Fibrinogen (Fg) - the precursor of a fibrin clot, is an abundant, multifunctional protein constituting approximately 4% of the total plasma protein (1-3). Fibrinogen not only plays an important role in blood coagulation, but also acts as an adhesive protein essential for blood platelet adhesion and aggregation (4, 5).

Recently, it has been shown that homocysteine (Hcy) and its metabolites, including homocysteine thiolactone (HTL), mediate the modifications of various hemostatic proteins, including plasma proteins (i.e., fibrinogen) and blood platelet proteins (6-8). The modifications of these hemostatic proteins induced by Hcy or its derivatives seem to be the main reason for biotoxicity of homocysteine in cardiovascular system and cardiovascular diseases (6-8). Our earlier studies reported that modifications of fibrinogen induced by Hcy or its thiolactone may be associated with changes in hemostasis, including the coagulation process (8) and blood platelet activation - platelet adhesion (9, 10). Increased concentration of total Hcy (> 15 µM) in plasma is called hyperhomocysteinemia. The aim of our study was to establish the influence of commercial phenolic extract - extract from berries of Aronia melanocarpa (Aronox®), on the changes of adhesive properties of fibrinogen in in vitro model of hyperhomocysteinemia (induced by Hcy and HTL). A. melanocarpa grows in many sites throughout Europe, has no special soil requirements or fertilization needs, is very resistant to pests (no chemical sprayers are needed to have healthy fruits), has fruit every year regardless of conditions, and produces large amounts of polyphenol (including anthocyanins) rich fruits. There are no data in the literature indicating toxic action of Aronia melanocarpa fruits, juice, extracts, or other aronia products, including

**Commercial Extract from Aronia as a Modulator of Adhesive Properties of Fibrinogen Treated with Homocysteine and Its Thiolactone In Vitro**

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**Abstract:** Research has confirmed the positive effect of berries of Aronia melanocarpa on the cardiovascular system. The protective effects of polyphenol-rich extract from berries of A. melanocarpa against changes in biological properties of fibrinogen were studied. In in vitro model of hyperhomocysteinemia the capability of fibrinogen to interact with human blood platelets was measured by platelet adhesion in the presence of extract from A. melanocarpa. We induced hyperhomocysteinemia using a reduced form of homocysteine (Hcy, at a final concentration of 0.01, 0.1 and 1 mM) and the most reactive form of Hcy – its cyclic thioester, homocysteine thiolactone (HTL, at a final concentration of 0.1, 0.5 and 1 µM). It was observed that Hcy or HTL-treated fibrinogen, in comparison with untreated molecule, had a distinct capability to mediate blood platelet adhesion. The experiments also indicate that polyphenol-rich extract from black chokeberries (at final concentrations of 2.5–10 µM/mL) reduced the toxic action of Hcy and HTL on the adhesive properties of fibrinogen. The possible protection exerted by black chokeberry extract, through restoring the platelet adhesion of Hcy or HTL treated fibrinogen, may be important for vascular diseases.

**Keywords:** aronia, fibrinogen, blood platelets, homocysteine, hyperhomocysteinemia

**Abbreviations:** fg – fibrinogen, Hcy – homocysteine, HTL – homocysteine thiolactone

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Aronox®. Moreover, research has confirmed the positive effect of berries of *A. melanocarpa* on the cardiovascular system (11). Aronox® is also rich source of valuable phytochemicals that are responsible for the biological action of this extract (i.e., anti-platelet or antioxidative activity).

**MATERIALS AND METHODS**

**Chemicals**

Reduced forms of D,L-homocysteine and D,L-homocysteine thiolactone were purchased from Sigma (St Louis, MO). Thrombin was purchased from Biomed (Lublin, Poland). The natural concentration of total Hcy in human plasma was 8–14 µM (12).

**Biological materials**

*Aronia melanocarpa* is 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. HPLC separation of phenolic rich extracts from berries of aronia was described previously (13, 14). 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: cyanidin 3-galactoside, cyanidin 3-glucoside, cyanidin 3-arabinoside, cyanidin 3-xylloside) – 110.7 mg/g and flavonoids (quercetin glycosides) – 49.7 mg/g of extract) (14). Stock solutions of aronia extract (the commercial product – Aronox® by Agropharm Ltd., Poland; batch no. 020/2007k) were made in H2O at a concentration of 5 mg/mL and kept frozen until used for experiments.

