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Effect of aronia on thiol levels in plasma of breast cancer patients

Research Article

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Abstract: The various specific biomarkers of oxidative stress in plasma from patients with breast cancer, as well as biomarkers (the level of lipid hydroperoxides, conjugated dienes, thiobarbituric acid reactive substances) have been described. The aim of our present study was to evaluate the amount of low-molecular-weight thiols (which are physiological free radical scavengers) and establish the effects of the extract from *A. melanocarpa* on the amount of these thiols in plasma obtained from patients with invasive breast cancer, patients with benign breast diseases and from healthy volunteers. We observed in patients the higher amounts of homocysteine in plasma from patients in comparison to plasma from the control group; however the total level of glutathione, cysteine, cysteinylglycine, and the amount of thiols in reduced and oxidized forms was changed (*e.g.*, in patients, the decrease of glutathione and cysteine reached about 50% of total values). Moreover, we showed that in the presence of the extract of *A. melanocarpa* (50 μg/mL, 5 min, 37°C), changes in amount of thiols in plasma from patients with invasive breast cancer and patients with benign breast diseases were significantly reduced *in vitro*. Considering the data presented in this study, we suggest that the extract from *A. melanocarpa* has an effect on thiol metabolism and the levels of all tested thiols observed in plasma obtained from breast cancer patients.

Keywords: *Breast cancer • Low-molecular-weight thiols • Homocysteine • Aronia melanocarpa (Rosaceae)*

1. Introduction

Human diet is rich in a great variety of micronutrients with antioxidant properties. Dietary antioxidants (vitamins, minerals and phenolic compounds) as well as endogenous antioxidants may protect against oxidative stress. An increased dietary intake of antioxidants is associated with a reduced risk of some diseases. The consumption of low levels of antioxidants in the form of fruit and vegetables has been shown to more than double the incidence of certain cancers [1-4]. Among these antioxidants, the extract from *Aronia melanocarpa* (Rosaceae) containing anthocyanidines, phenolic acids and quercetine glycosides [5-7] may play an important protective role. This extract containing active components may be present in human diet and is a good source of phenolic compounds. The extract from *A. melanocarpa* has antimutagenic activity and exhibits a distinct immunomodulatory activity in human lymphocyte cultures and in patients with breast cancer

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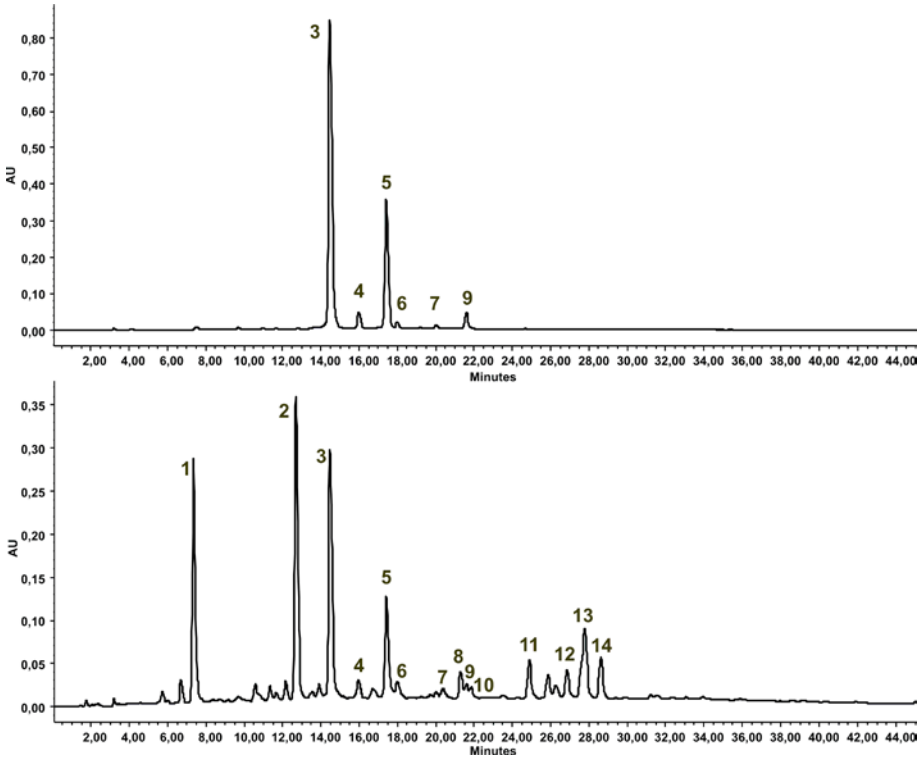

