Peer-reviewed version available at *Nutrients* **2018**, *11*, 21; doi:10.3390/nu11010021

Article

Extract from *Aronia melanocarpa* L. Berries Prevents Cadmium-induced Oxidative Stress in the Liver: a Study in a Rat Model of Low-level and Moderate Lifetime Human Exposure to this Toxic Metal

Magdalena Mężyńska*, Malgorzata M. Brzóska*, Joanna Rogalska

Department of Toxicology, Medical University of Bialystok, Adama Mickiewicza 2C street, 15-222 Bialystok, Poland; joanna.rogalska@umb.edu.pl
*Correspondence: magdalena.mezynska@.umb.edu.pl; malgorzata.brzoska@umb.edu.pl; tel.: +48 85 7485604; fax: +48 85 7485834

Abstract: The study investigated, in a rat model of low-level and moderate environmental exposure to cadmium (Cd; 1 or 5 mg Cd/kg diet, respectively, for 3-24 months), whether the co-administration of 0.1% extract from *Aronia melanocarpa* L. berries (AE) may protect against oxidative stress in the liver. The intoxication with Cd, dose- and duration-dependently, weakened the enzymatic antioxidative barrier (superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, and glutathione S-transferase), decreased the concentrations of non-enzymatic antioxidants (reduced glutathione and total thiol groups), and increased the concentrations of oxidized glutathione, hydrogen peroxide, xanthine oxidase, and myeloperoxidase in this organ. These resulted in a decrease in the total antioxidative status (TAS), an increase in the total oxidative status (TOS), and development of oxidative stress in the liver (evaluated based on the index of oxidative stress calculated as the ratio of TOS and TAS). The administration of AE at both levels of Cd treatment significantly improved the enzymatic and non-enzymatic antioxidative barrier, decreased the concentration of pro-oxidants, and protected from the development of oxidative stress in the liver. In conclusion, consumption of aronia products may prevent Cd-induced destroying the oxidative/antioxidative balance and development of oxidative stress in the liver protecting against this organ damage.

Key words: *Aronia melanocarpa* berries, cadmium, liver, oxidative/antioxidative balance, oxidative stress, protection

1. Introduction

Nowadays, the growing interest of the scientific community, including nutritionists, has been focused on the possibility of using various compounds naturally occurring in plants, not only in the prevention and treatment of civilization diseases, but also in the protection against the unfavourable outcomes of exposure to environmental pollutants, including toxic heavy metals such as cadmium (Cd) [1–5]. Especially interesting in this regard are fruits, vegetables, and herbs, which are a rich source of substances characterized by antioxidative properties and could be included in the human diet [1–5].

Cd belongs to the most toxic pollutants in industrialized and developing countries and forecasts show that exposure to this xenobiotic will increase [5,6]. For the general population food is the main source of intoxication with this element [5–7]. Moreover, a significant source of chronic exposure to this metal is also habitual tobacco smoking [8,9]. Environmental exposure to Cd at levels nowadays occurring in numerous countries has been reported to create a risk of damage to the kidneys [2,10], skeleton [2,11], and cardiovascular system [2,12], as well as deterioration of the sight and hearing [13,14] and development of cancer [2,15,16]. Moreover, some data show that even low-level exposure to this element may also lead to the injury to the liver [2,17,18].

The liver, apart from the kidneys, is the main place of Cd accumulation in the organism and the critical target organ for its toxicity (for review see [1]). This organ, due to its role in the biotransformation (detoxification or metabolic activation) and storage of numerous xenobiotics that enter the body from various sources (mainly contaminants of food and drinking water, medicines, and ethyl alcohol) and some endogenous substances, is especially subjected to damage by various noxious compounds and poisonous products of their biotransformation, including free radicals (FR; for review see [1]). Because the liver plays a key role in the proper functioning of the organism, its dysfunction has numerous negative outcomes [19-21]. Thus, it is very important to find effective agents allowing to improve the function of this organ and protect it from damage by various xenobiotics, including Cd which is accumulated in the hepatic tissue during a lifetime.

Oxidative stress has been recognized as one of the main mechanisms of the toxic action of Cd, including its damaging impact on the liver [1,2,22–25]. Although this heavy metal is not capable of generating FR and reactive oxygen species (ROS) directly, it can mediate their production indirectly by weakening the enzymatic and non-enzymatic antioxidative barrier, damage to the mitochondria, and induction of the activity of oxidases [1,2,22–25]. Due to the pro-oxidative properties of Cd, there has been growing interest of the scientific community in the possibility of using natural agents with antioxidative potential in order to prevent from the unfavourable effects of exposure to this xenobiotic and for treatment of them [1–3,5].

Based on the recent findings of our research team [25–29] and some data by other authors [30], it seems that a very promising natural agent in the protection from harmful effects of exposure to Cd are berries of *Aronia melanocarpa* L. (*A. melanocarpa*, (*Michx.*) Elliott, *Rosaceae*). Aronia berries (chokeberries) are a rich source of polyphenolic compounds, which are one of the most powerful and widespread groups of natural antioxidants [4,31]. Owing to the large number of hydroxyl groups (-OH groups), these compounds are capable of chelating metal ions, including Cd ions (Cd^{2+}) [31–33]. Apart from polyphenols, chokeberry is also abundant in other ingredients characterized by an ability to detoxify FR and ROS, such as vitamins, essential bioelements, carotenoids, phytosterols, and triterpenes [4].

Our own studies, conducted in a female rat model of low-level and moderate (1 and 5 mg Cd/kg diet, respectively, for up to 24 months) lifetime human exposure to Cd, revealed that a 0.1% extract from *A. melanocarpa* berries (AE) decreased the body burden of Cd (Tables S1 and S2), including its accumulation in the liver (Figure S1, Tables S1 and S2) and kidneys [27], and offered significant protection against this heavy metal-induced damage to the skeleton [25,28,29]. The extract improved the oxidative/antioxidative balance of the serum and bone tissue [25] and prevented against this xenobiotic-caused disturbances in zinc (Zn) and copper (Cu) metabolism, including the liver status of these bioelements [26]. Moreover, the consumption of aronia anthocyanins under exposure to Cd was shown to diminish the storage of this toxic metal in the liver and kidneys and to decrease the serum activities of enzymatic markers of liver injury [30].

Taking into account strong antioxidative potential of AE [4,25] and the findings of our research team on the protection of the extract against the excessive body burden of Cd, including especially this metal accumulation in the liver, and some effects of its unfavourable action [25–29], we have hypothesized that administration of the chokeberry extract under chronic low-level and moderate exposure to this heavy metal may also improve the oxidative/antioxidative status of the liver and in this way prevent from the development of oxidative stress in this organ. The aim of the present study was to investigate this hypothesis. For this reason numerous markers of oxidative and antioxidative status and the level of oxidative stress were determined in the liver tissue in the experimental model of exposure to Cd (1 and 5 mg Cd/kg diet for up to 24 months) and/or the administration of 0.1% AE that we used previously [25–29]. To the best of our knowledge such investigation has not been conducted until now.

2. Materials and Methods

2.1. Ethics Statement

The study gained approval of the Local Ethics Committee for Animal Experiments in Bialystok (Poland; approval numbers 60/2009 on 21 September 2009 and 34/2015 on 25 March 2015). All procedures were performed according to the ethical principles and institutional guidelines, as well as the International Guide for the Use of Animals in Biomedical Research.

2.2. Cd Diets

Diets containing 1 and 5 mg Cd/kg (Labofeed H and Labofeed B diets) were prepared by the addition of cadmium chloride ($CdCl_2 \times 2½\ H_2O$; POCh; Gliwice, Poland) into the components of the standard Labofeed H diet (breeding diet ensuring the proper growth and development of young animals) and Labofeed B diet (maintenance diet) at the stage of their production by Label Food "Morawski'' Kcynia. Cd concentration in the fodder was re-quantified in our laboratory (using the atomic absorption spectrometry method) and it amounted to 1.09 ± 0.13 mg/kg in the 1 mg Cd/kg diet (mean ± standard deviation—SD) and 4.92 ± 0.53 mg/kg in the 5 mg Cd/kg diet [27]. This metal concentration determined in the standard Labofeed diets was 0.0584 ± 0.0049 mg/kg [27].

2.3. A. melanocarpa Extract

A certified (Certificate KJ 4/2010) powdered AE was provided by Adamed Consumer Healthcare. According to the producent's declaration the content of polyphenols in the extract reached 65.74%, therein 18.65% of anthocyanins. The total concentration of polyphenolic compounds in the AE, as well as the concentration of phenolic acids (including chlorogenic acid), flavonoids, proanthocyanidins, and anthocyanins (including cyanidin 3-O-β-galactoside, cyanidin 3-O-α-arabinoside, and cyanidin 3-O-β-glucoside) were determined by us [28]. The polyphenolic profile of the extract is presented in Table S3. According to the declaration of the producer and available literature data [4], the extract contained also other components such as carotenoids, pectins, sugar, sugar alcohols (sorbitol, parasorboside), triterpenes, and phytosterols, as well as minerals and vitamins.

The 0.1% aqueous solution of AE was prepared daily by dissolving 1 g of the powdered extract in 1 L of redistilled water. The total concentration of polyphenols in the 0.1% AE was 0.612 ± 0.003 mg/mL (mean ± standard error–SE) [27,28] and the concentration of Cd did not exceed 0.05 µg/L [27].

2.4. Animal Model

The investigation was carried out on 192 young (3–4 weeks old) female Wistar rats [Crl :WI (Han)] obtained from the certified Laboratory Animal House in Brwinów (Poland). Throughout the experiment all animals were kept in stainless-steel cages in controlled standard conditions (12-h light/dark cycle, temperature 22 ± 2 °C, relative humidity 50 ± 10%) and had free access to food (the Labofeed H diet throughout the first 3 months of the study and next the Labofeed B diet without and with Cd addition) and drinking water (redistilled water or the 0.1% AE).

After 5 days of acclimatization, the rats were randomly allocated to 6 experimental groups of 32 animals each. One group received 0.1% AE as the only drinking fluid (AE group), two groups were exposed to Cd via diet at the concentration of 1 and 5 mg Cd/kg (Cd_1 group and Cd_5 group, respectively), while the next two groups received the diet containing Cd (1 or 5 mg Cd/kg) and 0.1% AE simultaneously (Cd_1 + AE group and Cd_5 + AE group) for 3, 10, 17, and 24 months. The last group, maintained on redistilled water (containing <0.05 µg Cd/L) and standard Labofeed diet (without the addition of Cd), served as a control. The experimental model has been described in details in our previous reports [25-29].

