JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 2008, 59, 4, 823–835
www.jpp.krakow.pl

B. OLAS[1*], B. WACHOWICZ[1], P. NOWAK[1], M. KEDZIERSKA[1], A. TOMCZAK[2], A. STOCHMAL[3], W. OLESZEK[3], A. JEZIORSKI[4], J. PIEKARSKI[4]

STUDIES ON ANTIOXIDANT PROPERTIES OF POLYPHENOL-RICH EXTRACT FROM BERRIES OF *ARONIA MELANOCARPA* IN BLOOD PLATELETS

[1]Department of General Biochemistry, University of Lodz, Poland,
[2]Agropharm S.A. Tuszyn, Poland, [3]Department of Biochemistry,
Institute of Soil Science and Plant Cultivation, State Research Institute, Pulawy, Poland,
[4] Department of Oncological Surgery, Medical University of Lodz, Poland

The antioxidant properties of extract from berries of *Aronia melanocarpa* (chokeberry) containing: anthocyanidines, phenolic acids and quercetine glycosides on oxidative/nitrative stress induced by peroxynitrite ($ONOO^-$, a powerful physiological oxidant, nitrating species and inflammatory mediator) in human blood platelets were studied *in vitro*. The extract from *A. melanocarpa* (5 – 50 μg/mL) significantly inhibited platelet protein carbonylation (measured by ELISA method) and thiol oxidation estimated with 5,5'-dithio-bis(2-nitro-benzoic acid) (DTNB) induced by peroxynitrite (0.1 mM) (IC_{50} - 35 μg/mL for protein carbonylation, and IC_{50} - 33 μg/mL for protein thiol oxidation). The tested extract only slightly reduced platelet protein nitration (measured by C- ELISA method). The extract also caused a distinct reduction of platelet lipid peroxidation induced by peroxynitrite. Moreover, in our preliminary experiments we observed that the extract (50 μg/mL) reduced oxidative/nitrative stress in blood platelets from patients with breast cancer. The obtained results indicate that *in vitro* the extract from *A. melanocarpa* has the protective effects against peroxynitrite – induced oxidative/nitrative damage to the human platelet proteins and lipids. The extract from *A. melanocarpa* seems to be also useful as an antioxidant in patients with breast cancer.

Keywords: *aronia, blood platelets, oxidative stress, peroxynitrite*

INTRODUCTION

There are many nutritive and non-nutritive compounds present in the different plants or vegetables origin affecting blood platelet functions – an important

process in hemostasis and in various cardiovascular, cancer and inflammatory diseases (1). Moreover, the *in vitro* studies demonstrated not only antiplatelet activity, but also antioxidative properties for a number of compounds present in vegetables and fruit juices (1). Therefore the compounds that inhibit platelet function and have antioxidative activity are of great interest, however, the molecular basis for this protective properties is unclear.

Aronia melanocarpa (A. melanocarpa), native to eastern North America, has become popular in Eastern Europe and Russia. *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, anticancer, hepatoprotective, gastroprotective and other activities (2, 3). Most of the effects of *A. melanocarpa* anthocyanins are due to their antioxidative activity. Our earlier preliminary experiments showed that the extract from berries of *A. melanocarpa* reduces *in vitro* different steps of platelet activation (platelets adhesion to collagen and platelet aggregation) and the production of reactive oxygen species in resting blood platelets and platelets activated by strong physiological agonist – thrombin (4). Although the molecular basis for the biological activity of natural extract form *A. melanocarpa* is not understood, its action seems to be due to the antioxidant properties (4, 5). Therefore the aim of our study was to assess if polyphenol-rich extract from berries of *A. melanocarpa* (which may be present in human diet and is good source of phenolic compounds) may protect platelet lipids and proteins against oxidative/nitrative damage induced by peroxynitrite ($ONOO^-$). Peroxynitrite is a highly reactive species, produced by the reaction between the superoxide anion ($O_2^{-\bullet}$) and nitric oxide ($NO^\bullet$); $ONOO^-$ may oxidize and covalently modify a wide range of biomolecules, including DNA, proteins and lipids. The toxic effects of $ONOO^-$ on blood platelets are associated with the modulation of their haemostatic function (6, 7). In our study we measured nitrotyrosine level as a marker of platelet protein nitration caused by $ONOO^-$. Oxidative damage to platelet proteins induced by peroxynitrite was monitored by estimation the level of carbonyl and thiol groups. We also measured the platelet lipid peroxidation by the level of thiobarbituric acid – reactive substances (TBARS). Moreover, we investigated the effects of polyphenol rich extract of *A. melanocarpa* on various biomarkers of oxidative/nitrative stress in platelets from patients with breast cancer and in healthy group.

MATERIALS AND METHODS

Plant material

The commercial extract of aronia (*Aronia melanocarpa)* berries (1 kg) was supplied by Agropharm Ltd, Poland. Voucher sample has been deposited in Agropharm. The berries were extracted with EtOH-H_2O (1:1). The extract was fractionated to obtain fraction rich with

anthocyanins that was dried by spray drying to produce a powder. The phenolic rich powder was obtained with the efficiency of 0.6% of berries.