Figure 1. HPLC profiles of aronia phenolic rich extract registered at 515 nm (upper) and at 254 nm (lower). 1 and 2 - chlorogenic acids; 3 - cyanidin 3-O-galactopyranoside; 4, 5, 6, 7, 9 – anthocyanin glycosides; 8, 10, 11, 12, 13, 14 – quercetin glycosides.

[8]. Moreover, this extract exerts multifunctional biological effects, including not only anti-cancer properties [7,8], but antioxidant [7,9,10] and anti-platelet properties as well [7,9,10]. Our preliminary results showed that the extract from *A. melanocarpa* reduced oxidative/nitrative stress in blood platelets in not only the healthy subject group, but also among breast cancer patients [6]. The concentration of the extract from *A. melanocarpa* used in our studies (50 μg/mL) was similar to that used in studies of other investigators [10]. Currently, there are no data in literature about any unwanted and toxic effects of *A. melanocarpa* fruits, juices and extracts. Because aronia extract is rich in anthocyanins, responsible for the inhibition of oxidative stress [7,9,10], the aim of our present experiments was to study the effects of the extract from *A. melanocarpa* on the level of low-molecular-weight thiols (glutathione (GSH), cysteine (CSH), cysteinylglycine (CGSH) and homocysteine (HCSH)) determined by HPLC method in plasma from healthy volunteers, patients with invasive breast cancer and patients with benign breast diseases. The HPLC method used (with 2-chloro-1-methylquinolinium tetrafluoroborate - CMQT) is a fast, sensitive and selective method for the quantifications of picomoles of GSH and oxidized form of glutathione - GSSG even in plasma, where concentration of GSH is very low. The sensitivity of this method is even higher than methods using electrochemical detection. In these studies we also compared the action of the extract form berries of *A. melanocarpa* with effects of the well-known commercial monomeric polyphenol, resveratrol (3, 4', 5-trihydroxystilbene).

2. Experimental Procedures

2.1 Chemicals

Resveratrol (*trans*-3,4',5-trihydroxystilbene) was purchased from Sigma. All other reagents were of analytical grade. Stock solution of resveratrol was made in 50% dimethylsulfoxide (DMSO) at the concentration of 5 mg/ml and kept frozen (-70˚C).

2.2 Plant material, extraction and isolation

The voucher specimen is deposited at the company - Agropharm Ltd, Poland (batch no. 020/2007k). The *Aronia melanocarpa* has been grown in Poland at large plantations to be used to produce phenolic-rich juices, jams and 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 revealed in the 254 nm profile

the presence of a number of peaks (Figure 1), and was described earlier [6]. Stock solutions of the extract of aronia (Aronox by Agropharm Ltd, Poland) was made in H_2O at the concentration of 5 mg/mL; kept frozen (-70˚C) and was used for plasma experiments.

2.3 Selection criteria for patients with breast cancer and volunteers

Blood samples were taken from 23 healthy female volunteers. The median age of healthy volunteers was 43 years (range, 25-57 years; mean, 44.7 year; standard deviation ±4.5).

Patients with breast cancer were hospitalized in the Department of Oncological Surgery, Medical University of Lodz, Poland.

All patients and volunteers expressed their written informed consent for participation in this study.