The administration of 0.1% AE allowed obtaining respectively higher than the recommended daily consumption of polyphenols. The daily intake of the extract by rats reached from 41.5 to 104.6 mg/kg b.w. (Table S4) [27]. The daily intake of Cd within the 24-month exposure to the 1 and 5 mg

Cd/kg diet ranged from 37.50 to 84.88 μg/kg b.w. and from 196.69 to 404.76 μg/kg b.w., respectively (Table S4) [27]. The mean intake of the extract and Cd throughout the study did not differ depending on whether they were administered in combination or separately (Table S4) [27]. Cd concentrations in the blood and urine (markers of exposure to this xenobiotic) of the rats treated with the 1 mg Cd/kg diet (0.103–0.324 μg/L and 0.085–0.354 μg/g creatinine, respectively) and 5 mg Cd/kg diet (0.584–1.332 μg/L and 0.284–0.820 μg/g creatinine, respectively), alone or together with AE (Table S1) [27] were within the range of this metal concentrations noted in the general population in industrialised countries [10–18], confirming that the used experimental model corresponds well with human environmental exposure to this xenobiotic. The treatment with the diet containing 1 and 5 mg Cd/kg corresponds to low-level and moderate environmental human exposure to this heavy metal, respectively [2,10–18,27].

Through the whole investigation, no statistically significant differences in the consumption of food and drinking water, or body weight gain were observed among the experimental groups [27]. Although during the study there were no symptoms of abnormalities in the health status, 3 cases of unprompted deaths were noted between the 17th and 24th month of the experiment in the AE, Cd_1, and Cd_5 groups (one case in each group).

At termination, after overnight fasting, the rats were subjected to barbiturate anaesthesia (Morbital, 30 mg/kg b.w., intraperitoneally). The whole blood was taken by cardiac puncture with and without anticoagulant (heparin; Biochemie GmbH, Kundl, Austria) and various organs and tissues, including the liver used in the present investigation, were dissected. Immediately after collection, the liver was rinsed with a cold physiological saline and next it was gently dried on filter paper. The liver was stored frozen at -80°C for further studies.

In order to evaluate the oxidative/antioxidative status of the liver, numerous biomarkers of enzymatic (superoxide dismutase–SOD, catalase–CAT, glutathione peroxidase–GPx, glutathione reductase–GR, and glutathione S-transferase–GST) and non-enzymatic (reduced glutathione–GSH, thioredoxin–Trx, and total thiol groups–TSH) antioxidative barrier were determined. Moreover, the concentration of oxidized glutathione (GSSG) was measured in order to evaluate the ratio of GSH/GSSG. The total antioxidative status (TAS) and total oxidative status (TOS) of the liver were assayed, and the oxidative stress index (OSI), as an indicator of the intensity of oxidative stress, was calculated (OSI = TOS/TAS). The concentrations of hydrogen peroxide (H_2O_2), xanthine oxidase (XOD), and myeloperoxidase (MPO) were evaluated as well. All the parameters measured in the liver were adjusted for protein concentration.

2.5. Determination of the Oxidative/antioxidative Status of the Liver

2.5.1. Preparation of Liver Tissue Homogenates

Pre-weighted slices of the liver tissue were homogenized in a cold potassium phosphate buffer (50 mM, pH = 7.4; prepared by mixing 1 M potassium dihydrogen phosphate, 1 M dipotassium hydrogen phosphate [POCh, Gliwice, Poland] and distilled water) with the addition of butyl-hydroxytoluene (as an antioxidant; Sigma-Aldrich, Gmbh, Steinheim, Germany) by using a high-performance homogenizer (Ultra-Turrax T25; IKA, Staufen, Germany) to obtain 10% homogenates. All homogenates were divided into two portions. One portion, used for the measurement of CAT, GST, GSH, GSSG, TSH, H_2O_2, Trx, XOD, MPO, TAS, and TOS, was centrifuged (MPW-350R centrifugator, Medical Instruments, Warsaw, Poland) at 700 × g for 20 min at 4°C, whereas the second one, used for the quantification of SOD, GPx, and GR, was centrifuged at 20000 × g for 30 min at 4°C [34]. After the centrifugation the aliquots were separated and used for the measurements. The aliquots were stored frozen at -80°C until all measurements were performed.

The quantification of investigated parameters was performed with the use of enzyme-linked immunosorbent assay (ELISA) universal microplate reader (BIO-TEK Instruments Inc, ELX_{800}, Winooski, USA) and U-3010 spectrophotometer by Hitachi (Tokyo, Japan).

2.5.2. Determination of the Parameters of the Oxidative/antioxidative Status and the Level of Oxidative Stress in the Aliquots of the Liver Homogenates

The activity of total SOD (Cu,Zn-SOD and Mn-SOD) was measured by using the commercial kit purchased from Cayman Chemical Company (Ann Arbor, MI, USA). The assay utilizes a tetrazolium salt for detection of superoxide radicals ($O_2^{\cdot-}$) generated by XOD and hypoxantine. Determination of CAT was made according to the spectrophotometric method by Aebi [35] based on the measurement of the amount of H_2O_2 (CHEMPUR, Piekary Śląskie, Poland) degraded by CAT. The vanishing of H_2O_2 was tracked spectrophotometrically at 240 nm. The activity of GPx was assayed by using the Bioxytech GPx-340 kit by OxisResearch (Foster City, CA, USA). In the assay, GPx is used to catalyse the oxidation of GSH to GSSG initiated by the addition of tertbutyl hydroperoxide. Next, GSSG is reduced by GR and during this process the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) is transformed into its oxidized form – $NADP^+$, which is accompanied by a decrease in the absorbance at 340 nm. The rate of the decrease in the absorbance is directly proportional to the activity of GPx in the sample. The activity of GR was estimated with the use of the Bioxytech GR-340 kit by OxisResearch. The method lean on the oxidation of NADPH to $NADP^+$ in the process of GSSG reduction catalysed by limited concentration of GR. One molecule of NADPH is utilized for the reduction of one molecule of GSSG. The depletion of GSSG is determined by the measurement of NADPH consumption and demonstrated as a decrease in the absorbance at 340 nm. The precision of the measurements, expressed as intra-assay coefficient of variation (CV), for SOD, CAT, GPx, and GR was <3.4%, 2.7%, 4.9%, and 6%, respectively, whereas the inter-assay CV for SOD was <3%.

The concentrations of GSH and GSSG were estimated colorimetrically using the Glutathione Assay Kit by Cayman Chemical Company (Ann Arbor, MI, USA). GSH was determined based on the absorbance of 5-thio-2-nitrobenzoic acid (TNT) at 405–412 nm. TNT is the product of the reaction between GSH and 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB). Quantification of GSSG was accomplished by first derivatizing GSH with 2-vinylpyridine. Liver TSH were determined with the method by Ellman [36]. The method is based on the colorimetric measurement of TNT formed in the reaction between –SH groups in the sample and DTNB at 412 nm [36]. The intra-assay CV was <4% for GSH and GSSG and <3.4% for TSH, while the inter-assay CV was <1.5% for GSH and <3% for GSSG.

The concentration of H_2O_2 was assayed using the Bioxytech H_2O_2-560 kit by OxisResearch (Portland, OR, USA) based on this compound-induced oxidation of ferrous iron (Fe^{2+}) to ferric ion (Fe^{3+}). The intra-assay CV was <5%. The liver concentrations of GST, Trx, XOD, and MPO were determined with the use of specific Rat(GST), Rat(Trx), Rat(XOD), and Rat(MPO) double-antibody sandwich ELISA kits by SunRed (Shanghai, China) with the intra-assay CV <5%, 2%, 2.3%, and 4%, respectively. The inter-assay CV was <5% for GST, <5% for Trx, <3.3% for XOD, and <4.4% for MPO.

The liver TAS was assayed using the ImAnOx (TAS) ELISA kit by Immundiagnostik AG (Bensheim, Germany). The method is based on the reaction of elimination of added H_2O_2 by antioxidants present in an investigated sample. The residual H_2O_2 generates products which absorb at 450 nm. Analytical quality of the assay was determined by the measurement of the TAS in control samples included in the kit. The certified values of TAS in the control samples provided by the producer were 172–232 and 239–323 μmol/L, while the values quantified by us reached 205.5 ± 12.5 and 271.5 ± 4.5 μmol/L (mean ± SD), respectively. The value of TOS was determined with the PerOx (TOS) ELISA kit by Immundiagnostik AG (Bensheim, Germany) based on the measurement of total lipid peroxides present in the investigated sample in the reaction with peroxidase at 450 nm. The values of TOS in the control samples provided with the kit were 169–282 and 407–678 μmol/L, while the values determined by us amounted to 218.3 ± 20 and 609.6 ± 27.2 μmol/L (mean ± SD), respectively. The intra-assay CV was <4.6% for TAS and 4.2% for TOS, whereas the inter-assay CV was <4% and <4.5%, respectively.

All analyses performed with the use of commercial kits were done strictly in accordance to the manufacturers' instructions.

2.5.3. Protein Measurement

The concentration of protein in the aliquots of the liver homogenates was assayed with the use of BioMaxima kit (Lublin, Poland) with the intra-assay CV <2.2%.

2.6. Statistical Analysis

Statistical analysis of the results was performed using the Statistica 10 package (StatSoft, Tulsa, USA). A one-way analysis of variance (ANOVA) with Duncan's multiple range post hoc test was carried out for comparisons between individual groups and to determine if the differences between these groups were statistically significant ($p < 0.05$). At the beginning, ANOVA was performed to determine whether there were statistically significant differences among the six experimental groups, and when the analysis has revealed an existence of statistically significant differences among the experimental groups, Duncan's multiple range post hoc test was performed to determine which two means differed ($p < 0.05$). In figures, statistically significant differences in relation to the control group, the respective group receiving Cd alone (Cd_1 + AE vs. Cd_1 and Cd_5 + AE vs. Cd_5), the group receiving AE alone (Cd_1 vs. AE, Cd_1 + AE vs. AE, Cd_5 vs. AE, Cd_5 + AE vs. AE), and the respective group exposed to the 1 mg Cd/kg diet alone or with AE (Cd_5 vs. Cd_1 and Cd_5 + AE vs. Cd_1 + AE) are marked. In the case when the post hoc analysis revealed any influence of the co-administration of Cd and AE on the investigated parameter, the possible interactive and independent effects of Cd and AE action were evaluated with the use of a two-way analysis of variance (ANOVA/MANOVA, test F). F values having $p < 0.05$ were taken to indicate a statistically significant effect. Moreover, in order to estimate the dependence between the liver burden of Cd and the extent of destroying the oxidative/antioxidative balance in this organ, the relationships between the main indices of oxidative stress measured in this study (TOS, OSI, and H_2O_2) and the concentration of Cd in the liver (Table S1), presented in our previous reports from studies in this experimental model [27], were evaluated with the use of Spearman rank correlation analysis. Correlations were considered statistically significant at $p < 0.05$.