The concentration of phenolics in the extract was determined by HPLC (Waters with 996 PAD detector, 616 Pump and Millenium software) using analytical column (4.6 x 25 cm, Eurospher 100, RP18, 5 µm, Säulentechnik) heated to 50°C, and gradient system (AcN-1%H_3PO_4). Solvent A 1%H_3PO_4, solvent B 40% $CNCH_3$ (linear gradient 20%B ›40%B over 70 min) was used and the determination was based on standard curves prepared for cyanidin 3-*O*-galactopyranoside for antocyanins, chlorogenic acid for phenolic acids and rutin for flavonoids, respectively.

Identification of phenolics was performed by LC-DAD-ESI/MS/MS. An LC system consisting of Finnigan Surveyor pump equipped with a gradient controller, an automatic sample injector and PDA detector was used. The separation was performed on a 250 x 4 mm i.d., 5 µm Eurospher 100 C_{18} column (Knauer, Germany). A mobile phase consisted of 5% acetic acid in water (B) and 5% acetic acid in acetonitrile (A) for the separation was used. The flow-rate was kept constant at 0,5 mL/min for a total run time of 90 min.

A Thermo Finnigan LCQ Adventage Max ion-trap mass spectrometer with an electrospray ion source was coupled to the HPLC system described above. The samples were introduced on column *via* automatic sampler injector. The spray voltage was set to 4.2 kV and a capillary offset voltage was –60 V. All spectra were acquired at a capillary temperature of 220 °C. The calibration of the mass range (400–2000 Da) was performed in negative ion mode. Nitrogen was used as sheath gas and the flow rate was 0.9 L/min. The maximum ion injection time was set to 200 ms.

Chemicals

Stock solution of the extract of aronia was made in H_2O at the concentration of 5 mg/mL; kept frozen and was used for platelet experiments. All other reagents were of analytical grade and were provided by commercial suppliers. 5,5'-dithio-bis(2-nitro-benzoic acid) (DTNB), rabbit anti-DNP antibody and 2,4-dinitrophenylhydrazine (DNPH) were purchased from Sigma. Peroxynitrite was synthesized according to the method of Pryor and Squadrito (8). Freeze fractionation (-70°C) of the peroxynitrite solution formed a yellow top layer, which was retained for further studies. The top layer typicall contained 80-100 mM peroxynitrite as determined spectrophotometrically at 302 nm in 0.1 M NaOH (ε_{302nm} = 1679 M^{-1} cm^{-1}). Some experiments were also performed with decomposed $ONOO^-$, which was prepared by allowing the $ONOO^-$ to decompose at neutral pH (7.4) in 100 mM potassium phosphate buffer (15 min, room temperature). All other reagents were of analytical grade.

Isolation of blood platelets

Human blood was taken from healthy volunteers aged 23 to 36 (average: 26.5; SD = 4.6 years) 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 platelet suspension was then prepared according to the washing procedure described previously (4).

The studied population of patients with breast cancer comprised 10 patients between the ages of 18 – 36 years (average: 34.2; SD = 5.9 years) who were hospitalized in Department of Oncological Surger, Medical University in Lodz, Poland. None of the patients received aspirin or any other anti-platelet drugs and none were alcohol/drug abuser and without clinic manifestation of allergy and infection.

The protocol was accepted by the Committee for Research on Human Subjects of the Medical University of Lodz number RNN/252/07/KB.

Blood platelets were finally suspended in Ca^{2+}/Mg^{2+} free Tyrode's buffer at the final concentration of 2.5 - 3 x 10^8 platelets/mL. Platelets suspensions were incubated with the extract of aronia at the final concentrations of 5; 7.5; 10; 25 and 50 µg/mL (5 min, 37°C) and then with $ONOO^-$ (0.1 mM, 5 min, 37°C). We also measured oxidative/nitrative stress (by the level of thiol groups and 3-nitrotyrosine in proteins) after incubation (5 min, 37°C) of blood platelet with the extract of aronia at the final concentration of 50 µg/mL, isolated from control group and patients with breast cancer. The platelets were counted by the photometric method as described by Walkowiak *et al.* (9).

Detection of thiol groups in blood platelet proteins

Washed human platelet suspensions in Tyrode's buffer were incubated with the extract of aronia at the final concentrations of 5; 7.5; 10; 25 and 50 µg/mL (5 min, 37°C) and then with $ONOO^-$ (0.1 mM, 5 min, 37°C). To frozen control or tested compounds-treated platelets (1 mL of platelet suspension) 1 mL of protein precipitating solution was added (30% NaCl, 0.85% H_3PO_4, 0.2% EDTA). Acid-soluble (glutathione) and acid-insoluble (proteins) platelet fractions were separated according to Ando and Steiner (10, 11) and then the amount of free thiol groups in the acid-insoluble (proteins) fraction was estimated with 5,5'-dithio-bis(2-nitro-benzoic acid) (DTNB) (10, 11). To the pellet (the acid-precipitable fraction) 5 mL of H_2O and 3 mL of 10% SDS were added. After solubilization, 0.5 mL of samples were taken and free thiol groups were determined (10, 11). A standard -SH curve was prepared for GSH.

Detection of carbonyl groups in blood platelet proteins by ELISA method

Detection of carbonyl groups by an ELISA method in blood platelets (control or incubated with the extract of aronia at the final concentrations of 5; 7.5; 10; 25 and 50 µg/mL (5 min, 37°C) and with $ONOO^-$ (0.1 mM, 5 min, 37°C) was carried out according to the procedure of Buss *et al.* (12), with modifications as described previously (13).