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

2.3.1 Study group – patients with invasive breast cancer

There were 21 women with invasive breast cancer in the study group. The median age of patients was 55 years (range, 40-80 years; mean, 57.2 year; standard deviation, ±10.2). In 11 patients, the breast cancer was located in the left breast; in the remaining 10 patients, the right breast. Ductal invasive carcinoma was a dominant histological type of breast cancer. It was diagnosed in 17 out of 21 studied patients. Histological types of the remaining four breast carcinomas were: lobular invasive (2 cancers), mixed ductal-lobular invasive (1 cancer), mucinous (1 cancer). In a subgroup of 17 ductal invasive breast cancers, nuclear grade was classified as G2 in 8 cases and G3 in the remaining 9 cases. Median diameter of the primary tumor was 24 mm (range, 9-150 mm; mean, 38.6 mm; standard deviation, ±34.2). In 8 patients no metastases were found in axillary lymph nodes; in the remaining 13 patients, axillary lymph nodes were cancer-positive. Number of surgically removed cancer-positive lymph nodes ranged from 1 to 9; however, in two patients, the surgical removal of axillary lymph nodes was impossible due to infiltration of nodal metastases on adjacent anatomical structures (axillary vein, *etc.*). For this reason it was impossible to calculate the median and mean numbers of cancer-positive axillary lymph nodes in these patients. The stage of breast cancers in the studied group was classified according to the TNM system (AJCC, 2003 edition). Seven cases were classified as stage I, 5 were classified as IIa, 5 as stage IIb, 2 as stage IIIa, and the remaining 2 as stage IV. Sixteen cancers were classified as estrogen-receptor-positive (ER+); 13 were classified as progesterone-receptor-positive (PR+). There were 5 cancers both ER-negative and PR-negative. Overexpression of HER2 receptor was found in 7 out of 21 studied breast cancers.

2.3.2 Comparison group – patients with benign breast diseases

There were 11 women with benign breast diseases in the comparison group. Median age of patients was 46 years (range, 25-67 years; mean, 45.4 year; standard deviation, ±14.1). The breast pathology was diagnosed in left breast in 5 cases, and in right breast in 6 cases. Fibrocystic disease was diagnosed in 4 patients, fibroadenoma in 3 patients, fat necrosis in 2 patients, papilloma in 1 patient and typical ductal hyperplasia in 1 patient.

2.4 Isolation of plasma

Human blood from patients and healthy volunteers was collected into sodium citrate (5 mmol/L final concentration) and immediately centrifuged (3000 × g, 15 min) to get plasma. Blood samples from patients with breast cancer or benign breast diseases were taken before surgery, and breast cancer patients did not have preadjuvant therapy.

Some samples of plasma were incubated with the extract of *A. melanocarpa* or resveratrol (50 µg/mL, 5 min, 37˚C) and then kept frozen (-70˚C).

2.5 Determination of low-molecular-weight thiols in plasma

The classical technique HPLC has been used to analysis of thiols (glutathione, cysteine, cysteinylglycine and homocysteine) in plasma (from patients and healthy volunteers). HPLC analysis was performed with a Hewlett-Packard 1100 Series system according to Głowacki *et al.* [11] and Bald *et al.* [12]. The analytes (plasma) were derivatized with thiol-specific ultraviolet labeling reagent, 2-chloro-1-methylquinolinium tetrafluoroborate (CMQT), and separated from each other by reversed-phase high performance liquid chromatography with detection at 355 nm. Oxidized forms were converted to their thiol counterparts by reductive cleavage with sodium borohydride prior to the derivatization step. In order to circumvent the loss of reduced fraction of thiols due to oxidation during sample preparation, the derivatization reagent was added to whole blood immediately after collection and before separation of plasma. This method measures total thiols, reduced thiols and oxidized thiols, and is linear within the physiological and pathological ranges of thiols.

2.6 Statistical analyses

All the values in this study were expressed as mean ± SEM. In order to eliminate uncertain data, Grubbs test was performed. The statistically significant difference between the control group and patients was analyzed with the variance analysis (ANOVA test) in combination with Bonferroni correction. The statistical analysis of difference between the control plasma (without aronia extract) and plasma treated with aronia extract was done with paired Student's t-test using StatSoft Inc. "Statistica" v. 6.0.