**Blood platelet and fibrinogen isolation**

Blood samples were taken from 6 healthy volunteers not taking any medications or addictive substances (including tobacco, alcohol and aspirin or any other anti-platelet drugs) and keeping a balanced diet (meat and vegetables), with similar socio-economic background, using no antioxidant supplementation. The protocol was passed by the Committee for Research on Human Subjects of the University of Lodz, number KBBN-UL/I/5/2011; and participants have signed written informed consent forms.

Fg was isolated from pooled citrated human plasma by the cold ethanol precipitation technique followed by ammonium sulfate fractionation at 26% saturation at 4°C, according to Doolittle et al. (15). The concentration was determined spectrophotometrically at 280 nm using an extinction coefficient 1.55 for a 1 mg/mL solution. The concentration of purified Fg in the reaction system was about 2 mg/mL.

Human blood was collected into ACD solution (citric acid/ citrate/dextrose; 5 : 1, v/v) and blood platelets were isolated by differential centrifugation of blood as described by Wachowicz and Kustrowi (16). The platelets were counted by the photometric method according to Walkowiak et al. (17).

We induced hyperhomocysteinemia using a reduced form of Hcy and the most reactive form of Hcy – its cyclic thioester, homocysteine thiolactone. Samples of human Fg (2 mg/mL) in 50 mM Tris/HCl, 140 mM NaCl, pH 7.4 were exposed to:

- D,L-homocysteine at a final concentration 0.01, 0.1 and 1 mM;
- D,L-homocysteine thiolactone at a final concentration 0.1, 0.5 and 1 µM;
- the extract from berries of *A. melanocarpa* at a final concentration between 2.5-10 µg/mL;
- the extract from berries of *A. melanocarpa* at a final concentration between 2.5-10 µg/mL and the reduced form of D,L-homocysteine at a final concentration of 0.1 or 1 mM;
- the extract from berries of *A. melanocarpa* at a final concentration between 2.5-10 µg/mL and D,L-homocysteine thiolactone at a final concentration of 1 µM.

Samples were incubated for 30 min at 37°C. The concentration of Hcy and HTL tested corresponds to levels found in human plasma during hyperhomocysteinemia *in vivo*. Other authors used very similar model for inducing hyperhomocysteinemia (i.e., blood samples were recovered for 10 min prior to their supplementing with Hcy or HTL, each at the final concentration of 25 µM, corresponding to mild hyperhomocysteinemia) (18).

**Blood platelet adhesion**

Adhesion of blood platelets to Fg was determined according to Tuszynski and Murphy (19). Wells of a 96-well microtiter dish (CLINIPLATE EB FB 50 PCS/CRS, Labsystems) were incubated for 2-3 h with 50 mL of Fg (dissolved in phosphate-buffered saline, pH 7.5 - PBS). The wells were aspirated, treated with 200 mL PBS containing 1% BSA for 1 h, and then washed three more times with 200 mL of PBS. Immediately after washing, the wells were supplemented with 50 mL of the test agonist: thrombin (final concentration 0.1 U/mL) in PBS. Then, 100 mL of platelet suspension (4 × 10⁸ platelets/mL) was added to each well and the plate
was incubated at 37°C for 1 h. Non-adherent cells were removed by aspiration and the wells were washed three times with 200 mL of PBS. The total cell-associated protein was determined by dissolving the attached blood platelets directly in the microtiter wells with 200 mL of the Sigma BCA working solution, and incubated at 37°C for 60 min. Plates were allowed to cool to room temperature, cover sheets were removed, and the absorbance of each well determined at 540 nm with a microtiter plate reader (BioRad, Model 550). The absorbance of control platelets (with native Fg) was expressed as 100%.

**Data analysis**

All the values in this study are expressed as the means ± SD. The statistical analysis (to calculate the effect of different concentrations of aronia extract) was performed using ANOVA and post hoc tests (Bonferroni). In order to eliminate uncertain data, the Q-Dixon test was performed.