3. Results

3.1. Effect of AE and/or Cd on the Enzymatic Antioxidative Barrier of the Liver

The administration of AE alone for up to 24 months had no impact on the activity of SOD, CAT, GPx, and GR and the concentration of GST in the liver (Figures 1 and 2), except for a decrease in the activities of GR after 3 months (Figure 2) and CAT after 10 months of the study (Figure 1). The consumption of AE alone also decreased the liver concentration of TSH after 3 and 10 months, but after 24 months of the experiment the value of this parameter was higher compared to the control group (Figure 2).

In the rats receiving the diet containing 1 mg Cd/kg, the activity of SOD in the liver was decreased (by 28–100%) after 3–17 months, while the activity of CAT was declined (by 50%) only after 24 months (Figure 1). The administration of AE under exposure to the 1 mg Cd/kg diet completely prevented the Cd-induced decrease in the activity of SOD after 17 months and the activity of CAT after 24 months of the investigation; however, it did not provide any protection regarding the Cd-decreased activity of SOD after 3 and 10 months and declined the activity of CAT unchanged by Cd after 10 and 17 months to the values lower (by 41% and 37%, respectively) compared to the control group (Figure 1). In the rats intoxicated with the diet containing 5 mg Cd/kg, the activity of SOD in the liver was decreased, compared to control group, only after 17 months (by 29%), whereas the activity of CAT was increased after 3 months (by 43%) and decreased (2.9-fold) after 17 months (Figure 1). The consumption of AE completely prevented the decrease in the activity of SOD after 17 months induced by the 5 mg Cd/kg diet and markedly increased the activity of this enzyme unchanged by Cd alone after 24 months compared to the control group and Cd_5 group (by 36% and 59%, respectively; Figure 1). The co-administration of AE for 3 and 17 months had no impact on the changed by the 5 mg Cd/kg diet activity of CAT, while the supplementation with the extract for 24

months enhanced the activity of this enzyme unaffected by Cd making it higher (by 38%) compared to the Cd_5 group (Figure 1). The ANOVA/MANOVA analysis revealed that the modifying effect of AE consumption during the exposure to Cd on the activity of SOD and CAT in the liver was the result of independent action of the extract ingredients ($F = 4.370–6.852$, $p < 0.05$) and/or their interaction with this metal ($F = 4.961–8.478$, $p < 0.05–0.01$), especially under the higher exposure to Cd (Table S5). However, the two-way analysis of variance revealed the lack of a statistically significant independent effect of AE and its interaction with Cd (Table S5) on the activity of SOD in the Cd_1 + AE group after 17 months, in spite of the total protective impact of the extract administration under the low-level exposure to this xenobiotic, recognised on the basis of the findings of the one-way analysis of variance (Duncan's multiple range test; Figure 1).

The intoxication with the 1 mg Cd/kg diet decreased the activities of GPx in the liver at each time point (by 51–81%; Figure 1) and GR after 10 and 24 months (by 58% and 47%, respectively; Figure 2), as well as the concentration of GST after 3–17 months (by 42–47%; Figure 2). The administration of AE to the animals maintained on the diet containing 1 mg Cd/kg totally protected from the decrease in the activity of GPx after 3 and 10 months, whereas after its longer application (17- and 24-month) the protection was only partial (Figure 1). The co-administration of the chokeberry extract under the exposure to 1 mg Cd/kg diet decreased (by 44%) the Cd alone-unchanged activity of GR in the liver after 3 months, compared to the control group, completely prevented the decrease in the activity of this enzyme after 10 and 24 months. The activity of GR in the Cd_1 + AE group after 24 months was higher compared to the control group (by 72%) and Cd_1 group (3.2-fold; Figure 2). Moreover, the use of AE during the low-level intoxication with Cd for 3, 10, and 17 months completely prevented the decrease in the concentration of GST induced by this xenobiotic (Figure 2). Like in the case of the treatment with the diet containing 1 mg Cd/kg, the intoxication with the 5 mg Cd/kg diet decreased the activity of GPx in the liver at each time point (by 64–77%; Figure 1) and the activity of GST during the first 17 months (by 24–58%; Figure 2). The activity of GR at this level of exposure to Cd was increased (by 58%) after 3 months and next it decreased reaching after 10 and 17 months values lower (by 60% and 47%, respectively) compared to the control group (Figure 2). The administration of AE to the rats maintained on the diet containing 5 mg Cd/kg for 10–24 months, but not for 3 months, completely prevented this metal-caused decline in the activity of GPx (Figure 1). The consumption of the extract under the moderate exposure to Cd provided entire protection from the decrease in the activity of GR after 10 months, increased its activity after 3 and 24 months to the values higher compared to both control (by 100% and 74%, respectively) and Cd_5 group (by 30% and 57%, respectively; Figure 2). Moreover, the supplementation with AE totally prevented from the decrease in the concentration of GST caused by the 17-month consumption of the diet containing 5 mg Cd/kg and increased its concentration (unchanged by Cd alone) after 24 months to the values higher compared to the control group and Cd_5 group (by 46% and 82%, respectively; Figure 2). The consumption of AE did not provide any protection against the decrease in the activity of GR induced by the 17-month feeding with the 5 mg Cd/kg diet and the concentration of GST after 3 and 10 months (Figure 2). The modifying impact of AE on the activities of GPx and GR and the concentration of GST in the liver of rats exposed to Cd was caused by the independent action of the extract ($F = 6.998–34.18$, $p < 0.05–0.001$) and/or its interaction with this toxic metal ($F = 6.242–30.31$, $p < 0.05–0.001$; Table S6). Nevertheless, the ANOVA/MANOVA analysis revealed the lack of a statistically significant independent effect of AE and/or its interaction with Cd (Table S6) on the activity of GR in the Cd_1 + AE and Cd_5 + AE groups after 10 months in spite of the complete protective impact of the extract administration under exposure to this heavy metal revealed by one-way analysis of variance (Duncan's multiple range test; Figure 2).

As can be evident from the data presented in Figures 1 and 2, the impact of Cd alone and the effect of AE co-administration on the enzymatic antioxidative barrier of the liver was, to some extent, dependent on the level of exposure to this toxic metal. It is important to underline that the values of the activities or concentrations of the determined antioxidative enzymes (SOD, CAT, GR, GPx, and GST) in the Cd_5 and/or Cd_5 + AE groups at some time points reached values higher than in the

respective groups maintained on the diet containing 1 mg Cd/kg alone or with the extract (Cd_1 and Cd_1 + AE groups; Figures 1 and 2).

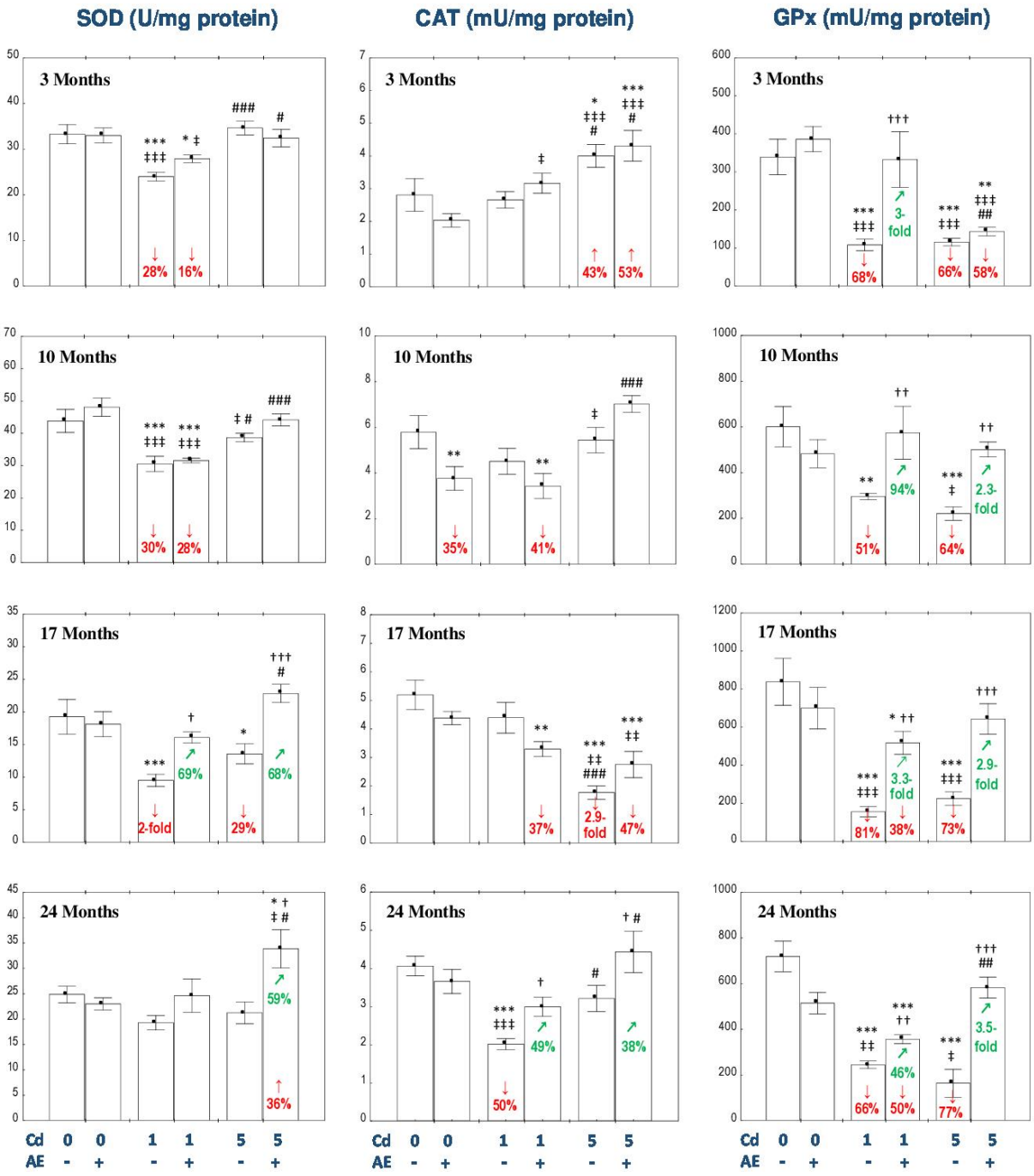

Figure 1. The effect of the extract from the berries of *Aronia melanocarpa* L. (AE) on the activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) in the liver of rats exposed to cadmium (Cd). The rats received Cd in the diet at the concentration of 0, 1, and 5 mg Cd/kg and/or 0.1% aqueous AE (+) or not (-). Data are presented as mean ± SE for 8 rats, except for 7 animals in the AE, Cd_1, and Cd_5 group after 24 months. Statistically significant differences (ANOVA, Duncan's multiple range test): * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control group; † $p < 0.05$, †† $p < 0.01$, ††† $p < 0.001$ vs. respective group intoxicated with Cd alone; ‡ $p < 0.05$, ‡‡ $p < 0.01$, ‡‡‡ $p < 0.001$ vs. group receiving AE alone; $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$ vs. respective group receiving the 1 mg Cd/kg diet (alone or with AE). Numerical values in bars disclose the percentage changes or factors of changes in comparison to the control group (↓, decrease; ↑, increase) or the respective group receiving Cd alone (↗, increase).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 7 November 2018 doi:10.20944/preprints201811.0160.v1