Determination of nitrotyrosine in the proteins of blood platelets by a competition ELISA method

Detection of nitrotyrosine-containing proteins by a competition ELISA (C-ELISA) method in blood platelets (control or the extract of aronia at the final concentrations of 5; 7.5; 10; 25 and 50 µg/mL (5 min, 37°C) and with $ONOO^-$ (0.1 mM, 5 min, 37°C) -treated platelets) was performed according to the procedure of Khan *et al.* (14) as described previously (13). The nitro-fibrinogen (at concentration of 0.5 µg/mL and 3-6 mol nitrotyrosine/mol protein) was prepared for use in the standard curve. The linearity of the method was confirmed by the construction of a standard curve ranging form 10 to 500 nM nitrotyrosine-fibrinogen equivalent. The concentrations of nitrated proteins that inhibit anti-nitrotyrosine antibody binding were estimated from the standard curve and are expressed as nitro-Fg equivalents. The amount of nitrotyrosine present in fibrinogen after treatment with peroxynitrite (at final concentration of 1 mM) was determined spectrophotometrically (at pH 11.5, ε_{430nm} = 4400 M^{-1} cm^{-1}).

Production of thiobarbituric acid reactive substances (TBARS) in blood platelets

Incubation of blood platelets suspensions (control and incubated with the extract of aronia at the final concentrations of 5; 7.5; 10; 25 and 50 µg/mL (5 min, 37°C) and then with $ONOO^-$ (0.1 mM, 5 min, 37°C)) was stopped by cooling the samples in an ice-bath. Samples of platelets were transferred to an equal volume of 20% (v/v) cold trichloroacetic acid in 0.6 M HCl and centrifuged

at 1200 × g for 15 min. One volume of clear supernatant was mixed with 0.2 volume of 0.12 M thiobarbituric acid in 0.26 M Tris at pH 7.0 and immersed in a boiling water bath for 15 min. Absorbance at 532 nm was measured and results were expressed as nmoles of TBARS (15).

Data analysis

In order to eliminate uncertain data, the Q-Dixon test was performed. All the values in this study were expressed as means ± SEM. The statistical analysis was performed with one-way ANOVA for repeated measurements. The statistically significant differences were also assessed by applying the paired Student's t-test. The significance of correlation coefficient r, was analysed by test t.

RESULTS

The HPLC separation of the phenolic rich extract from berries of aronia revealed in the 254 nm profile the presence of a number of peaks (*Fig. 1*). They were tentatively identified based on UV spectra as phenolic acids (2 compounds), antocyanins (6 compounds) and flavonoids (6 compounds). Profile at 515 nm

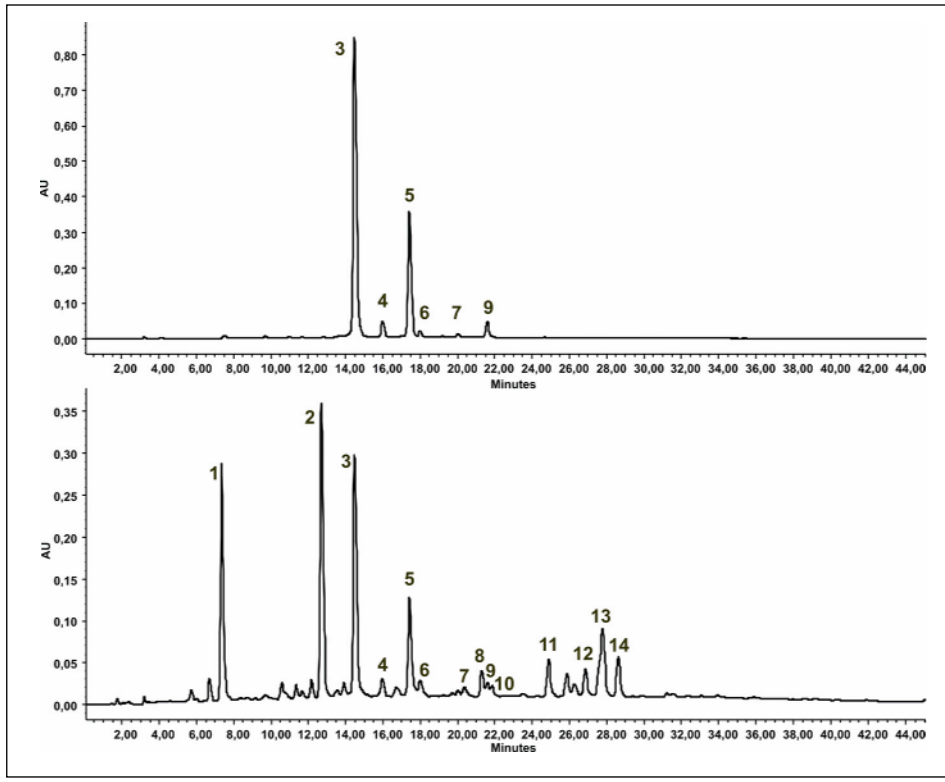

Fig. 1. HPLC profiles of aronia phenolic rich extract registered at 515 nm (upper) and at 254 nm (lower).

