)—149.2 mg/g of extract, anthocyanins (anthocyanin glycosides: cyaniding 3-galactoside, cyaniding 3-glucoside, cyaniding 3-arabinoside, cyaniding 3-xyloside)—110.7 mg/g and flavonoids (quercetin glycosides)—49.7 mg/g of extract) [6, 10, 24].

The extract of grape seeds was supplied by Bionorica (Germany). High-performance liquid chromatography (HPLC) was used to separate and determine individual phenolic compounds in GSE. The total concentration of phenolics was 500 mg/g of extract (including total flavanols—250 mg/g of extract and resveratrol—2 mg/g of extract) [12, 13]. Stock solution of aronia extract, GSE and resveratrol was made in 50 % dimethylsulfoxide (DMSO) at the concentration of 25 mg/ml and kept frozen. The final concentration of DMSO in samples was lower than 0.05 %, and in all experiments, its effects were determined.

Analysis of endogenous total Hcy, the reduced form of Hcy and its thiolactone

The natural concentration of total Hcy in plasma was 8–14 μM. The endogenous concentration of reduced form

of Hcy and HTL was about 100 ± 11.2 nM and 0–35 nM, respectively. The classical technique HPLC has been used to the determination of Hcy [25] or HTL [26] in human plasma. The HPLC analysis was performed with a Hewlett-Packard 1100 Series system according to Glowacki et al. [26] and Bald et al. [25].

Isolation of blood platelets

Human blood was taken from healthy volunteers ($n = 10$) aged 22–36 (average, 24; SD = 6.2 years) not taking any medications or addictive substances (including tobacco, alcohol and aspirin or any other antiplatelet drugs) and keeping a balanced diet (meat and vegetables), with similar socioeconomic background, using no antioxidant supplementation.

Human blood was collected into ACD solution (citric acid/citrate/dextrose; 5:1 v/v), and platelets were isolated by differential centrifugation of blood as described by Wachowicz and Kustron [27]. The platelets were counted by the photometric method according to Walkowiak et al. [28]. The entire platelets washing procedure was performed in plastic tubes and carried out at room temperature. Washed human platelet suspensions (3×10^8 platelets/ml) in the modified Tyrode's Ca^{+2}/Mg^{+2} free buffer (127 mM NaCl, 2.7 mM KCl, 0.5 mM NaH_2PO_4, 12 mM $NaHCO_3$, 5 mM HEPES, 5.6 mM glucose, pH 7.4) were exposed (5 min, 37 °C) to:

- D, L-homocysteine at a final concentration 100 μM
- D, L-homocysteine thiolactone at a final concentration 1 μM
- Aronia extract at a final concentration between 2.5 and 10 μg/ml
- Aronia extract at a final concentration between 2.5 and 10 μg/ml and the reduced form of D, L-homocysteine at a final concentration 100 μM
- Aronia extract at a final concentration between 2.5 and 10 μg/ml and D, L-homocysteine thiolactone at a final concentration 1 μM
- GSE at a final concentration between 2.5 and 10 μg/ml
- GSE at a final concentration between 2.5 and 10 μg/ml and the reduced form of D, L-homocysteine at a final concentration 100 μM
- GSE at a final concentration between 2.5 and 10 μg/ml and D, L-homocysteine thiolactone at a final concentration 1 μM.
- Resveratrol at a final concentration 10 μg/ml
- Resveratrol at a final concentration 10 μg/ml and the reduced form of D, L-homocysteine at a final concentration 100 μM
- Resveratrol at a final concentration 10 μg/ml and D, L-homocysteine thiolactone at a final concentration 1 μM.

The protocol was passes by the Committee for Research on Human Subjects of the University of Lodz number KBBN-UŁ/I/5/2011.

Platelet adhesion

Adhesion of blood platelets to collagen and fibrinogen was determined according to Tuszynski and Murphy [29] as described earlier [30]. The absorbance of control platelets (without tested compounds) was expressed as 100 %.

Platelet aggregation

The platelet aggregation was determined by turbidimetry method using a Chrono-log Lumi-aggregometer. After incubation of platelets (3×10^8 platelets/ml) with tested compounds at 37 °C for 5 min, the sample was incubated for 2 min at 37 °C in the aggregation cuvette with stirring, 5 μl of thrombin was added (final concentration 0.1 U/ml) and aggregation was measured.