3. Results

Using HPLC, we determined in human plasma the level of different thiols: glutathione, cysteine, cysteinylglycine and homocysteine. Our studies have shown that the level of total glutathione, cysteine and cysteinylglycine in plasma from patients with invasive breast cancer and patients with benign breast diseases was significantly lower than the level of these thiols in the control plasma obtained from healthy volunteers (Figure 2A, B and C). The decrease of glutathione and cysteine reached about 50% of total values (Figure 2A and B). The difference (for glutathione, cysteine, cysteinylglycine) between the control group and patients with benign breast diseases was statistically significant (ANOVA test - P<0.05). The same difference between the control group and patients with invasive breast cancer was observed (ANOVA test - P<0.05).

Moreover, we established that the levels of low-molecular-weight thiols in reduced forms (GSH, CSH, CGSH) and in oxidized forms (GSSG, CSSC and CGSSGC) in plasma from patients with invasive breast cancer and patients with benign breast diseases were changed compared to healthy group (ANOVA test - P<0.05) (Table 1). Contrary, the level of homocysteine in plasma from patients with invasive breast cancer was significantly higher (about 15%) than in plasma obtained from healthy volunteers (ANOVA test - P<0.05) (Figure 2D).

We observed that *in vitro* the extract of *A. melanocarpa* (50 μg/mL, 5 min, 37˚C) reduced the differences of total low-molecular-weight thiols, thiols in reduced and oxidized forms in plasma from patients with invasive breast cancer and patients with benign breast diseases (Figure 2A and B, Table 1). Table 2 presents