(*Fig. 1*) revealed six peaks corresponding to anthocyanins, out of which cyanidin 3-*O*-galactopyranoside was the dominant one. The identities of the compounds were confirmed by LC-ESI/MS/MS analyses. Two phenolic acids showed the pseudomolecular ion at *m/z* 353 $(M-H)^-$, characteristic for isomers of chlorogenic acid. The compounds 3 (*m/z* 494), 4 (*m/z* 449), 5 (*m/z* 419), 6 (*m/z* 517), 7 (*m/z* 419), 9 (*m/z* 627) were classified as anthocyanin glycosides. The compounds 8 (*m/z* 625), 10 (*m/z* 625), 11 (*m/z* 463), 12 (*m/z* 595, 13 (*m/z* 609), 14 (*m/z* 463) showing in MS/MS fragmentation an ion at *m/z* 301, characteristic for quercetin were identified as quercetin glycosides. The concentrations of phenolics in the extract were determined as: phenolic acids - 149.2 mg/g, anthocyanins 110.7 mg/g and flavonoids 49.7 mg/g. Thus, the total concentration of phenolics in the rich phenolic powder used in this studies was 309.6 mg/g.

The extract from aronia significantly (ANOVA test - $p<0.005$) inhibited peroxynitrite -induced carbonylation of platelet proteins. The inhibitory effects of the extract were dose-dependent (analysis of correlation – $r = -0.885$ ($p<0.02$)) (*Fig. 2A* and *Table 1*), with IC_{50} of 35 μg/mL. The strongest inhibition of peroxynitrite-induced platelet protein carbonylation at the highest concentration of the extract (50 μg/mL) was observed (Student's t-test – $p<0.01$) when inhibition reached about 61% (*Table 1*). Formation of carbonyl groups in the platelet proteins (in the presence of the extract from aronia and peroxynitrite) was assayed by ELISA method, which is based on the covalent reaction of the carbonyl groups with 2,4-dinitrophenylhydrazine.

We have shown that exposure of blood platelets to peroxynitrite at the concentration of 0.1 mM resulted in a distinct depletion of free thiol groups in platelet proteins (*Fig. 2B*). After 5 min treatment of blood platelets with $ONOO^-$ (0.1 mM) the amount of protein thiol groups decreased by about 50 % (*Fig. 2B*). The presence of the tested extract from aronia protected platelet protein thiols from oxidation induced by $ONOO^-$ and this effect was dose-dependent (ANOVA test – $p<0.005$; *Fig. 2B*), with IC_{50} of 33 μg/mL. At lowest concentration of the extract used in the experiments (5 μg/mL) the level of thiol groups in platelet proteins was reduced only by about 5% (Student's t-test – $p<0.05$; *Table 1*).

Our studies demonstrated that the exposure of blood platelets to peroxynitrite resulted in the formation of nitrotyrosine in platelet proteins, as determined by C-ELISA method (*Fig. 3A*); the extract from aronia at the concentrations between 5 and 50 μg/mL slightly diminished tyrosine nitration in platelet proteins (ANOVA test – $p>0.05$; *Fig. 3A*). The maximum inhibition (about 15%) of protein nitration was observed when the extract at the concentration of 50 μg/mL was used (Student's t-test – $p<0.01$; *Table 1*).

Moreover, we observed that the extract from aronia suppresses peroxynitrite toxicity measured by the thiobarbituric acid technique (expressed as TBARS) and this effect was dose-dependent (ANOVA test – $p<0.05$; *Fig. 3B, Table 1*).

In control experiments, we have observed that the decomposed $ONOO^-$ did not change the level of TBARS. It also did not cause the tyrosine nitration in platelet