Monitoring of $O_2^{-\bullet}$ generation in blood platelets

Generation of superoxide anion radicals ($O_2^{-\bullet}$) in control platelets and in platelets incubated with homocysteine, with homocysteine thiolactone or with tested extracts was measured by cytochrome c reduction, as described earlier [31]. For that, an equal volume of Ca^{2+}/Mg^{2+} free Tyrode's buffer, containing cytochrome c (160 μM) was added to a 1-ml suspension of platelets. After incubation, the platelets were sedimented by centrifugation at 2,000 × g for 5 min, and the supernatants were transferred to cuvettes. Reduction of cytochrome c was measured spectrophotometrically at 550 nm. To calculate the molar concentration of $O_2^{-\bullet}$, an extinction coefficient for cytochrome c of 18,700 M^{-1} cm^{-1} was used [32].

Data analysis

All the values in this study were expressed as means ± SD. The statistical analysis (to calculate the differences among the effect of different concentration of aronia extract) was performed with one-way ANOVA test. The statistically significant differences were also assessed by applying the unpaired Student's t test, and the significance level was $p < 0.05$. In order to eliminate uncertain data, the Q-Dixon test was performed.

Results

We observed that the adhesion to collagen or fibrinogen of resting platelets incubated with Hcy (100 μM) was

changed, but this process was not statistically significant ($p > 0.05$) (Table 1). Incubation of platelets with HTL (1 μM) had stimulatory effects on the adhesion of resting platelets ($p < 0.001$) (Table 1). The tested plant extracts from berries of aronia and GSE did not change the adhesion of resting platelets, but they reduced the adhesion of resting platelets in the presence of HTL (1 μM) (Table 1).

Our comparative studies demonstrated that homocysteine and its thiolactone modulated the adhesion of thrombin-stimulated platelets to collagen and fibrinogen (Fig. 1a, b). Moreover, our results (in the model system—in vitro) showed that adhesion to collagen and to fibrinogen of blood platelets incubated with two different polyphenol-rich extracts (from the aronia extract and GSE) and stimulated by thrombin (0.1 U/ml) was changed (Fig. 1a, b). Incubation of platelets with the aronia extract had inhibitory effect on adhesion of thrombin-stimulated platelets to collagen and fibrinogen (Fig. 1a, b), and the effect of its action was concentration-dependent (Fig. 1a, b). The aronia extract reduced also the platelet adhesion in the presence of Hcy (100 μM) or HTL (1 μM), and the action of the aronia extract on this process was concentration-dependent (Fig. 1a, b). Another tested plant extract—GSE was able to prevent adhesion of thrombin (0.1 U/ml)—activated platelets to collagen and fibrinogen in a statistically significant manner (Fig. 1a, b). The inhibitory effect of GSE seems to be concentration-dependent in both adhesion tests. Table 2 reports the percent inhibition of adhesion to collagen of activated platelets induced by all tested concentrations of extracts (2.5–10 μg/ml). The effect of resveratrol (10 μg/ml) is also reported, for comparison, and indicates that the extent of inhibition induced by the polyphenol-rich extracts is higher to that of resveratrol. Table 2 also reports the percent inhibition of adhesion to fibrinogen of activated platelets induced by all tested concentrations of extracts (2.5–10 μg/ml), compared to the

Table 1 The effects of polyphenol-rich extracts from black chokeberry and grape seeds (at final concentrations of 2.5–10 μg/ml) on adhesion of resting platelets to collagen and to fibrinogen and on the level of $O_2^{-\bullet}$ in resting blood platelets during model of hyperhomocysteinemia