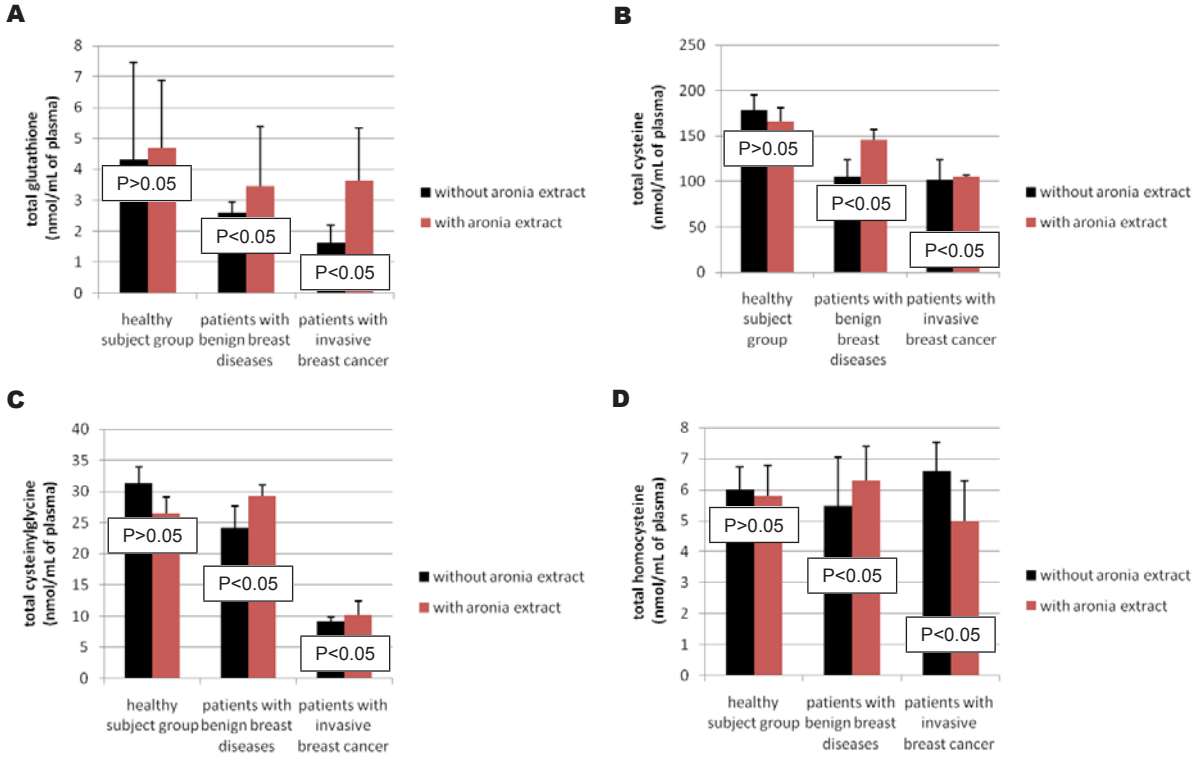

Figure 2. Changes of the total level of thiols (glutathione (A) cysteine (B), cysteinylglycine (C) and homocysteine (D)) in plasma of healthy subjects (n=23), patients with invasive breast cancer (n=21) and patients with benign breast diseases (n=11) in the presence of the extract from berries of *A. melanocarpa* (50 μg/mL, 5 min, 37˚C). The results are representative of independent experiments in triplicate and expressed as a mean ± SEM. The statistical analysis of difference between the control plasma (without the extract) and plasma treated with the extract was done using paired Student's t-test.

Treatment of plasma	the level of GSH (nmol/mL of plasma)	the level of GSSG (nmol/mL of plasma)	the level of CSH (nmol/mL of plasma)	the level of CSSC (nmol/mL of plasma)	the level of CGSH (nmol/mL of plasma)	the level of CGSSGC (nmol/mL of plasma)
healthy subject group (n=23)						
No treatment (control)	3.4 ± 1.1	1.1 ± 0.2	8.4 ± 1.9	51.4 ± 6.1	3.1 ± 1.0	8.8 ± 1.9
A. melanocarpa	3.6 ± 1.0 (P>0.05)	1.0 ± 0.3 (P>0.05)	8.1 ± 1.9 (P>0.05)	49.6 ± 5.1 (P>0.05)	3.1 ± 0.7 (P>0.05)	8.2 ± 1.9 (P>0.05)
patients with benign breast diseases (n=11)						
No treatment (control)	1.7 ± 0.5	1.2 ± 0.3	5.2 ± 0.8	31.2 ± 6.7	2.4 ± 0.9	7.4 ± 1.7
A. melanocarpa	2.5 ± 0.7 (P<0.05)	1.0 ± 0.24 (P<0.05)	7.1 ± 0.9 (P<0.05)	40.4 ± 9.7 (P<0.05)	3.4 ± 1.0 (P<0.05)	8.4 ± 0.7 (P<0.05)
patients with invasive breast cancer (n=21)						
No treatment (control)	0.9 ± 0.4	0.9 ± 0.4	3.9 ± 0.8	28.2 ± 3.4	1.1 ± 0.5	3.8 ± 0.5
A. melanocarpa	2.7 ± 1.0 (P<0.02)	1.0 ± 0.2 (P<0.05)	5.1 ± 1.0 (P<0.05)	26.4 ± 4.2 (P<0.05)	1.3 ± 0.6 (P<0.05)	3.1 ± 0.6 (P<0.05)

Table 1. The effects of phenolic compounds present in the extract from berries of *A. melanocarpa* (50 μg/mL, 5 min, 37˚C) on the level of low-molecular-weight thiols in the reduced forms: GSH, CSH, CGSH; and in the oxidized forms: GSSG, CSSC, CGSSGC in plasma from the healthy group and patients with invasive breast cancer and patients with benign breast diseases.

The results are representative of independent experiments in triplicate and expressed as a mean ± SEM.

The statistical analysis of difference between the control plasma (without the extract) and plasma treated with the extract was done using paired Student's t-test.

the changes of the GSH/GSSG ratio and other thiols (cysteine and cysteinylglycine) in plasma from healthy group and patients in the presence of the extract from berries of *A. melanocarpa* (50 μg/mL, 5 min, 37˚C). The extract from berries of *A. melanocarpa* reduces the changes of the tested thiols ratio in plasma from patients with benign breast diseases (P<0.05) and in plasma from patients with invasive breast cancer (P<0.05) (Table 2).