Peer-reviewed version available at Nutrients 2018, 11, 21; doi:10.3390/nu11010021

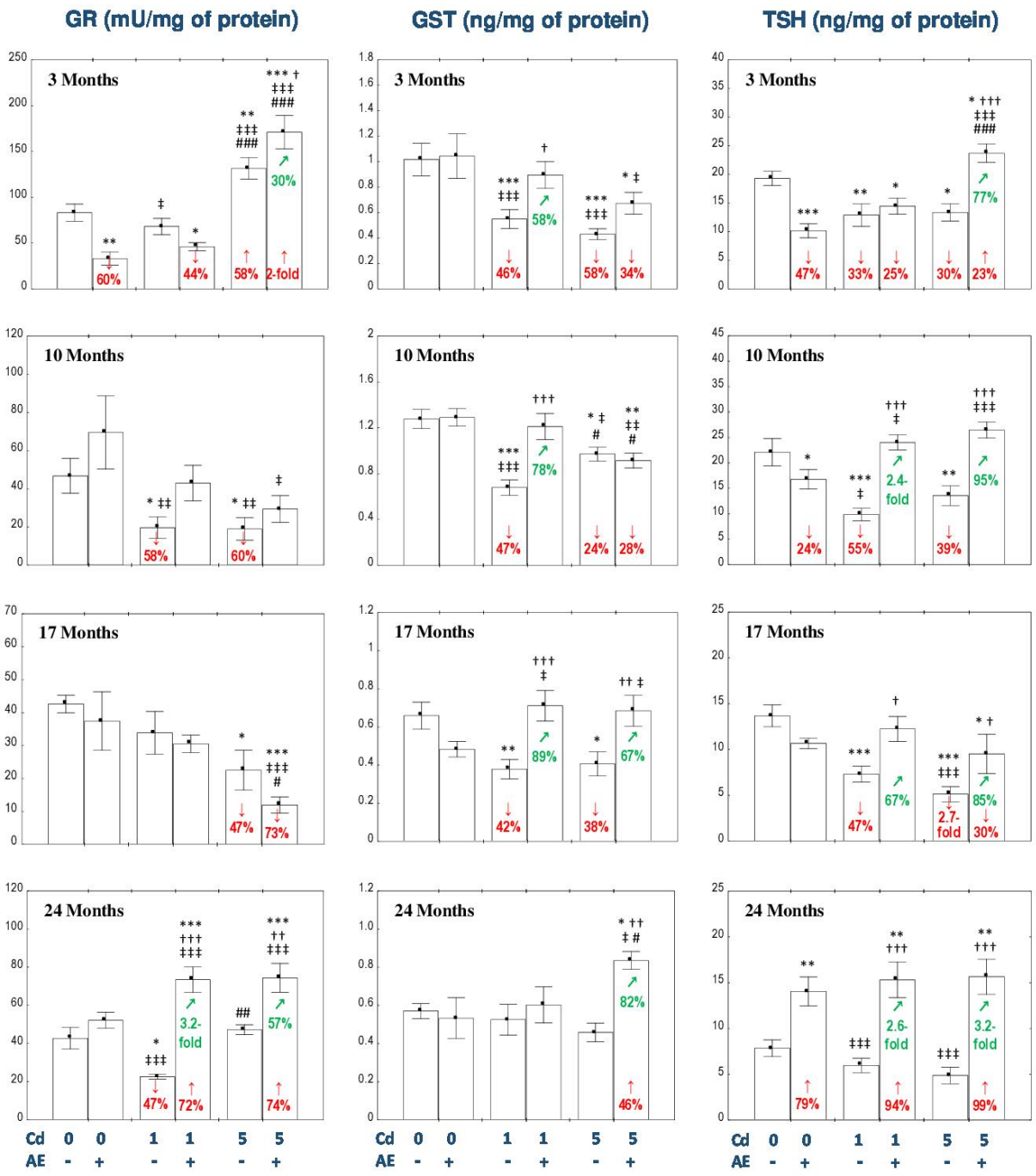

Figure 2. The effect of the extract from the berries of *Aronia melanocarpa* L. (AE) on the activity of glutathione reductase (GR) and the concentrations of glutathione S-transferase (GST) and total thiol groups (TSH) in the liver of rats exposed to cadmium (Cd). The rats received Cd in the diet at the concentration of 0, 1, and 5 mg Cd/kg and/or 0.1% aqueous AE (+) or not (-). Data are presented as mean ± SE for 8 rats, except for 7 animals in the AE, Cd_1, and Cd_5 group after 24 months. Statistically significant differences (ANOVA, Duncan's multiple range test): * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control group; † $p < 0.05$, †† $p < 0.01$, ††† $p < 0.001$ vs. respective group intoxicated with Cd alone; ‡ $p < 0.05$, ‡‡ $p < 0.01$, ‡‡‡ $p < 0.001$ vs. group receiving AE alone; $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$ vs. respective group receiving the 1 mg Cd/kg diet (alone or with AE). Numerical values in bars disclose the percentage changes or factors of changes in comparison to the control group (↓, decrease; ↑, increase) or the respective group receiving Cd alone (↗, increase).

3.2. Effect of AE and/or Cd on the Non-enzymatic Antioxidative Barrier of the Liver

The only changes in the concentrations of GSH and GSSG and the ratio of GSH/GSSG in the liver of rats consuming AE alone were an increase in GSH after 3 months, decrease in GSSG after 24 months, and an enhancement of the GSH/GSSG ratio also after 24 months (Figure 3).

The exposure to the 1 mg Cd/kg diet resulted in a decrease in the concentration of GSH after 17 and 24 months (by 26% and 35%, respectively) and an increase (by 29–57%) in the concentration of GSSG after 10, 17, and 24 months of the treatment (Figure 3). The ratio of GSH/GSSG in the Cd_1 group was reduced (by 41%) only after 17 months of the intoxication (Figure 3). The consumption of AE under the low-level treatment with Cd totally prevented this metal-provoked decrease in the concentration of GSH after 17 and 24 months and increased its concentration after 10 months to the value higher (by 50 %) than in the control group (Figure 3). The co-administration of the extract and the 1 mg Cd/kg diet completely prevented this metal-caused increase in the concentration of GSSG after 10, 17, and 24 months, as well as the decrease in the ratio of GSH/GSSG after 17 months (Figure 3). After 24 months, the concentration of GSSG in the Cd_1 + AE group was even lower (by 35%) compared to the control group (Figure 3). Moreover, the intake of AE increased the GSH/GSSG ratio compared to the control group (by 53%) and Cd_1 group (by 100%) after 10 months (Figure 3). Apart from that, the ratio of GSH/GSSG increased (4-fold), compared to the Cd_1 group, as a result of the extract consumption under the 24-month maintenance on the 1 mg Cd/kg diet; however, it did not differ compared to the control group (Figure 3). The exposure to the 5 mg Cd/kg diet led to a decrease in the concentration of GSH after 17 and 24 months (by 37% and 31%, respectively), increased the concentration of GSSG at each time point (by 31–61%), and decreased the ratio of GSH/GSSG after 10 and 17 months (by 49% and 52%, respectively; Figure 3). The supplementation with AE under the feeding with the 5 mg Cd/kg diet totally prevented the decrease in the concentration of GSH induced by this toxic metal after 17 and 24 months of exposure and increased (by 45%) its concentration compared to the Cd_5 group after 10 months (Figure 3). The administration of the extract completely prevented the increase in the concentration of GSSG in the liver caused by the 5 mg Cd/kg diet throughout the study and after 17 and 24 months the values of this parameter in the Cd_5 + AE group were even lower (by 46%) than in the control group (Figure 3). The consumption of AE during the moderate treatment with Cd increased the ratio of GSH/GSSG at each time point (from 92% after 3 months up to 5.5-fold after 24 months) completely preventing the decrease in the ratio caused by the 10- and 17-month intoxication with this toxic metal and making it higher compared to the control group throughout the study, except for 10 months (Figure 3). The two-way analysis of variance showed that the impact of AE administration on the concentrations of GSH and GSSG, as well as the ratio of GSH/GSSG was the result of independent action of the extract ingredients ($F = 4.752–81.91$, $p < 0.05–0.001$) and/or their interaction with this metal ($F = 4.312–15.52$, $p < 0.05–0.001$; Table S7). However, the ANOVA/MANOVA analysis revealed the lack of a statistically significant independent effect of chokeberry extract and its interaction with Cd (Table S7) on GSH concentration in the Cd_1 + AE group after 10 and 17 months, in spite of the clear impact of AE administration under the exposure to Cd recognised on the basis of the findings of one-way analysis of variance (Duncan's multiple range test; Figure 3).