	Platelet adhesion to collagen (%)	Platelet adhesion to fibrinogen (%)	Production of $O_2^{-\bullet}$ (nmol $O_2^{-\bullet}/10^8$ platelets)
Control	100	100	0.899 ± 0.174
Hcy (100 μM)	105.9 ± 6.9 (n.s.)	106.9 ± 8.0 (n.s.)	1.254 ± 0.193 (**)
HTL (1 μM)	121.5 ± 7.8 (**)	116.4 ± 4.1 (**)	1.174 ± 0.144 (**)
Aronia extract (2.5 μg/ml)	98.3 ± 12.8 (n.s.)	99.9 ± 12.1 (n.s.)	0.742 ± 0.123 (***)
Aronia extract (5 μg/ml)	97.7 ± 15.8 (n.s.)	98.9 ± 11.0 (n.s.)	0.605 ± 0.156 (***)
Aronia extract (10 μg/ml)	95.8 ± 13.5 (n.s.)	95.9 ± 10.8 (n.s.)	0.534 ± 0.099 (***)
GSE (2.5 μg/ml)	98.9 ± 20.4 (n.s.)	104.9 ± 11.1 (n.s.)	0.749 ± 0.078 (***)
GSE (5 μg/ml)	95.5 ± 12.9 (n.s.)	100.5 ± 9.8 (n.s.)	0.645 ± 0.165 (***)
GSE (10 μg/ml)	104.8 ± 13.5 (n.s.)	99.9 ± 14.2 (n.s.)	0.621 ± 0.087 (***)
Resveratrol (10 μg/ml)	63.6 ± 8.8 (**)	59.9 ± 8.8 (**)	0.754 ± 0.076 (***)
Aronia extract (2.5 μg/ml) + Hcy	99.0 ± 9.2 (n.s)	98.9 ± 5.5 (n.s.)	1.123 ± 0.145 (*)
Aronia extract (5 μg/ml) + Hcy	98.9 ± 7.7 (n.s.)	99.5 ± 6.1 (n.s.)	0.940 ± 0.089 (**)
Aronia extract (10 μg/ml) + Hcy	99.3 ± 9.1 (n.s.)	97.8 ± 5.9 (n.s.)	0.848 ± 0.065 (**)
Aronia extract (2.5 μg/ml) + HTL	106.3 ± 4.0 (*)	107.7 ± 5.9 (*)	1.056 ± 0.076 (*)
Aronia extract (5 μg/ml) + HTL	105.0 ± 3.6 (*)	103.8 ± 5.2 (*)	0.939 ± 0.087 (**)
Aronia extract (10 μg/ml) + HTL	97.4 ± 4.5 (*)	95.5 ± 6.6 (*)	0.851 ± 0.078 (**)
GSE (2.5 μg/ml) + Hcy	105.6 ± 9.5 (n.s.)	100.4 ± 9.9 (n.s.)	1.147 ± 0.099 (*)
GSE (5 μg/ml) + Hcy	107.2 ± 9.1 (n.s.)	107.2 ± 14.3 (n.s.)	0.999 ± 0.097 (**)
GSE (10 μg/ml) + Hcy	106.2 ± 9.5 (n.s.)	99.8 ± 8.1 (n.s.)	0.890 ± 0.154 (**)
GSE (2.5 μg/ml) + HTL	108.4 ± 3.9 (*)	103.8 ± 3.4 (*)	1.028 ± 0.087 (*)
GSE (5 μg/ml) + HTL	99.6 ± 5.1 (*)	90.2 ± 4.8 (*)	0.934 ± 0.123 (**)
GSE (10 μg/ml) + HTL	98.6 ± 4.6 (*)	89.7 ± 4.6 (*)	0.842 ± 0.078 (**)
Resveratrol (10 μg/ml) + Hcy	104.4 ± 1.9 (n.s.)	108.0 ± 2.9 (n.s.)	0.987 ± 0.134 (**)
Resveratrol (10 μg/ml) + HTL	105.4 ± 0.8 (*)	98.6 ± 4.3 (*)	0.923 ± 0.145 (**)

Hyperhomocysteinemia was induced by a reduced form of Hcy (at final dose of 100 μM) or its thiolactone (1 μM). Data represent means ± SD of 10 experiments. Blood platelets (3×10^8 platelets/ml) were preincubated (5 min, 37 °C) with tested compounds (Hcy, HTL and/or plant extracts). Data represent means ± SD of 10 experiments. The effects were statistically significant according to the unpaired Student's *t* test (* $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$; n.s.—$p > 0.05$)