The comparison effects of the aronia extract (50 μg/mL, 5 min, 37˚C) and resveratrol (50 μg/mL,

Treatment of plasma	GSH/GSSG ratio	CSH/CSSC ratio	CGSH/CGSSCG ratio
healthy subject group (n=23)			
No treatment (control)	3.182	0.163	0.352
A. melanocarpa	3.600	0.163	0.378
Resveratrol	3.854	0.171	0.325
patients with benign breast diseases (n=11)			
No treatment (control)	1.426	0.167	0.328
A. melanocarpa	2.576	0.183	0.408
Resveratrol	2.370	0.171	0.374
patients with invasive breast cancer (n=21)			
No treatment (control)	0.968	0.139	0.303
A. melanocarpa	2.609	0.193	0.424
Resveratrol	1.454	0.174	0.356

Table 2. Changes the GSH/GSSG ratio and other thiols (cysteine and cysteinylglycine) in plasma from the healthy group and patients with invasive breast cancer and patients with benign breast diseases in the presence of the extract from berries of *A. melanocarpa* (50 μg/mL, 5 min, 37˚C) and resveratrol (50 μg/mL, 5 min, 37˚C).

5 min, 37˚C) on the GSH/GSSG ratio and cysteine or cysteinylglycine in plasma from tested groups is presented in Table 2. We observed that the extract from *A. melanocarpa* has an even stronger effect than resveratrol alone *in vitro* (Table 2).

4. Discussion

Reactive oxygen species and damage caused by these species are implicated in the pathogenesis of a variety of diseases, including cancers. Although ROS may be generated as byproducts of aerobic metabolism, they are essential for various defense mechanisms in the cells or are involved in signalling processes. They can also cause oxidative damage to DNA, proteins and lipids. Moreover, during cancer therapy (radiotherapy or chemotherapy), ROS may be generated. The toxic side effects of chemotherapy may be associated also with damage to different molecules caused by ROS.

For the first time, our present results showed the changes of the total level of low-molecular-weight thiols, such as glutathione, cysteine and cysteinylglycine and their reduced and oxidized forms in plasma from patients with invasive breast cancer and patients with benign breast diseases. We observed that the thiol levels (GSH, CSH, CGSH) were lower in breast cancer patients than in control subjects (Figure 2A-C, Table 1 and 2). This is an interesting observation, but might be connected to the multiple various factors, including oxidative stress, because our earlier experiments showed the increased generation of superoxide anion radicals and oxidative/nitrative modifications of proteins in blood platelets from breast cancer patients [6,13]. On the other hand, we observed that the total level of homocysteine was significantly elevated in plasma of patients with invasive breast cancer (Figure 2D). This observation is consistent with the other earlier observations demonstrating that cultured breast cancer cells produce more homocysteine than normal cells [14]. Gatt *et al*. [15] showed also that hyperhomocysteinemia in women with advanced breast cancer exists. These observations may explain the high rate of venous thrombosis in women with metastatic breast malignancy, because increased concentration of homocysteine in the blood may be an independent risk factor for atherosclerotic disease, deep vein thrombosis and thromboembolism. Moreover, homocysteine induces changes in hemostasis, as well blood clotting as fibrynolysis [16]. Elevated level of homocysteine may disrupt functions of the vascular endothelium, changing the character of its surface from anticoagulant to procoagulant. Nishinaga *et al.* [17] have shown that homocysteine reduces antithrombin binding activity of endothelial cells. Hcys may alter properties of endothelial cells by impairing the production or bioavailability of vasoactive mediators, including nitric oxide, but also endothelin-1 [18,19].