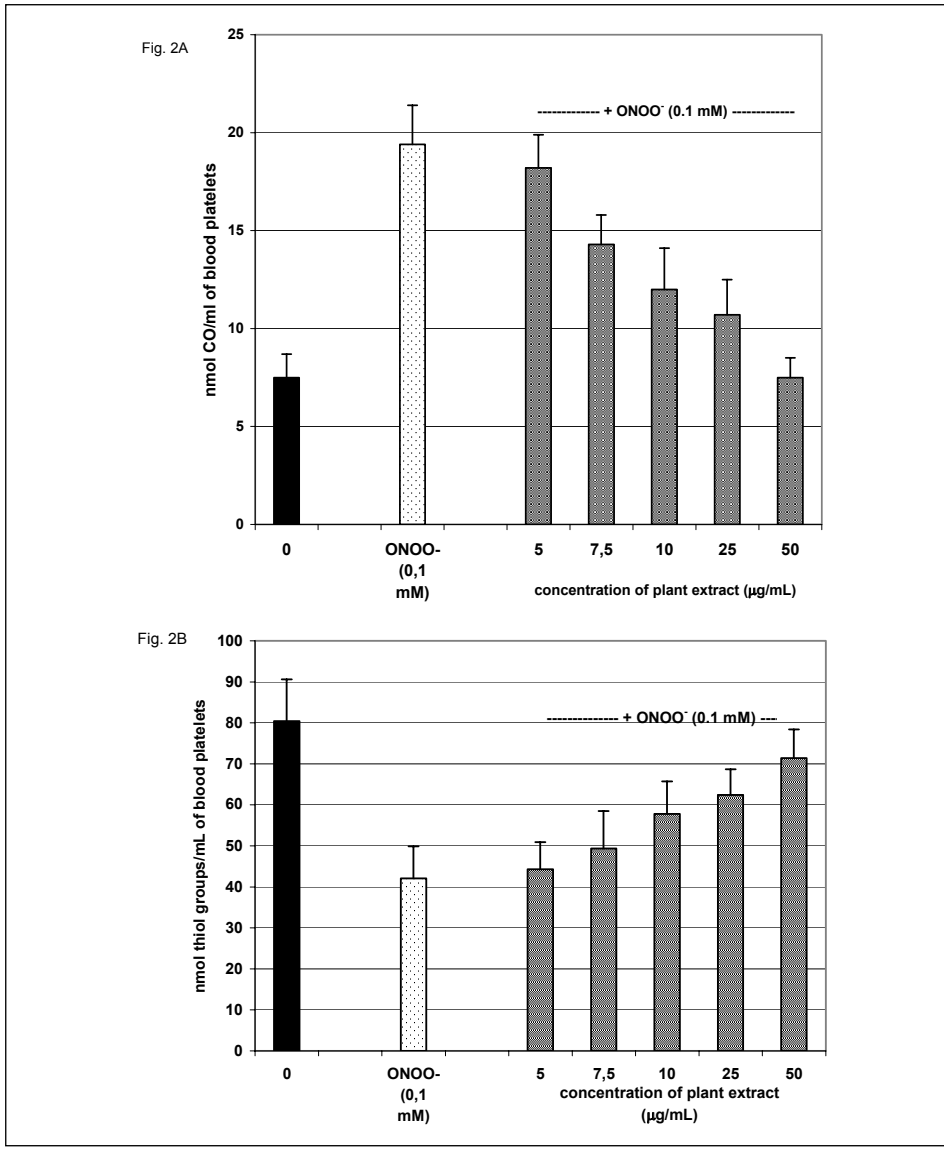

Fig. 2A and *B*. The effect of the extract from berries of *A. melanocarpa* (5-50 µg/mL, 5 min, 37°C) on protein oxidation (CO group formation (A) and the level of thiol groups (B)) in blood platelets treated with $ONOO^-$ (0.1 mM, 5 min, 37°C). The results are expressed as nmol CO/mL of blood platelets and as nmol thiol groups/mL of blood platelets. Data represent means ± SEM of six experiments done in triplicate. The effects of five different concentrations of tested extract were statistically significant according to one-way ANOVA test, $p<0.005$ (for the level of CO groups or thiol groups). Statistical analysis of correlation: for the level of CO groups - $r = -0.885$ ($p<0.02$); for the level of thiol groups - $r = 0.911$ ($p<0.02$). Positive control for $ONOO^-$ treated blood platelets is platelets without $ONOO^-$; positive control for plant extract and $ONOO^-$ treated blood platelets is platelets with only $ONOO^-$.