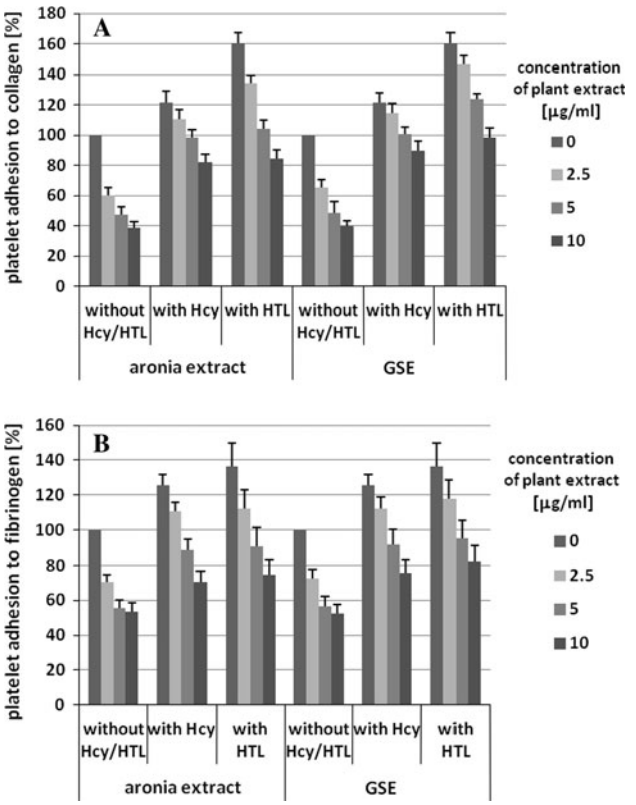

Fig. 1 The effects of polyphenol-rich extracts from black chokeberry and grape seeds (at final concentrations of 2.5–10 μg/ml) on adhesion of thrombin (0.1 U/ml)-activated platelets to collagen (**a**) and to fibrinogen (**b**) during model of hyperhomocysteinemia. Hyperhomocysteinemia was induced by a reduced form of Hcy (at final dose of 100 μM) or its thiolactone (1 μM). Data represent means ± SD of 10 experiments done in quadruplicate. The effect of three different concentrations of aronia extract (2.5, 5 and 10 μg/ml) was statistically significant according to one-way ANOVA test, $p < 0.02$ (for platelets treated with Hcy), $p < 0.001$ (for platelets treated with HTL and platelets treated without Hcy/HTL)

effect of resveratrol (10 μg/ml) during a model of hyperhomocysteinemia.

Using the turbidimetry method, we found that homocysteine (100 μM) and homocysteine thiolactone (1 μM) alone did not induce aggregation of blood platelets in vitro (data are not presented). In another set of experiments, we noted that Hcy and its thiolactone used at tested concentrations increased platelet aggregation induced by the physiological agonist—thrombin (0.1 U/ml) (Fig. 2), but the aronia extract and GSE (for all used concentrations—2.5, 5 and 10 μg/ml) inhibited this process. The aronia extract and GSE (at all tested concentration) also reduced the stimulatory action of Hcy or HTL on blood platelet aggregation induced by thrombin (Fig. 2). In Table 2, the inhibitory effect of tested plant extracts and of resveratrol at 10 μg/ml is reported on platelet aggregation when thrombin was used as platelet agonist during a model of hyperhomocysteinemia.

Hcy and its derivative HTL at tested concentrations stimulated the generation of $O_2^{-\bullet}$ in resting blood platelets and platelets activated by thrombin in vitro (Fig. 3, Table 1). On the other hand, we showed that the aronia extract and GSE (at all tested concentrations) induced a decrease of $O_2^{-\bullet}$ in resting blood platelets and platelets activated by thrombin (Fig. 3, Table 1), and the effect was concentration-dependent (Fig. 3). Moreover, the experiments demonstrated that the aronia extract and GSE (2.5, 5 and 10 μg/ml) reduced the increase of $O_2^{-\bullet}$ in blood platelets (unstimulated and stimulated cells) treated with Hcy or its thiolactone (Fig. 3, Tables 1, 2).

In control experiments, we observed that DMSO, the solvent of the extracts and resveratrol, added to platelet suspensions at a final concentration lower than 0.05 %, did not change the platelet activation in the studied tests (data not shown).