Changes in glutathione content have been reported in several malignancies [20-22]. Kumar *et al.* [21] showed significantly lower levels of plasma glutathione in invasive cancer. In cancer patients the content of reduced glutathione in erythrocytes is also decreased. We suggest that decreased concentration of glutathione in plasma may promote oxidative stress in patients with invasive breast cancer and patients with benign breast diseases (Figure 2A, Table 1 and 2), because glutathione is an important endogenous antioxidant and regulator of the redox status in plasma, and other tissues. Moreover, glutathione has other variety of physiologically important functions in defense and metabolism, including detoxication of electrophilic compounds, or synthesis and transport of biologically active, endogenous substances. Our present results showed that not only plasma glutathione, but also other thiols (cysteine and cysteinylglycine) are highly unstable and may be changed under oxidative stress in patients with invasive breast cancer and patients with benign breast diseases (Figure 2A-C, Table 1 and 2). Our results are consistent with the literature [23-25]. Rajneesh *et al.* [25] observed the significant increase of plasma lipid peroxidation and the decrease of antioxidant enzymes activity in plasma from breast cancer patients compared to controls.

Epidemiological and clinical studies suggest that diets rich in fruits and vegetables decrease the risk of premature mortality from major clinical conditions, including heart diseases and cancers. However, the data on using of antioxidant supplements during breast cancer are controversial. Review article of Greenlee *et al.* [26] showed that 22 articles did not support any conclusions regarding the effects of individual antioxidant supplements during conventional breast cancer treatment on toxicities, tumor response, recurrence, or survival, but a few studies suggested that antioxidants may decrease side effects associated with treatment, including vitamin E for hot flashes due to hormonal therapy and glutamine for oral mucositis during chemotherapy. Sharhar *et al.* [27] observed that poor antioxidant status (the low level of vitamin A, E and selenium in plasma) and high oxidative stress are associated with breast cancer risk in Malaysian women. However, many reports suggest anti-proliferative effects of chokeberries and/or chokeberry extracts against colon cancer on the basis of *in vitro* studies [28-30] and in one animal study [31]. Experiments of Yaneva *et al.* [32] indicate on the immunomodulatory activity of *A. melanocarpa* in combination with apple pectin in

patients with breast cancer undergoing postoperative radiation therapy. Results of Lala *et al.* [31] show also the protective role of the extract from *A. melanocarpa* in colon carcinogenesis *in vitro* by multiple mechanism of action. In the present *in vitro* study, we analyzed the ability of the extract from *A. melanocarpa* (which may be integral part of human diet) to reduce the level of the low-molecular-weight thiols measured in plasma of patients with breast cancer, because the extract from *A. melanocarpa* reduces oxidation and nitration of platelet proteins from healthy subject group and breast cancer patients [6]. Moreover, in our earlier paper, we showed that the extract from *A. melanocarpa* added to blood platelets significantly reduced the production of superoxide anion radicals in platelets from breast cancer patients [13].

Here, we have shown for the first time, that the extract from *A. melanocarpa* distinctly reduced differences in level of the low-molecular-weight thiols glutathione, cysteine and cysteinylglycine in the plasma of patients with invasive breast cancer and benign breast diseases (Figure 2A-C, Table 1 and 2). We have observed that an increase in free thiols is accompanied by a decrease in disulfides in plasma treated with aronia extract (in patients with benign breast diseases and patients with invasive breast cancer) (Figure 2A-C). We may suggest that the free thiols are released from protein-bound thiols (disulfides) by the treatment of aronia (at model system *in vitro*), or *in vivo* aronia extract may induce the changes of activities of blood cell enzymes, which are involved in the metabolism of thiols [33]. We have also demonstrated that the extract from *A. melanocarpa* significantly decrease the level of homocysteine in plasma from patients with invasive breast cancer (Figure 2D). The obtained results are consistent with those in the literature [34]. We may suggest that polyphenols, which are presented in the tested extract, may induce an increase antioxidant capacity through enhancement of plasma superoxide dismutase or other enzymes. Moreover, our presented comparative studies on the effect of the extract from berries of *A. melanocarpa* and resveratrol on the level of the low-molecular-weight thiols in plasma of patients with invasive breast cancer, patients with benign breast diseases and the healthy subject group gave very similar results (Table 2).

Our present results may provide a molecular basis for the well-documented oxidative stress (measuring not only by lipid peroxidation, but also by the redox status) in patients with breast cancer. However, further studies are needed evaluate the impact of pharmacologic treatment on modification of plasma proteins and thiols in breast cancer.

Acknowledgements

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