The concentration of TSH in the AE group was decreased after 3 and 10 months and increased after 24 months (Figure 2). In the animals maintained on the diet containing 1 and 5 mg Cd/kg from 3 to 17 months the concentration of TSH in the liver was decreased (from 30% to 2.7-fold; Figure 2). The administration of AE under the intoxication with Cd completely or partially prevented all changes in the concentration of TSH caused by this toxic metal, except for the decrease in its concentration in the Cd_1 + AE group after 3 months (Figure 2). Furthermore the extract markedly increased the concentration of GSH unchanged by the 24-month intoxication with Cd alone (Figure 2). The concentration of TSH in the Cd_5 + AE group after 3 months and in the Cd_1 + AE and Cd_5 + AE groups after 24 months was higher not only compared to the respective Cd group (by 77%, 2.6-fold, and 3.2-fold, respectively), but even compared to the control group (by 23%, 94%, and 99%, respectively; Figure 2). The ANOVA/MANOVA analysis revealed that the beneficial effect of AE consumption on the concentration of TSH during the exposure to 1 mg Cd/kg diet was caused by

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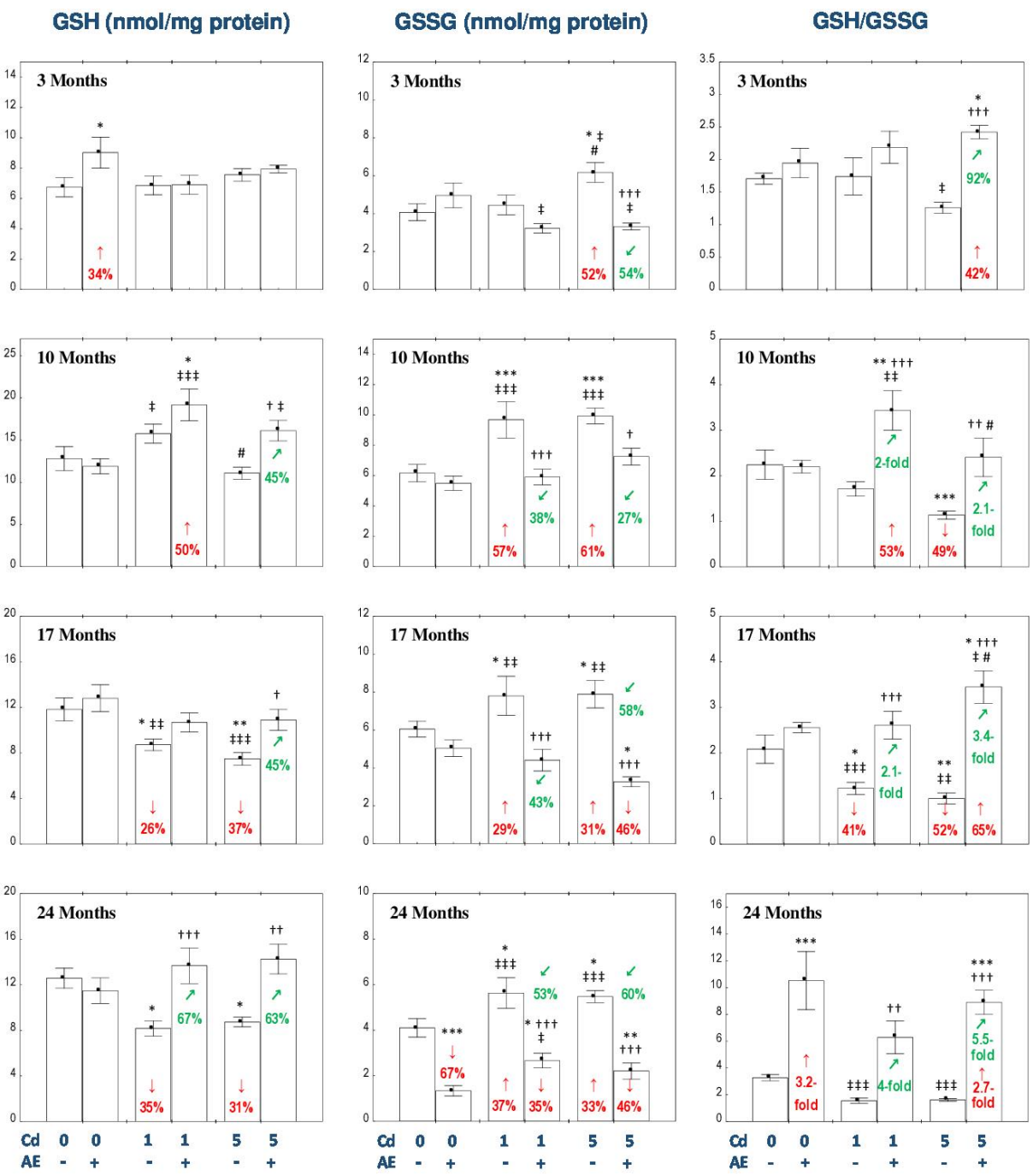

Figure 3. The effect of the extract from the berries of *Aronia melanocarpa* L. (AE) on the concentrations of reduced glutathione (GSH) and oxidized glutathione (GSSG), and the ratio of GSH/GSSG in the liver of rats exposed to cadmium (Cd). The rats received Cd in the diet at the concentration of 0, 1, and 5 mg Cd/kg and/or 0.1% aqueous AE (+) or not (-). Data are presented as mean ± SE for 8 rats, except for 7 animals in the AE, Cd_1, and Cd_5 group after 24 months. Statistically significant differences (ANOVA, Duncan's multiple range test): * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control group; † $p < 0.05$, †† $p < 0.01$, ††† $p < 0.001$ vs. respective group intoxicated with Cd alone; ‡ $p < 0.05$, ‡‡ $p < 0.01$, ‡‡‡ $p < 0.001$ vs. group receiving AE alone; # $p < 0.05$ vs. respective group receiving the 1 mg Cd/kg diet (alone or with AE). Numerical values in bars or above the bars disclose the percentage changes or factors of changes in comparison to the control group (↓, decrease; ↑, increase) or the respective group receiving Cd alone (↙, decrease; ↗, increase).

independent action of the extract ingredients after 10 and 24 months (F = 5.346, $p < 0.05$ and F = 30.21, $p < 0.001$, respectively) and their interaction with this metal after 10 and 17 months of the study (F = 26.05, $p < 0.05$ and F = 14.45, $p < 0.001$, respectively; Table S7). The influence of AE on

the concentration of TSH under the higher intoxication with Cd was the result of its interaction with this heavy metal (F = 7.760–47.82, $p < 0.05$–0.01), except for 24 months, where this effect was a result of independent action of chokeberry extract (F = 35.90, $p < 0.001$; Table S7).

The concentration of Trx in the liver of control animals ranged from 0.152 ± 0.009 to 0.236 ± 0.017 ng/mg protein at particular time points and did not change significantly in all experimental groups throughout the study, except for an increase (by 59% and 46%, respectively) in the Cd_5 + AE group compared to the control and Cd_5 group, after 24 months of the investigation (Table S8).

In the Cd_5 group, the concentration of GSH after 10 months of the experiment was lower (by 30%) than in the Cd_1 group, while the concentration of GSSG after 3 month was higher (by 39%) than at the low-level exposure (Figure 3). Moreover, the ratio of GSH/GSSG in the Cd_5 + AE group was lower (by 30%) after 10 months and higher (by 32%) – after 17 months compared to the Cd_1 + AE group (Figure 3).

3.3. Effect of AE and/or Cd on the Concentration of H_2O_2 in the Liver

The administration of AE alone had no impact on the concentration of H_2O_2 in the liver throughout the 24-month experimental period (Figure 4).

The intoxication with the 1 and 5 mg Cd/kg diet for 10, 17, and 24 months resulted in an increase in the concentration of H_2O_2 (from 73% to 3.4-fold), while the co-administration of AE totally prevented against this effect of Cd action (Figure 4). Apart from all that, the consumption of the extract during the 3-month exposure to Cd decreased the concentration of H_2O_2 compared to the respective group treated with Cd alone at both levels of exposure and towards the control group at the low-level intoxication (Figure 4).

According to the results of the ANOVA/MANOVA analysis, the beneficial impact of AE on the concentration of H_2O_2 in the liver of rats exposed to Cd was an effect of both independent action of the extract ingredients (F = 9.818–103.3, $p < 0.01$–0.001) and/or their interaction with Cd (F = 21.02–137.6, $p < 0.001$; Table S9).

There were no differences in the concentration of H_2O_2 in the liver between the respective groups receiving the 1 and 5 mg Cd/kg diet alone or with AE (Cd_1 vs. Cd_5 and Cd_1 + AE vs. Cd_5 + AE; Figure 4).

3.4. Effect of AE and/or Cd on the Concentration of MPO and XOD in the Liver

The concentrations of MPO and XOD in the liver were not influenced by the consumption of AE alone (Figure 4).

The exposure to the 1 and 5 mg Cd/kg diet increased the concentrations of MPO and XOD in this organ after 10, 17, and 24 months (by 26–88%; Figure 4), while AE co-administration completely prevented from the growth in the concentrations of these enzymes mediated by this xenobiotic (Figure 4). Moreover, the concentration of XOD in the Cd_1 + AE and Cd_5 + AE groups after 17 months was even lover (by 23% and 27%, respectively) compared to the control group (Figure 4). The consumption of the extract under the 3-month maintaining of the rats on the diet containing 1 and 5 mg Cd/kg decreased the concentrations of MPO and XOD compared to the control group (by 29–34%) and respective group administered with Cd alone (by 27–40%; Figure 4).

The ANOVA/MANOVA analysis revealed that the favourable effect of AE consumption on the concentrations of MPO and XOD in the liver during the exposure to Cd was the result of independent action of the extract ingredients (F = 5.003–58.18, $p < 0.05$–0.001) and/or their interaction with this heavy metal (F = 5.008–36.78, $p < 0.05$–0.001; Table S9). However, the analysis displayed the lack of a statistically significant independent effect of AE and its interaction with Cd (Table S9) on the concentration of MPO after 3 months in spite of the clear impact of AE consumption under the low-level exposure to Cd recognized on the basis of the findings of the ANOVA analysis (Duncan's multiple range test; Figure 4).