Discussion

Different observations have proposed that Hcy and its derivatives, including HTL may act as an oxidant in the model system in vitro and in vivo [30–37], but diet phenolic antioxidants can inhibit the oxidative stress [30] and may also reduce the toxic action of homocysteine and its thiolactone on various elements of hemostasis (fibrinogen, plasminogen, endothelial cells and blood platelets) [14, 38–40]. Fu et al. [38] reported that red wine prevents homocysteine—induced endothelial dysfunction in porcine coronary arteries. Our earlier results showed that resveratrol, strongly, but not completely reduced the platelet apoptosis induced by Hcy or HTL [41]. Moreover, it showed that resveratrol may protect plasma proteins against modifications (measured by level of thiol and ε-amino groups of Lys) caused by homocysteine or its derivatives—HTL [39]. Our preliminary results in vitro also demonstrated that resveratrol reduced the toxicity action of Hcy and HTL on blood platelets [40]. Other studies showed that red wine polyphenolic compounds supplementation at low dose significantly decreased plasma Hcy levels and restored the hepatic and plasma-decreased paraoxonase-1 activity induced by chronic hyperhomocysteinemia [42]. Moreover, Noll et al. [42] observed that aortic expression of proinflammatory cytokines and adhesion molecules and levels of soluble lectin-like oxidized low-density lipoprotein receptor-1 were reduced in hyperhomocysteinemic mice fed the red wine polyphenolic extract supplementation.

In the present study, we examined the defence properties of polyphenol-rich extracts from black chokeberries and grape seeds, which are an integral part of the human diet, and this study provides more information about biological activity of the aronia extract and GSE during

Table 2 The inhibitory effects of polyphenol-rich extracts from black chokeberry and grape seeds (at final concentrations of 2.5–10 μg/ml) and resveratrol (at final concentration of 10 μg/ml) on the platelet adhesion to collagen or fibrinogen, the platelet aggregation and the production of $O_2^{-\bullet}$ in blood platelets activated by thrombin during model of hyperhomocysteinemia

	The inhibition of platelet adhesion to collagen (%)	The inhibition of platelet adhesion to fibrinogen (%)	The inhibition of aggregation (%)	The inhibition of $O_2^{-\bullet}$ production (%)
Aronia extract (2.5 μg/ml) + Hcy	9.0 ± 2.2 (**)	12.0 ± 5.5 (*)	18.4 ± 2.9 (**)	6.9 ± 0.9 (**)
Aronia extract (5 μg/ml) + Hcy	18.9 ± 3.7 (**)	29.5 ± 6.1 (**)	40.5 ± 4.1 (**)	22.7 ± 1.4 (**)
Aronia extract (10 μg/ml) + Hcy	32.3 ± 4.1 (**)	43.8 ± 5.5 (**)	48.9 ± 4.7 (**)	34.8 ± 2.4 (**)
Aronia extract (2.5 μg/ml) + HTL	16.3 ± 4.0 (**)	17.7 ± 5.9 (**)	26.3 ± 3.3 (**)	9.3 ± 1.0 (**)
Aronia extract (5 μg/ml) + HTL	35.0 ± 3.6 (**)	33.8 ± 5.2 (**)	39.4 ± 4.2 (**)	22.0 ± 2.8 (**)
Aronia extract (10 μg/ml) + HTL	47.4 ± 4.5 (**)	45.5 ± 6.6 (**)	51.3 ± 4.7 (**)	39.7 ± 3.1 (**)
GSE (2.5 μg/ml) + Hcy	5.6 ± 3.5 (*)	10.4 ± 2.9 (**)	14.7 ± 3.3 (**)	2.9 ± 2.0 (n.s.)
GSE (5 μg/ml) + Hcy	17.2 ± 4.1 (**)	27.2 ± 4.3 (**)	35.9 ± 6.7 (**)	17.0 ± 3.7 (**)
GSE (10 μg/ml) + Hcy	26.2 ± 3.5 (**)	39.8 ± 5.1 (**)	43.6 ± 7.1 (**)	26.0 ± 3.9 (**)
GSE (2.5 μg/ml) + HTL	8.4 ± 3.9 (*)	13.8 ± 3.4 (**)	22.8 ± 4.4 (**)	3.1 ± 2.0 (n.s.)
GSE (5 μg/ml) + HTL	22.6 ± 5.1 (**)	30.2 ± 4.8 (**)	34.8 ± 5.8 (**)	13.7 ± 3.9 (**)
GSE (10 μg/ml) + HTL	38.6 ± 4.6 (**)	39.7 ± 4.6 (**)	42.1 ± 6.4 (**)	23.9 ± 3.9 (**)
Resveratrol (10 μg/ml) + Hcy	4.4 ± 1.9 (*)	8.0 ± 2.9 (*)	10.1 ± 2.4 (*)	5.4 ± 3.9 (n.s.)
Resveratrol (10 μg/ml) + HTL	5.4 ± 0.8 (*)	8.6 ± 2.3 (*)	8.9 ± 3.0 (*)	9.1 ± 2.7 (*)