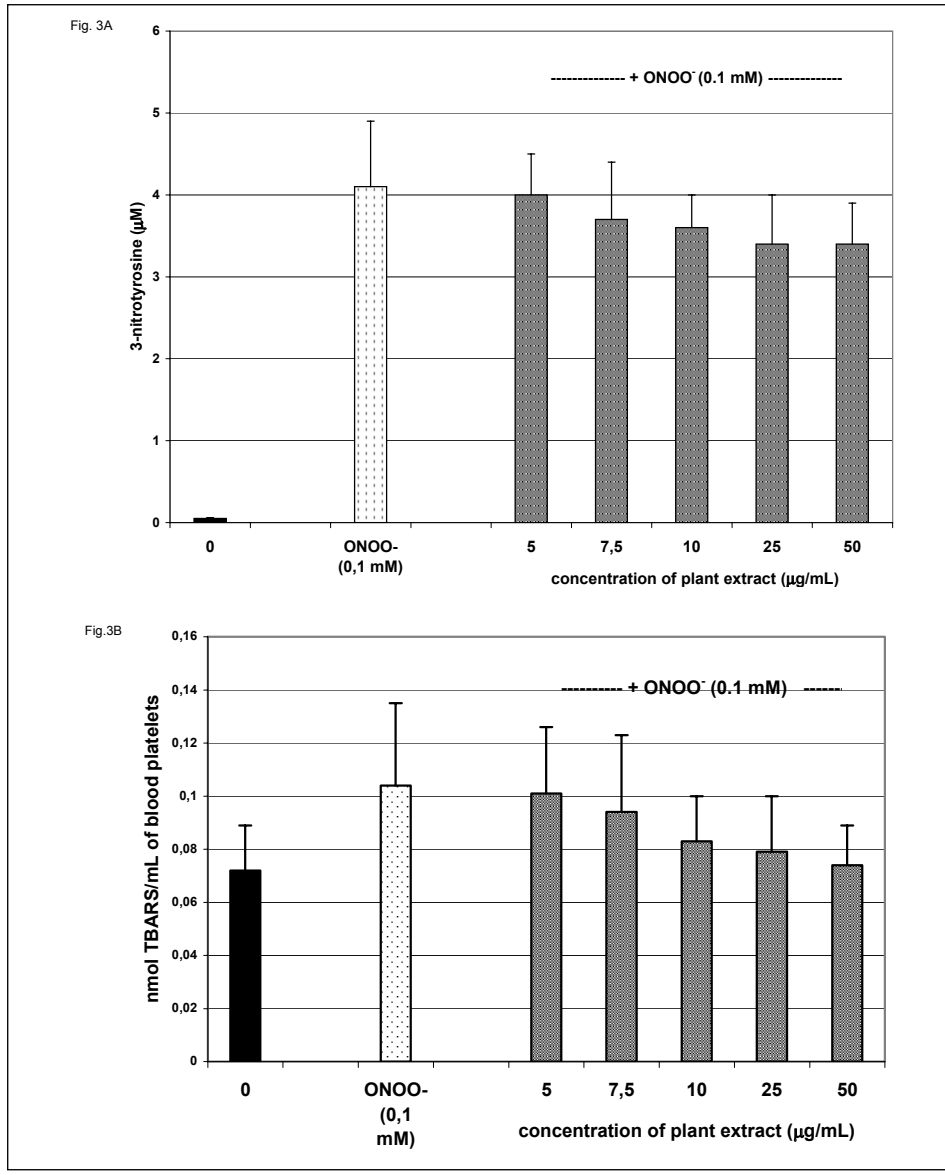

Fig. 3A and *B.* The effect of the extract from berries of *A. melanocarpa* (5-50 μg/mL, 5 min, 37°C) on 3-nitrotyrosine formation (protein nitration) (A) and on the level of TBARS (lipid peroxidation) (B) in blood platelets treated with $ONOO^-$ (0.1 mM, 5 min, 37°C). Data represent means ± SEM of six experiments done in triplicate. The effects of five different concentrations of tested extract were statistically significant according to one-way ANOVA test, $p>0.05$ (for protein nitration) and $p<0.05$ (for lipid peroxidation). Statistical analysis of correlation: for protein nitration - $r = -0.741$ ($p>0.05$); for lipid peroxidation - $r = -0.858$ ($p<0.05$). Positive control for $ONOO^-$ treated blood platelets is platelets without $ONOO^-$; positive control for plant extract and $ONOO^-$ treated blood platelets is platelets with only $ONOO^-$.

Table 1. The effects of phenolic compounds present in the extract from berries of *A. melanocarpa* (5-50 μg/mL, 5 min, 37°C) on different markers of oxidative stress in blood platelets treated with $ONOO^-$ (0.1 mM, 5 min, 37°C). Inhibition of oxidative stress by the extract from berries of *A. melanocarpa* was expressed as percentage of that recorded for blood platelets treated with $ONOO^-$. The results are representative of six independent experiments, and are expressed as means ± SEM. The effect of the extract from berries of *A. melanocarpa* was statistically significant according to the paired Student's t-test.

The concentration of the extract from berries of *A. melanocarpa* (μg/mL)	Inhibition of protein oxidation measured by the level of carbonyl groups (%)	Inhibition of protein oxidation measured by the level of thiol groups (%)	Inhibition of protein nitration measured by the level of 3-nitrotyrosine (%)	Inhibition of lipid peroxidation measured by the level of TBARS (%)
5	6.2 ± 1.1 ($p<0.05$)	5.2 ± 0.9 ($p<0.05$)	2.5 ± 0.7 ($p>0.05$)	2.9 ± 0.5 ($p<0.05$)
7.5	26.3 ± 1.9 ($p<0.05$)	17.3 ± 2.2 ($p<0.05$)	7.5 ± 1.7 ($p<0.05$)	9.6 ± 1.1 ($p<0.05$)
10	38.1 ± 2.4 ($p<0.02$)	37.3 ± 3.1 ($p<0.05$)	12.5 ± 2.1 ($p<0.05$)	20.2 ± 2.5 ($p<0.05$)
25	44.8 ± 3.1 ($p<0.02$)	48.2 ± 2.8 ($p<0.02$)	15.0 ± 2.9 ($p<0.05$)	24.0 ± 2.9 ($p<0.05$)
50	61.3 ± 5.5 ($p<0.01$)	69.6 ± 5.7 ($p<0.01$)	15.1 ± 3.1 ($p<0.05$)	28.8 ± 3.1 ($p<0.05$)

proteins, nor did protein oxidation (measured by carbonyl groups and thiol groups) (data are not presented).

In another set of experiments, we have shown an increased level of biomarkers of oxidative/nitrative stress such as 3-nitrotyrosine in platelet proteins from breast cancer patients. In patients with breast cancer the amount of thiol groups in platelet proteins was decreased. We have demonstrated that the extract at the concentration of 50 μg/mL added to blood platelets significantly reduce oxidative/nitrative stress in tested cells from patients with breast cancer (*Table 2*).

DISCUSSION

Recently, attention has been focussed on preventive strategies for cancer and cardiovascular diseases. A large number of natural compounds present in the human diet has been linked to a possible decrease in the incidence of platelet-related disorders and to the risk of developing cancers. Research of some authors indicated that the extract from *A. melanocarpa* rich in polyphenols has antimutagenic activity and exhibits a distinct immunomodulatory activity in human lymphocyte cultures and in patients with breast cancer (3). Our earlier experiments *in vitro* showed that

Table 2. The effects of phenolic compounds present in the extract from berries of *A. melanocarpa* (50 µg/mL, 5 min, 37°C) on the oxidative/nitrative stress (measured by the level of thiol groups and 3-nitrotyrosine in proteins) in platelets from patients with breast cancer and in healthy group. Patients group n = 10 and healthy subjects group n = 10. The results are representative of 10 independent experiments in triplicate and expressed as a mean ± SEM. The statistical analysis of difference between the control platelets (without the extract) and platelets treated with the extract was done with paired Student's t-test.