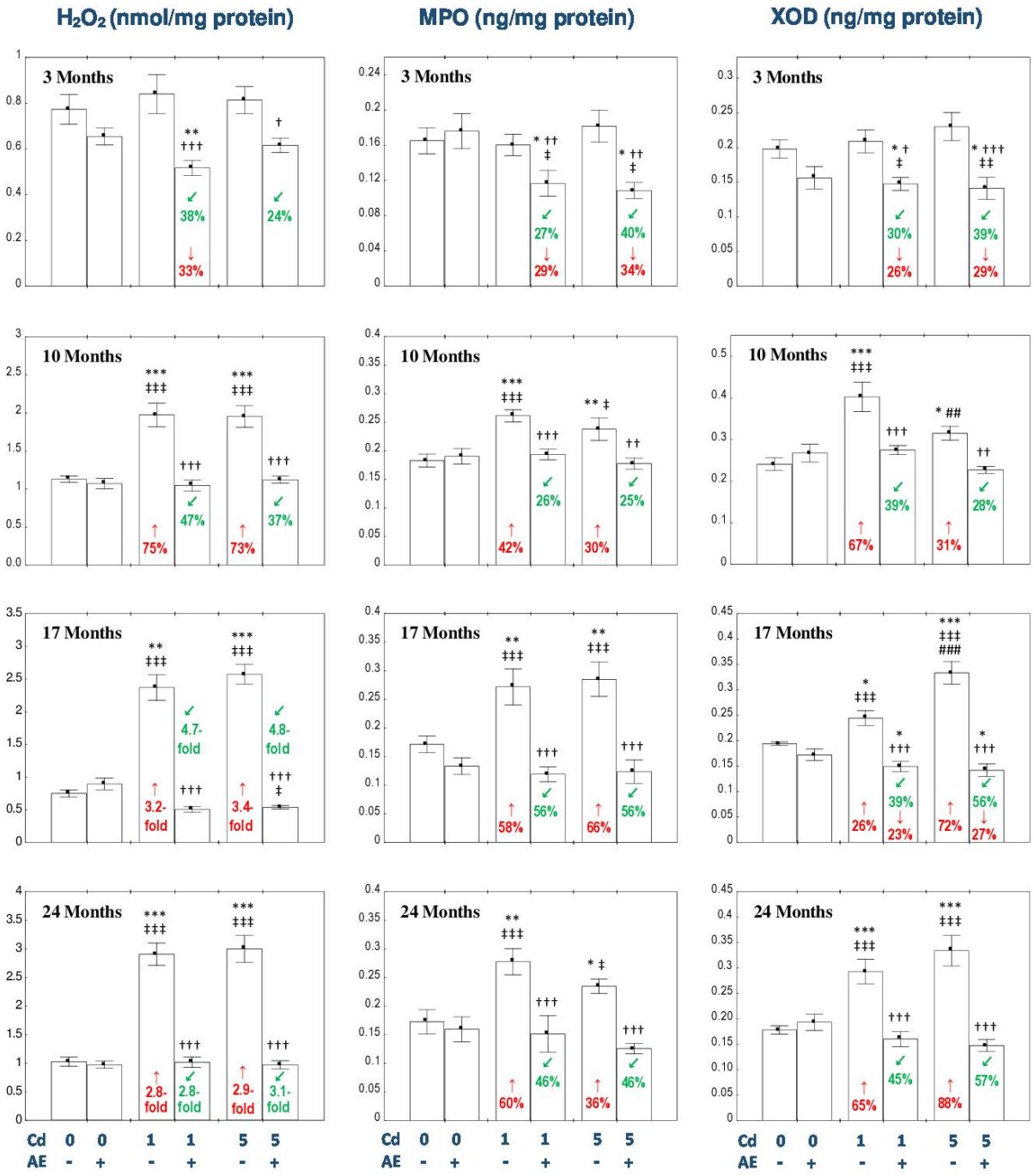

Figure 4. The effect of the extract from the berries of *Aronia melanocarpa* L. (AE) on the concentrations of hydrogen peroxide (H_2O_2), myeloperoxidase (MPO), and xanthine oxidase (XOD) in the liver of rats exposed to cadmium (Cd). The rats received Cd in the diet at the concentration of 0, 1, and 5 mg Cd/kg and/or 0.1% aqueous AE (+) or not (-). Data are presented as mean ± SE for 8 rats, except for 7 animals in the AE, Cd_1, and Cd_5 group after 24 months. Statistically significant differences (ANOVA, Duncan's multiple range test): * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control group; † $p < 0.05$, †† $p < 0.01$, ††† $p < 0.001$ vs. respective group intoxicated with Cd alone; ‡ $p < 0.05$, ‡‡ $p < 0.01$, ‡‡‡ $p < 0.001$ vs. group receiving AE alone; $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$ vs. respective group receiving the 1 mg Cd/kg diet (alone or with AE). Numerical values in bars or above the bars disclose the percentage changes or factors of changes in comparison to the control group (↓, decrease; ↑, increase) or the respective group receiving Cd alone (↙, decrease).

There were no differences in the concentrations of MPO and XOD in the liver between the respective groups receiving the 1 and 5 mg Cd/kg diet alone or with AE (Cd_1 vs. Cd_5 and Cd_1 + AE vs. Cd_5 + AE), except for lower (by 22%) concentration of XOD in the Cd_5 group compared to the Cd_1 group after 10 months and higher (by 36%) value of this parameter in the Cd_5 group after 17 months (Figure 4).

3.5. Effect of AE and/or Cd on TAS and TOS and the Level of Oxidative Stress in the Liver

The administration of AE alone for up to 24 months had no impact on TAS, TOS, and OSI in the liver (Figure 5).

The exposure to the 1 mg Cd/kg diet resulted in a decrease in TAS and an increase in OSI after 10 and 17 months (by 28–92%), while TOS remained unchanged during the whole experiment (Figure 5). The administration of AE under the 10- and 17-month exposure to the 1 mg Cd/kg diet had no impact on the Cd-induced changes in TAS and decreased, compared to the control group (by 38% and 29%, respectively) and Cd_1 group (by 40% and 32%, respectively), the Cd alone-unchanged TOS (Figure 5); however, it completely prevented the increase in the value of OSI (Figure 5). Moreover, the 24-month consumption of AE under the low-level exposure to Cd resulted in an increase in TAS and a decrease in OSI compared to both control (by 18% and 23%, respectively) and Cd_1 group (by 36% and 24%, respectively; Figure 5). In the animals treated with the 5 mg Cd/kg diet, TAS was decreased (by 19%) after 10 months and TOS was increased (by 51%) after 24 months of the investigation (Figure 5). The supplementation with AE during the moderate exposure to Cd totally prevented this xenobiotic-caused decrease in the liver TAS after 10 months, whereas after 24 months of co-administration it enhanced the parameter to the value higher compared to the control group and Cd_5 group (by 43% and 54%, respectively; Figure 5). Moreover, the consumption of the extract by the animals maintained on the 5 mg Cd/kg diet decreased the Cd alone-unchanged TOS after 10 and 17 months (by 19% and 20%, respectively) and partially prevented this xenobiotic-induced increase in TOS after 24 months of the experiment (Figure 5). After 24 months of co-administration of the 5 mg Cd/kg diet and AE, TOS remained elevated (by 36%) compared to the control group (Figure 5). In the rats intoxicated with 5 mg Cd/kg diet, the value of OSI was increased (by 17–61%) at each time point, whereas the co-administration of the extract completely protected against the development of oxidative stress evaluated based on the value of OSI (Figure 5).

The ANOVA/MANOVA analysis showed that the effect of the consumption of chokeberry extract on TAS, TOS, and OSI in the liver tissue under intoxication with the 1 and 5 mg Cd/kg diet, was the result of independent action of the extract ingredients ($F = 6.094–101.2$, $p < 0.05–0.001$) and/or their interaction with this heavy metal ($F = 5.934–54.51$, $p < 0.05–0.001$; Table S10). Nevertheless, the ANOVA/MANOVA analysis revealed the lack of a statistically significant independent effect of AE and its interaction with Cd (Table S10) on TAS in the Cd_5 + AE group as well as TOS in the Cd_1 + AE and the Cd_5 + AE groups after 10 months of the investigation, in spite of the evident impact of AE administration under the exposure to this metal recognised on the basis of one-way analysis of variance (Duncan's multiple range test; Figure 5).

The liver TAS after 10–24 months in the Cd_5 + AE group was higher (by 21–36%) than in the Cd_1 + AE group (Figure 5). Moreover, after 17 months TAS in the Cd_5 group was higher (by 62%) compared to the Cd_1 group (Figure 5). TOS in the Cd_5 + AE group after 10 and 24 months was higher (by 37% and 41%, respectively) than in the Cd_1 + AE group. Furthermore, the value of TOS in the Cd_5 group after 24 months was higher compared to the Cd_1 group (Figure 5). The extent of oxidative stress, evaluated based on the value of OSI in the Cd_5 group after 10 and 17 months was more enhanced than in the Cd_1 group (by 9% and 37%, respectively), while after 24 months oxidative stress in the Cd_5 group and Cd_5 + AE group was more severe (by 60% and 24%) than in the respective groups maintained on the diet containing 1 mg Cd/kg alone or with the extract (Figure 5).

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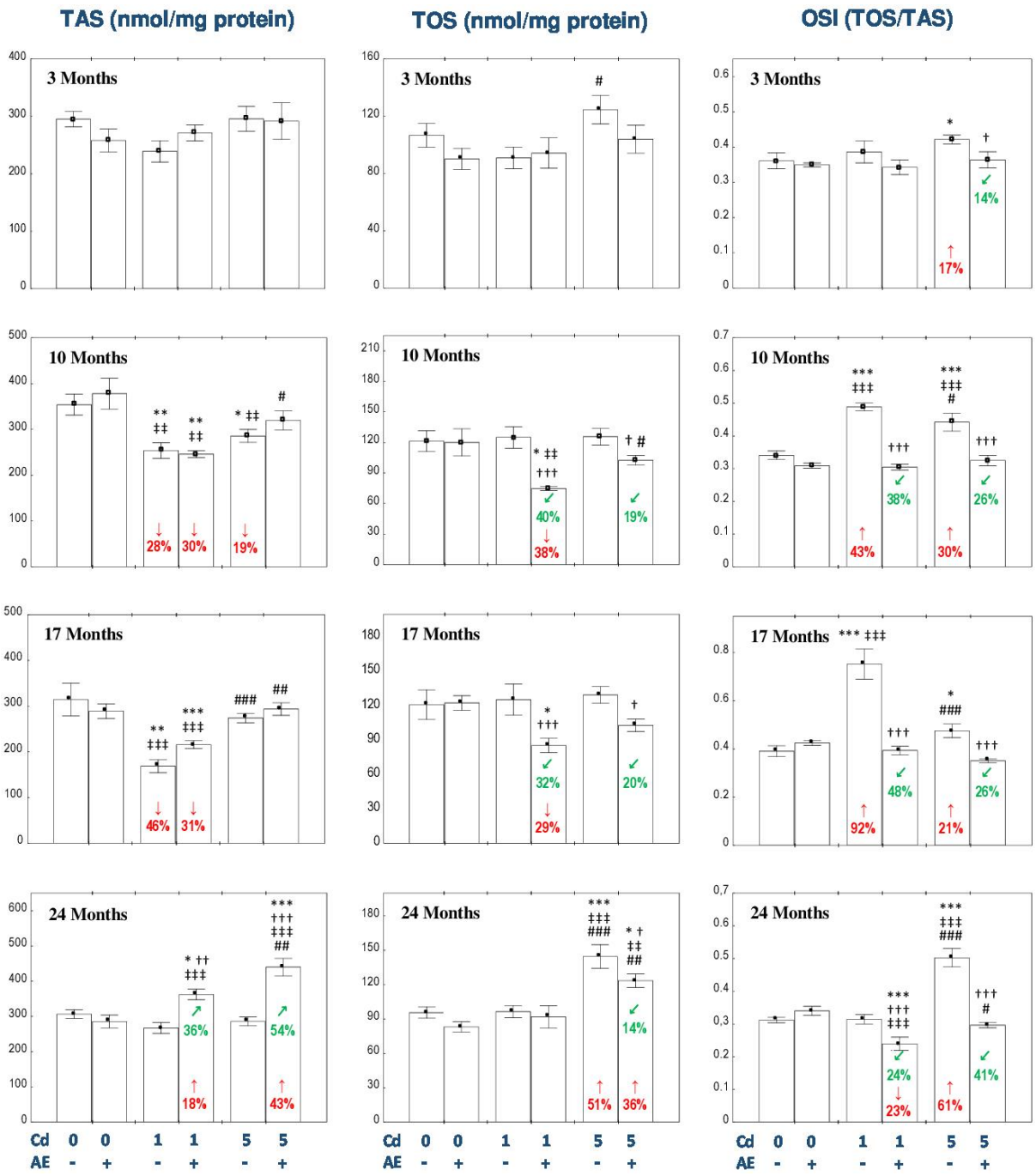