Hyperhomocysteinemia was induced by a reduced form of Hcy (at final dose of 100 μM) or its thiolactone (1 μM). Blood platelets (3×10^8 platelets/ml) were preincubated (5 min, 37 °C) with tested compounds (Hcy, HTL and/or plant extracts). Data represent means ± SD of 10 experiments. The effects were statistically significant according to the unpaired Student's *t* test (* $p < 0.05$; ** $p < 0.001$; n.s.—$p > 0.05$)

hyperhomocysteinemia. In the literature, there are no data about the unwanted and toxic properties of aronia fruits and commercial aronia products (juices, jams and extracts). For the first time, our present results indicate that the aronia extract (Aronox; the same extract used in our previous study [11] and by others [43, 44]) reduced the toxicity action of Hcy and HTL on blood platelet adhesion and aggregation (Figs. 1, 2, Table 1). The range of aronia concentrations, like GSE (2.5–10 μg/ml), is similar to that used in studies of other authors [45, 46], and tested concentrations of aronia extract or GSE are achievable in human plasma during supplementation with these extracts. The in vitro experiments demonstrated that the aronia extract and GSE had anticoagulant properties [47]. Results of Zhang et al. [48] showed the antithrombotic effect of GSE in a rat model of deep vein thrombosis. The present study provides more information about biological activity of GSE during a model of hyperhomocysteinemia and for the first time demonstrated that this extract reduced blood platelet activation induced by the strong physiological agonist—thrombin and also by thrombin during a model of hyperhomocysteinemia (Figs. 1, 2, Table 1).

In blood platelets, reactive oxygen species are produced in the receptor-mediated signaling pathways during platelet activation due to arachidonic acid metabolism by cyclooxygenase or lipooxygenase, the glutathione cycle, metabolism of phosphoinositides and due to xanthine oxidase [21, 22]. Reactive oxygen species induce changes in intraplatelet Ca^{2+} and may behave as second messengers in thrombin- or collagen-activated platelets. Reactive oxygen species generation has been proposed as critical signals regulating platelet activity. Some natural substances with antiplatelet and antioxidative activities, including plant phenolics, can modify this process. Our earlier [12, 13] and present results indicate that the aronia extract and GSE have not only antiplatelet, but also antioxidative properties. It should be underlined that in blood platelets, the oxidative stress induced by Hcy and another important Hcy form—HTL may stimulate functional properties of these cells [35]. Different experiments have provided evidence that

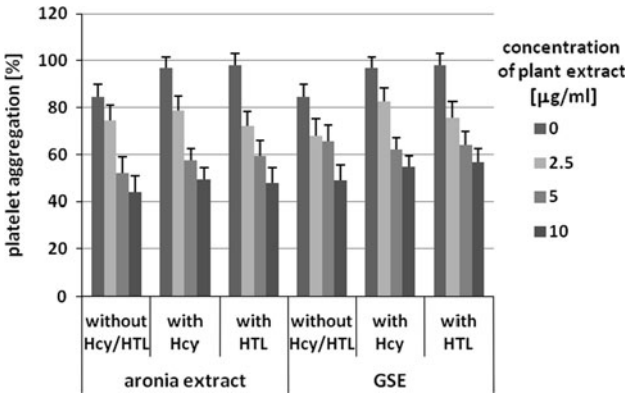

Fig. 2 The effects of polyphenol-rich extracts from black chokeberry and grape seeds (at final concentrations of 2.5–10 μg/ml) on the platelet aggregation stimulated by thrombin during model of hyperhomocysteinemia. Hyperhomocysteinemia was induced by a reduced form of Hcy (at final dose of 100 μM) or its thiolactone (1 μM). Blood platelets (3×10^8 platelets/ml) were preincubated (5 min, 37 °C) with tested compounds (Hcy, HTL and/or plant extracts). After incubation of platelets with these compounds at 37 °C for 5 min with stirring, 5 μl of thrombin (final concentration 0.1 U/ml) was added, and aggregation was measured. Data represent means ± SD of 10 experiments done in triplicate. The effect of three different concentrations of plant extracts (2.5, 5 and 10 μg/ml) was statistically significant according to one-way ANOVA test, $p < 0.001$