	The level of thiol groups in blood platelet proteins [mmol/mg of platelet proteins]	The level of 3-nitrotyrosine in blood platelet proteins [µ M]
Healthy subject group		
Blood platelets were not incubated with the extract from berries of *A. melanocarpa* (control)	86.5 ± 27.5	0.019 ± 0.002
Blood platelets were incubated with the extract from berries of *A. melanocarpa*	80.5 ± 20.4 ($p>0.05$)	0.018 ± 0.003 ($p>0.05$)
Patients with breast cancer		
Blood platelets were not incubated with the extract from berries of *A. melanocarpa* (control)	43.4 ± 23.4	2.784 ± 0.533
Blood platelets were incubated with the extract from berries of *A. melanocarpa*	75.1 ± 19.8 ($p<0.001$)	0.192 ± 0.074 ($p<0.0002$)

the aronia extract reduces not only generation of $O_2^{-\bullet}$ in blood platelets, but also platelet adhesion and aggregation (4). Results of Ryszawa *et al.* (5) *in vivo* demonstrated that the extract from *A. melanocarpa* caused an decrease in superoxide production only in patients with cardiovascular risk factors, whereas the inhibitory effect was not observed in control groups without risk factors for arteriosclerosis; this extract exerted anti-aggregatory effects on platelets in both studied groups. Moreover, $NO^{\bullet}$ derived from platelets was not involved in protective, direct anti-aggregatory effects of *A. melanocarpa* on platelets (5). Kowalczyk *et al.* (16) showed that administration of 240 mg of aronia anthocyanins (Aronox by Agropharm – the same extract used in our earlier and present studies) for 30 days in men with hypercholesterolemia, caused an increase of glutathione peroxidase and catalase activities in red blood cells. Combination therapy of statin with flavonoids rich extract from chokeberry fruits enhanced reduction in cardiovascular risk markers in patients after myocardial infraction (17, 18). Our preliminary results, for the first time showed that the extract from *A. melanocarpa* reduced oxidative/nitrative stress in blood platelets from breast cancer patients (*Table 2*). Some studies demonstrated that aronia crude extract has also anti-

inflammatory effect that is due to the direct blocking of the expression of the inducible nitric oxide synthase in a mouse macrophage cell line (RAW 264.7) (19, 20). In the present study we analysed the ability of the extract from *A. melanocarpa* to prevent peroxynitrite-mediated oxidation/nitration of different biomolecules in blood platelets. The concentrations of the extracts of *A. melanocarpa* (5-50 μg/mL) were similar to that used in studies of other investigators (5). The concentration of peroxynitrite (0.1 mM) used in our experiments was relatively high. The lifetime of peroxynitrite at physiological pH is very short; its half-time being of the order of 1 s. Exposure to a bolus of 250 μM peroxynitrite is equivalent of 7 min exposure to a steady-state $ONOO^-$ concentration of 1 μM. This concentration could be readily formed at sites of inflammation, where production of rates of $NO^\bullet$ and superoxide radicals considerably increases (21). Peroxynitrite forming in vascular system may cause oxidative/nitrative stress and a damage to some biological molecules in platelets such as proteins and lipids (22). These modifications of platelet biomolecules may be responsible for inhibition of hemostatic function after exposure of platelets to $ONOO^-$ (6, 7, 22). There is no information about the role of the extract from *A. melanocarpa* in the inhibition of oxidative/nitrative stress in these cells treated with peroxynitrite as an inflammatory mediator. Our present studies, for the first time, reported that the extract from *A. melanocarpa* suppresses the peroxynitrite toxicity measured as platelet protein nitration (*Fig. 3A*) and protein oxidation (measured by the level of carbonyl or thiol groups (*Fig. 2A* and *B*)). Moreover, our results show that this extract also suppresses the peroxynitrite toxicity expressed as platelet lipid peroxidation (*Fig. 3B*). The present study provides evidence of antioxidant properties of the extract and its ability to scavenge a highly reactive oxidant – peroxynitrite. It seems that antioxidative activities of the extract may be responsible for medicinal properties of this plant. The mechanisms of the protectory action of the extract from *A. melanocarpa* containing different compounds against oxidative/nitrative modifications induced by $ONOO^-$ or its intermediates remain unknown. The presented results may suggest that compounds present in the extract of *A. melanocarpa* may react with peroxynitrite and form products less toxic than $ONOO^-$ alone, however the interaction of $ONOO^-$ with the phenolic (present in aronia extract) seems to be slow to play a role under physiological conditions. In biological systems, at the presence of CO_2 the decomposition of peroxynitrite yields 30 - 35% of carbonate radical ($CO_3^{\bullet-}$) and nitrogen dioxide ($NO_2^\bullet$), which are also strong oxidants; natural phenols are the efficient scavengers of $CO_3^{\bullet-}$ and $NO_2^\bullet$ (23).

We suggest, that polyphenol-rich extract from berries of *A. melanocarpa* seems to be a promising candidate for future evaluations of its antioxidative activity, and may be a good candidate for scavenging peroxynitrite and protecting platelets against changes their haemostatic function caused by peroxynitrite. Moreover, the tested extract due to its antioxidative activity and anti-platelet effects may play an important role as a component of human diet in

prevention of cardiovascular, cancer or inflammatory diseases, where blood platelets are involved.