Figure 5. The effect of the extract from the berries of *Aronia melanocarpa* L. (AE) on the total antioxidative status (TAS), total oxidative status (TOS), and the oxidative stress index (OSI) in the liver of rats exposed to cadmium (Cd). The rats received Cd in the diet at the concentration of 0, 1, and 5 mg Cd/kg and/or 0.1% aqueous AE (+) or not (-). Data are presented as mean ± SE for 8 rats, except for 7 animals in the AE, Cd_1, and Cd_5 group after 24 months. Statistically significant differences (ANOVA, Duncan's multiple range test): * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control group; † $p < 0.05$, †† $p < 0.01$, ††† $p < 0.001$ vs. respective group intoxicated with Cd alone; ‡‡ $p < 0.01$, ‡‡‡ $p < 0.001$ vs. group receiving AE alone; $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$ vs. respective group receiving the 1 mg Cd/kg diet (alone or with AE). Numerical values in bars disclose the percentage changes in comparison to the control group (↓, decrease; ↑, increase) or the respective group receiving Cd alone (↙, decrease; ↗, increase).

3.5. Dependences Between the Indices of Oxidative Status and Cd Concentration in the Liver of Rats

Positive correlations were noted between Cd concentration in the liver and the main indices of the oxidative status of this organ such as TOS ($r = 0.217$, $p < 0.01$), OSI ($r = 0.145$, $p < 0.05$), and the concentration of H_2O_2 ($r = 0.262$, $p < 0.001$).

4. Discussion

The present paper is the first study indicating the beneficial influence of a polyphenol-rich extract from *A. melanocarpa* L. berries on the oxidative/antioxidative balance in the liver and its ability to protect against the development of oxidative stress in this organ at the conditions of low-level and moderate chronic exposure to Cd well corresponding to environmental human exposure to this xenobiotic nowadays occurring in industrialized and developing countries [2,10-18]. A very important finding of this investigation is also revealing that Cd may induce oxidative stress in the liver even at low repeated exposure.

It has already been reported by numerous authors that Cd may destroy the oxidative/antioxidative balance and induce oxidative stress in the liver contributing in this way to the damage to this organ [1,22–24,37–42]. However, in the current study it has been revealed for the first time that this xenobiotic may induce oxidative stress in the liver at low-level exposure (10-month exposure to the 1 mg Cd/kg diet in our experimental model) and at its low concentration in this organ (0.199 ± 0.028 μg/g), blood (0.1792 ± 0.0198 μg/L), and urine (0.1809 ± 0.0194 μg/g of creatinine) is comparable to the concentrations nowadays noted in the inhabitants of industrialized countries [10-18]. This is a very important finding of the study, because it shows that the general population may be at a real risk of destroying the oxidative/antioxidative balance and development of oxidative stress in the liver due to common presence of Cd in the environment and food and its accumulation in the human body through the lifetime [5–7]. At the same time it confirms the necessity of looking for effective ways to protect against oxidative stress and oxidative damage to various organs, including the liver.

The mechanisms of pro-oxidative action of Cd are widely reported (for revive see [1]) and thus they are not discussed in the present paper. Our findings show that Cd at both low-level and moderate exposure, may induce oxidative stress in the liver by weakening its enzymatic (SOD, CAT, GR, GPx, and GST) and non-enzymatic (TSH and GSH) antioxidative barrier and increasing the concentrations of pro-oxidants (GSSG, H_2O_2, MPO, and XOD). These result in a decrease in TAS and an increase in TOS of the liver and development of oxidative stress in this organ evaluated based on the value of OSI. It is important to underline that the extent of destroying the oxidative/antioxidative balance and the intensity of oxidative stress depended, to some extent, on the level of exposure to Cd. At the moderate exposure oxidative stress developed earlier (already after 3 months) than at the low-level intoxication (after 10 months) and after 24 months it was more severe at the higher treatment. This xenobiotic-induced changes in the enzymatic antioxidants (the activities of SOD and GPx and the concentration of GST) and the concentration of non-enzymatic radical scavengers (TSH) appeared already after 3 months of the exposure to 1 mg Cd/kg diet.

SOD, CAT, GPx, GR, and GST are the main enzymatic free-radical scavengers in cells [1,43]. SOD is the enzyme which catalyses the dismutation of $O_2^{\cdot-}$, whereas CAT and GPx are engaged in the detoxification of H_2O_2 [43]. GPx, GR, and GST are enzymes involved in the metabolism of GSH. GPx promotes the oxidation of GSH to GSSG, while GR is accountable for the reduction of GSSG [43]. As the main non-enzymatic antioxidant GSH plays an important function in the inactivation of organic and inorganic FR and xenobiotics, including heavy metals such as Cd [43]. Due to the presence of –SH group, GSH may bind ions of this metal (Cd^{2+}) into an inert complex excreted in the bile [43]. Trx being a protein with two –SH groups plays similar role as GSH [43].

The decrease in the activities of antioxidative enzymes might result from interactions between Cd and metals such as Zn, Cu, manganese – Mn (Zn,Cu-SOD, Mn-SOD), selenium – Se (Se-GPx), and iron – Fe (Fe-CAT) inhered in their active centres [1,37,38,44,45]. Such explanation seems very probable in the case of SOD as recent findings of our research team have revealed that the rats

maintenance on the 1 and 5 mg Cd/kg diet may disturb the liver homeostasis of Cu and Zn [26]. The fact that some indices of the enzymatic antioxidative barrier (SOD, CAT, GR, and GST) at some time points reached higher values in the animals fed the diet containing 5 mg Cd/kg than in the case of the 1 mg Cd/kg diet, and sometimes even higher than in the control group (CAT and GR) may be explained by starting the defense mechanisms [1,46]. It is vital to notice that antioxidants seem to be more susceptible to the unfavourable impact of Cd than oxidases since the activities of SOD and GPx and the concentration of GPx were decreased already after 3 months of exposure to the 1 mg Cd/kg diet, whereas the concentrations of MPO and XOD were elevated only after exposure longer than 3 months. MPO is an enzyme occurring in the membranes of neutrophils which catalyses the synthesis of podchloric acid with strong oxidative and antibacterial abilities [47], whereas XOD takes part in the metabolism of purines [48]. Reactions catalysed by both MPO and XOD are the source of reactive oxidants, including H_2O_2 and $O_2^{\cdot-}$ [47,48].

The increase in the concentration of H_2O_2 in the liver might be the result of the decreased activities of enzymes which detoxifies this compound such as CAT and GPx, as well as the increased concentration of XOD. Enhanced concentration of H_2O_2 in cells has very negative consequences, because this compound in the presence of ions of transitive metals such as Fe^{2+} and Cu^{+} may generate a highly reactive hydroxyl radical ($OH^{\cdot}$) via the Fenton reaction [43]. The accumulation of H_2O_2 and other ROS and FR may have detrimental effects on cellular metabolism, respiration, energy production, signalling pathways, and may lead to the damage to the cellular and intracellular membranes and key macromolecules (proteins, lipids, and DNA) [1,22,25,38–40,42,43]. Moreover, the Cd-induced decline in the concentration of GST, an enzyme of phase II detoxification which couples toxic metabolites with GSH, may change the metabolism of various substances, and increase their toxicity [43,49].

As it was above discussed the present study provided new and important data on the impact of Cd on the oxidative/antioxidative status of the liver and risk of the development of oxidative stress in this organ at low-level exposure to this xenobiotic. However, our interest was focused first of all on the possible protective impact of prolonged (from 3 up to 24 months) consumption of AE on the oxidative/antioxidative status of the liver under chronic low-level and moderate treatment with Cd. Furthermore, the study allowed us to estimate the influence of the extract intake on the oxidative/antioxidative balance in this organ at a very low exposure to this xenobiotic resulting from its trace, but unavoidable, presence in the standard diet (0.06 mg/kg in our experimental model [27,28]). Detailed analysis of the results of the present study in the animals receiving AE and fed with the diet without Cd addition has revealed that prolonged, even a lifetime, consumption of the extract in the daily dose of 31.1–154.7 mg/kg b.w. (corresponding to aronia polyphenols consumption ranging from 41.5 to 101.7 mg/kg b.w.) generally (except for temporary changes of some parameters) did not influence negatively the oxidative/antioxidative status of the liver. In the contrary, after 24 months of the extract consumption, the concentration of GSSG was lower, while the concentration of TSH and the ratio of GSH/GSSG were higher, which may indicate improved antioxidative properties of the liver. The risk of disturbing the balance between antioxidants and pro-oxidants is always a very important question related to the enhanced consumption of antioxidants, including polyphenol-rich products. Our findings show that prolonged consumption of chokeberry extract did not create a risk of destroying the oxidative/antioxidative balance of the liver. However, the present study has revealed, first of all, that the intake of AE, which did not change the balance between antioxidants and pro-oxidants in the liver under the conditions of feeding with the diet containing only trace amounts of Cd is capable of improving the oxidative/antioxidative status and completely prevent the development of oxidative stress in this organ under both low-level and moderate lifetime exposure to this toxic metal. The beneficial impact of the consumption of AE was connected not only with an enhanced antioxidative potential of the liver, but also with decreased concentrations of oxidases and H_2O_2 in this organ. It is important to underline that although the impact of Cd on the values of OSI, reflecting the intensity of oxidative stress, differed depending on the duration and the level of exposure, the extract co-administration was capable of completely protecting the liver from the development of oxidative stress irrespective of the intensity of intoxication with this xenobiotic.