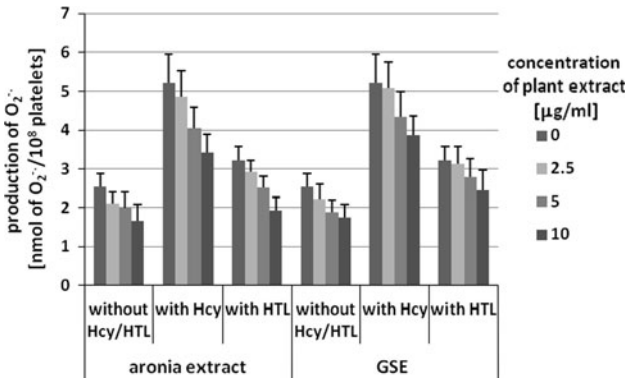

Fig. 3 The effects of polyphenol-rich extracts from black chokeberry and grape seeds (at final concentrations of 2.5–10 μg/ml) on the level of $O_2^{-\bullet}$ in blood platelets activated by thrombin during model of hyperhomocysteinemia. Hyperhomocysteinemia was induced by a reduced form of Hcys (at final dose of 100 μM) or its thiolactone (1 μM). After preincubation (5 min, 37 °C) of blood platelets with tested compounds (Hcy, HTL and/or plant extracts) was added an equal volume of Ca^{2+}/Mg^{2+} free Tyrode's buffer, containing cytochrome c (160 μM), and samples were sedimented by centrifugation at 2,000×*g* for 5 min. The level of $O_2^{-\bullet}$ in the supernatants was measured spectrophotometrically at 550 nm. Data represent means ± SD of 10 experiments done in triplicate. The effect of three different concentrations of plant extracts (2.5, 5 and 10 μg/ml) was statistically significant according to one-way ANOVA test, $p < 0.02$ (for platelets treated without Hcy/HTL and platelets treated with HTL), $p < 0.002$ (for platelets treated with Hcy)

Hcy and HTL may modulate signaling transduction and the response of blood platelets to physiological agonists (thrombin, ADP and collagen) [49–55]. Very important finding of our present results is that tested plant extracts (the aronia extract and GSE) causing the changes in the level of reactive oxygen species, including superoxide anion radicals (Fig. 3, Table 1), may be responsible for the inhibition of platelet activation during hyperhomocysteinemia. However, it seems that during hyperhomocysteinemia, the mechanisms of the aronia extract action may be partly associated not only with the decrease of reactive oxygen species level in platelets, but also with changes of $\alpha_{IIb}\beta_3$ expression on the surface of platelets, because McGarrigle et al. [56] and our earlier results [41] reported that Hcy and HTL cause the expression of $\alpha_{IIb}\beta_3$ (the receptor, which is involved in the platelet aggregation) on the surface of platelets. On the other hand, the aronia extract reduced the expression of $\alpha_{IIb}\beta_3$ [5]. Moreover, our earlier results showed that GSE may inhibit partly the proteolytic activity of thrombin, and GSE may reduce the activation cascade downstream of G-proteins [13].

Experiments presented here showed that not only resveratrol has antioxidative and antiplatelet action during a model of hyperhomocysteinemia, but also the tested phenolic-rich extracts (from aronia fruits and grape seeds) have the same properties, even higher than resveratrol. The basic strategy of these tested extracts action in blood platelets during hyperhomocysteinemia may serve—the aronia extract and GSE cause the changes in reactive oxygen species level, which may be responsible for the modification of platelet reactivity. However, additional mechanism of the aronia extract or GSE may also exist, because our preliminary results have indicated that the aronia extract (at final dosed of 2.5–10 μg/ml) reduced the toxicity action of Hcy and HTL on adhesive properties of fibrinogen [57].

In conclusion, present experiments suggest that anthocyanins and possibly other phenolic compounds, which are presented in the aronia extract and GSE may reduce toxic effect of Hcy and its thiolactone on biological properties of blood platelets, and therefore, the aronia extract and grape seeds extract may become new sources of compounds potentially useful during diseases of blood circulation system induced by hyperhomocysteinemia.

Acknowledgments This work was supported by grant (No 0365/B/PO1/2011/40) from National Science Centre (Poland) and statutory activities of Institute of Soil Science and Plant Cultivation, Pulawy (Poland).

Conflict of interest None to declare.

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