Acknowledgement: This work was supported by grant 505/373 from University of Lodz

Conflicts of interest statement: None declared.

REFERENCES

1. Dutta-Roy AK. Dietary components and human platelet activity. *Platelets* 2002; 13: 67-75.
2. Kahkonen MP, Hopia AI, Vuorela HJ, *et al.* Antioxidant activity of plant extracts containing phenolics compounds. *J Agric Food Chem* 1999; 47: 3954-3962.
3. Valcheva-Kuzmanova SV, Belcheva A. Current knowledge of Aronia melanocarpa as a medicinal plant. *Folia Med* 2006; 48: 11-17.
4. Olas B, Wachowicz B, Tomczak A, Erler J, Stochmal A, Oleszek W. Comparative anti-platelet and antioxidant properties of polyphenol-rich extracts from: berries of Aronia melanocarpa, seeds of grape, bark of Yucca schidigera in vitro. *Platelets* 2008; 19: 70-77.
5. Ryszawa N, Kawczynska-Drozdz A, Pryjma J, *et al.* Effects of novel plant antioxidants on platelet superoxide production and aggregation in arteriosclerosis. *J Physiol Pharmacol* 2006; 57: 611-626.
6. Nowak P, Wachowicz B. Studies on pig blood platelet responses to peroxynitrite action. *Platelets* 2001; 12: 376-381.
7. Nowak P, Wachowicz B. The effects of peroxynitrite on pig platelet lipid peroxidation and the secretory process induced by thrombin. *Cytobios* 2001; 106: 179-187.
8. Pryor WA, Squadrito GL. The chemistry of peroxynitrite: a product from the reaction of nitric oxide with superoxide. *Am J Physiol* 1995; 268: L699-L722.
9. Walkowiak B, Michalak E, Koziołkiewicz W, Cierniewski CS. Rapid photometric method for estimation of platelet count in blood plasma or platelet suspension. *Thromb Res* 1989; 56: 763-766.
10. Ando Y, Steiner M. Sulfhydryl and disulfide groups of platelet membranes: determination of disulfide groups. *Biochim Biophys Acta* 1973; 311: 26-37.
11. Ando Y, Steiner M. Sulfhydryl and disulfide groups of platelet membranes: determination of sulfhydryl groups. *Biochim Biophys Acta* 1973; 311: 38-44.
12. Buss H, Chan TP, Sluis KB, Domigan NM, Winterbourn CC. Protein carbonyl measurement by a sensitive ELISA method. *Free Radic Biol Med* 1997; 23: 361-366.
13. Olas B, Nowak P, Kolodziejczyk J, Ponczek M, Wachowicz B. Protective effects of resveratrol against oxidative/nitrative modifications of plasma proteins and lipids exposed to peroxynitrite. *J Nutr Biochem* 2006; 17: 96-102.
14. Khan J, Brennan DM, Bradley N, Gao B, Brukdorfer R, Jacobs M. 3-Nitrotyrosine in the proteins of human plasma determined by an Elisa method. *Biochem J* 1998; 330: 795-801.
15. Wachowicz B. Adenine nucleotides in thrombocytes of birds. *Cell Biochem Funct* 1984; 2: 167-170.
16. Kowalczyk E, Fijalkowski P, Kura M, *et al.* The influence of anthocyanins from Aronia melanocarpa on selected parameters of oxidative stress and microelements contents in men with hypercholesterolemia. *Pol Merkur Lekarski* 2005; 19: 651-653.

17. Naruszewicz M, Laniewska I, Millo B, Dłużniewski M. Combination therapy of statin with flavonoids rich extract from chokeberry fruits enhanced reduction in cardiovascular risk markers in patients after myocardial infraction (MI). *Atherosclerosis* 2007; 194: 179-184.
18. Naruszewicz M, Dluzniewski M. Flavonoids from choheberry fruits (Aronia melanocarpa) reduce oxidative stress and inflammation in patients with coronary artery disease treated with statins (Short lectures). *Planta Medica* 2007; 73.
19. Ohgami K, Ilieva I, Shiratori K, *et al.* Anti-inflammatory effects of aronia extract on rat endotoxin-induced uveitis. *Invest Opthalmol Vis Sci* 2005; 46: 275-281.
20. Pampura A, Beuscher N, Smirnova M, Horoszkiewicz-Hassam M, Schonknecht K. Clinical evaluation of the efficacy and safety of Bioaron C in children with recurrent bacterial and viral infections of the upper respiratory tract (Poster). *Planta Medica* 2007; 73.
21. Bartosz G. Peroxynitrite: mediator of the toxic action of nitric oxide. *Acta Biochim Pol* 1996; 43: 645-659.
22. Olas B, Nowak P, Kołodziejczyk J, Wachowicz B. The effects of antioxidants on peroxynitrite-induced changes in platelet proteins. *Thromb Res* 2004; 113: 399-406.
23. Zhao CY, Shi YM, Yao SD, *et al.* Scavenging effects of natural phenols on oxidizing intermediates of peroxynitrite. *Pharmazie* 2003; 58: 742-749.

Received: February 28, 2008
Accepted: June 10, 2008

Author's address: Prof. Beata Olas, Department of General Biochemistry, Institute of Biochemistry, University of Lodz, Banacha 12/16, 90-237 Lodz, Poland. Tel/Fax: 0-42-6354484; e-mail: olasb@biol.uni.lodz.pl