**RESULTS**

In order to evaluate the altered functionality of Hcy- or HTL-modified Fg in the presence of aronia extract, its ability to mediate platelet adhesion was
assessed. Our studies demonstrate that aronia extract (at all tested concentrations: 2.5, 5 and 10 µg/mL) did not change the ability of Fg to interact with blood platelets (by measuring platelet adhesion) (Fig. 1). On the other hand, as shown in Figure 1, both unstimulated (A) and thrombin-activated platelets (B) showed a reduced ability to adhere to Hcy (0.1 mM) treated Fg. Unstimulated platelets showed a lower capacity to adhere to Hcy (0.1 mM) treated Fg than thrombin-activated platelets. We also demonstrate an inhibitory effect on platelet adhesion to modified Fg when Fg was treated with homocysteine thiolactone (1 µM), and blood platelets were stimulated by thrombin (Fig. 1B). The results presented in Figure 1B demonstrate the protective action of aronia extract on platelet adhesion to modified Fg when it was treated with plant extracts and Hcy or HTL, and blood platelets were stimulated by thrombin. Aronia extract had the same properties when we measured the binding of adherent resting platelets to Hcy-modified Fg (Fig. 1A).

DISCUSSION

Some results showed that homocysteine and its thiolactone may induce the oxidative stress (20, 21). Other experiments indicate that elevated homocysteine and folate deficiency have been associated with increased oxidative stress. Oxidant injury has been proposed as a potential mechanism of atherogenesis in hyperhomocysteinemia (22). Results of Kolling et al. (23) demonstrated that supplementation with folic acid can be used as an adjuvant therapy in cardiovascular alterations caused by Hcy. They observed that Hcy induces oxidative-nitrative stress in the heart of rats, but folic acid has protective properties.

Epidemiologic studies suggest that the regular consumption of polyphenolic antioxidants, secondary metabolites of plants, is correlated with a decrease in the risk of cardiovascular disease, diabetes, arthritis and cancer. Moreover, dietary polyphenolic antioxidants can reduce oxidative stress stimulated by hyperhomocysteinemia (20, 22-30). Our earlier results (29, 31) also suggested that changes in the level of reactive oxygen species (including superoxide anion radicals in blood platelets), caused by plant phenolic extracts (aronia extract and extract from grape seeds) may be responsible for the inhibition of platelet activation (stimulated by thrombin) during hyperhomocysteinemia. Kolodziejczyk et al. (32) observed that grape seed extracts reduced the biotoxicity action of Hcy and HTL on fibrinolysis.

Present experiments have demonstrated that commercial extract from berries of *A. melanocarpa* (Aronox® by Agropharm – used in our earlier tests (13, 14, 31) and by other authors (33-35)) reduces the biotoxicity of Hcy and HTL on the adhesive properties of Fg (measured by the capability of Fg to interaction with blood platelets), suggesting a possible protective role in hyperhomocysteinemia. The range of tested concentrations of aronia extract (2.5–10 µg/mL) is similar to that used in experiments by other authors (34, 35) and concentrations of tested extract in our work (2.5–10 µg/mL) can be achieved in plasma during supplementation with this commercial extract (35). However, the protective action of the aronia extract on Fg during hyperhomocysteinemia is still unclear. It should be underlined that modifications of many of the plasma proteins, including Fg, induced by Hcy and its derivatives, may stimulate various functional changes (Fig. 1). Because our earlier experiments showed that different antioxidants (resveratrol, grape seeds extract and aronia extract) protected plasma and platelet proteins against modifications induced by hyperhomocysteinemia (28-32), now we suggest that the same protective mechanisms caused by aronia extract may exist in our present study, when we used Hcy- and HTL-Fg in the purified system. Moreover, our previous results indicate that aronia extracts decreased the biotoxic action of Hcy and HTL on the other hemostatic properties of Fg and plasma (i.e., clot formation and fibrin lysis) (37). Results of Arts et al. (38) reported that tea polyphenols, including flavonoids react with thiols and –SH groups of proteins. We may also suggest that the interaction between the thiol group of Hcy and polyphenols may play an important function in the protective action of aronia extract on Fg – treated with Hcy or its thiolactone. Therefore, aronia extract seems to be a promising dietary supplement to prevent the cardiovascular or the vascular system during hyperhomocysteinemia.

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Statement of interest

None to declare.
REFERENCES


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