The favourable effect of AE on the oxidative/antioxidative status of the liver under treatment with Cd may be explained by a direct effect of the extract resulting from its strong antioxidative potential [4,25] and from an indirect effect related to interactions between ingredients of the extract and this toxic element. Both the direct and indirect effects might be due to first of all the high abundance of polyphenolic compounds, characterized by strong antioxidative properties and the ability to chelate Cd^{2+} [4,26,27,32,33]. The polyphenolic profile of aronia berries includes mainly anthocyanins (cyanidin 3-O-α-arabinoside, cyanidin 3-O-β-galactoside, cyanidin 3-O-β-glucoside, cyanidin-3-xyloside, pelargonidin-3-galactoside, pelargonidin-3-arabinoside), proanthocyanidins (oligomeric and polymeric catechins), flavonols (quercetin, quercetin-3-galactoside, quercetin-3-glucoside, quercetin-3-rutinoside, quercetin-3-vicianoside, quercetin-3-robinobioside, and kaempferol) as well as chlorogenic acid and neochlorogenic acid [4,25,27]. Owing to the occurrence of -OH groups, polyphenols may serve as hydrogen donors able to terminate radical chain reactions such as lipid peroxidation and play an important function in the regeneration of non-enzymatic antioxidants such as vitamin C and E [3,45,51]. It is vital to underline that the beneficial influence of AE may also be due to the presence in the extract of ingredients other than polyphenols with proven ability to combat Cd-induced oxidative stress such as vitamin C and E, β-carotene, triterpens, and essential microelements such as Fe, Zn, and Se [3,4,38,50,51].

Taking into account the recent findings of our research team on the impact of AE on the body status of Zn and Cu at the conditions of low-level and moderate intoxication with Cd, and these bioelements binding to metallothionein (MT) [26], the beneficial effect of the extract might also be mediated by this low molecular weight (6–7 kDa), rich in -SH groups (thiol groups), metal binding protein and the ability to maintain the pool of MT-unbound Zn and Cu in this organ at the proper level. Such explanation seems to be possible as the extract administration for 17 months under the both low-level and moderate exposure to Cd completely prevented this heavy metal induced inhibition of the activity of SOD, being the Zn- and Cu-dependent enzyme, and even enhanced its activity in the case of the higher 24-month treatment.

Our previous findings on the impact of AE on the body burden of Cd, including mainly the accumulation of this xenobiotic in the liver (Figure S1; Tables S1 and S2) [27], together with the positive correlations between Cd concentration in this organ and the values of the main indices of the oxidative status (TOS, OSI, and the concentration of H_2O_2), noted in the present study, allow for the conclusion that the favourable effects of AE may also be explained by the ability of chokeberry ingredients to reduce the body and liver burden of Cd by decreasing this xenobiotic absorption from the gastrointestinal tract and increasing its urinary excretion [27]. The presence of -OH groups and carbonyl groups in polyphenols structure, especially the occurrence of 3'4'-dihydroxyl groups in the B ring, simultaneous occurrence of 5-hydroxyl group in the A ring and 4-carbonyl group in the C ring as well as the 3-hydroxyl and 4-carbonyl groups in the C ring, enables polyphenols to bind ions of metals, including Cd^{2+} [32,33,52]. Moreover, chokeberries are also rich in other compounds such as fibres and pectin which are capable to bind Cd^{2+} and in this way alleviate the toxicity of this metal [53,54].

The present paper is the only data on the influence of chokeberry extract on the oxidative/antioxidative balance in the liver under the conditions of low-level and moderate exposure to Cd corresponding to human lifetime exposure to this xenobiotic. However, some experimental investigations revealed the preventive effect of rich in polyphenolic compounds extracts from green tea, strawberry, blueberry, ginger, and olive oil [22–24,40,41], as well as single polyphenols like naringenin and curcumin [39,42] in protection from this metal-induced oxidative stress in the liver and hepatocytes damage.

Liver, as the organ of the biggest Cd accumulation is especially exposed to this heavy metal toxicity [1,2,5,27]. The mechanisms of the pro-oxidative Cd action and this xenobiotic-induced oxidative damage to this organ are well-known and widely described (for review see [1,2]) and thus they are not discussed in details in this paper. Since oxidative stress has been recognized as the crucial mechanism of this xenobiotic-induced damage to the liver [1,22–24,37–42], the improvement in the antioxidative potential of hepatocytes seems to be the key mechanism in combating the negative

effects of the action of this heavy metal. The fact that AE prevent the developments of Cd-induced oxidative stress in the liver allows to suspect that the consumption of chokeberry extract may protect this organ from oxidative damage. Therefore, our current research are focused on investigating the possibility of using AE in the liver protection from the outcomes of oxidative stress in this organ. We have already reported that aronia ameliorated Cd-generated changes in the liver morphology and decreased the number of apoptotic cells in this organ [55]. Detailed results of this study will be published as soon as possible.

The present study is a part of comprehensive researches evaluating the possibility of using *A. melanocarpa* L. berries in protection of the organism from detrimental effects of the exposure to Cd. The results of the present investigation together with the previously reported findings that AE prevented from the destruction of the body status of Zn and Cu, decreased Cd accumulation in the liver, kidneys, and bones, as well as protected bone tissue from damage induced by this xenobiotic under the condition of low-level and moderate exposure [25–29] suggest that consumption of chokeberry products may provide protection for the target organs of this metal toxicity. These results have not only scientific value but also an important practical application, since the consumption of aronia berries is widely recommended due to their multidirectional health-promoting properties [4]. Moreover, the beneficial influence of AE on the liver under the condition of exposure to Cd allows us to suspect that the consumption of the extract may also protect this organ from the damaging influence of other xenobiotics characterized by pro-oxidative properties.

We are aware not only of the accomplishments, but also of the limitations of our investigation. Since women are more susceptible to Cd toxicity than man the experimental model used in this study consists with female rats, therefore our results refer to the female liver. Thus, the further research involving an evaluation of AE on the male liver is needed. Moreover, it is necessary to examine to what extent chokeberry extract can protect against oxidative damage to the liver and explain the involvement of antioxidative properties of the extract in the mechanisms of its protective action on the liver at the conditions of low-level and moderate chronic exposure to Cd. We have undertaken research in this direction and their results will be published soon.

5. Conclusion

In conclusion, this study demonstrated for the first time that the extract from the berries of *A. melanoccarpa* L. offers protection against Cd-induced disruption in the oxidative/antioxidative balance and protects from the development of oxidative stress in the liver of rats under the conditions of low-level and moderate treatment with this toxic metal reflecting the environmental exposure of humans in industrialized and developing countries. The protective action of AE may be explained by high occurrence of numerous beneficial compounds, mainly polyphenols, able to scavenge FR and ROS, and suppress the activity of oxidases as well as by the presence in the extract ingredients possessing the ability to chelate Cd^{2+}. Although, the further studies have to be undertaken in this subject, based on the results of this investigation it can be concluded that aronia berries and their products may be very promising natural agents to be applied in the protection from oxidative damage of the liver in humans exposed to this toxic metal. This is the most important and practically useful, but not the only achievement of our research. Very significant and also of practical usefulness finding of the study is revealing the risk of the development of oxidative stress in the liver even at low-level repeated exposure to Cd, as well as providing evidence that prolonged enhanced consumption of AE under exposure to only trace amounts of Cd do not create a risk of destroying the balance between antioxidants and pro-oxidants.

Supplementary Materials: Table S1: The concentration of cadmium (Cd) in the blood, liver, and urine of rats receiving the extract from the berries of *Aronia melanocarpa* L. (AE) and/or Cd, Table S2: The effect of the extract from the berries of *Aronia melanocarpa* L. (AE) on the concentration of cadmium (Cd) in the blood, liver and urine of rats, Table S3: Polyphenolic composition of the extract from the berries of *Aronia melanocarpa* L. (AE), Table S4: The intake of cadmium (Cd) and the extract from the berries of *Aronia melanocarpa* L. (AE) in particular experimental groups, Table S5: Main and interactive effects of cadmium (Cd) and the extract from the berries of

Aronia melanocarpa L. (AE) on the activities of superoxide dismutase (SOD) and catalase (CAT) in the liver of rats, Table S6: Main and interactive effects of cadmium (Cd) and the extract from the berries of *Aronia melanocarpa* L. (AE) on the activities of glutathione peroxidase (GPx) and glutathione reductase (GR) and the concentration of glutathione S-transferase (GST) in the liver of rats, Table S7: Main and interactive effects of cadmium (Cd) and the extract from the berries of *Aronia melanocarpa* L. (AE) on the concentrations of reduced glutathione (GSH), oxidized glutathione (GSSG), and the ratio of GSH/GSSGS, as well as the concentration of total thiol groups (TSH) in the liver of rats, Table S8: Effect of the extract from the berries of *Aronia melanocarpa* L. (AE) on the concentration of thioredoxin (Trx) in the liver of rats exposed to cadmium (Cd), Table S9: Main and interactive effects of cadmium (Cd) and the extract from the berries of *Aronia melanocarpa* L. (AE) on the concentrations of hydrogen peroxide (H_2O_2), myeloperoxidase (MPO), and xanthine oxidase (XOD) in the liver of rats, Table S10: Main and interactive effects of cadmium (Cd) and the extract from the berries of *Aronia melanocarpa* L. (AE) on total antioxidative status (TAS), total oxidative status (TOS) and the index of oxidative stress (OSI) in the liver of rats, Figure S1: The effect of the extract from the berries of *Aronia melanocarpa* L. (AE) on the concentration and content of cadmium (Cd) in the liver of rats exposed to this metal.

Acknowledgments: This study was financially supported, in part, by the Grant (No. N N405 051140) from the National Science Centre (NCN, Poland) and the Grant (No. 136/KNOW/15) from Leading National Research Centre (KNOW, Poland). The study was conducted with the use of equipment purchased by the Medical University of Bialystok as part of the OP DEP 2007-2013, Priority Axis I.3, contract No. POPW.01.03.00-20-001/12.

Author Contributions: M.M. took part in designing the research, conducted the study, performed statistical analysis and interpreted the results, and wrote the paper; M.M.B. took part in designing the research, supervised the study, supervised analysis and interpretation of the results and the manuscript preparation, as well as corrected the final version of the article; J.R. participated in the study. All authors approved the final version of the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

AE	extract from the berries of *Aronia melanocarpa* L.
CAT	catalase
Cd	cadmium
Cd^{2+}	divalent ion of cadmium
Cu	copper
CV	coefficient of variation
DTNB	5,5′-dithio-bis-2-nitrobenzoic acid
ELISA	enzyme-linked immunosorbent assay
FR	free radicals
GPx	glutathione peroxidase
GR	glutathione reductase
GSH	reduced glutathione
GSSG	oxidized glutathione
GST	glutathione S-transferase
H_2O_2	hydrogen peroxide
MPO	myeloperoxidase
MT	metallothionein
NADPH	reduced nicotinamide adenine dinucleotide phosphate
$NADP^+$	oxidized nicotinamide adenine dinucleotide phosphate
-OH group	hydroxyl group
OSI	oxidative stress index
$O_2^{\cdot-}$	superoxide radicals
ROS	reactive oxygen species
SD	standard deviation
SOD	superoxide dismutase
-SH group	thiol group

TAS	total antioxidative status
TNT	5-thio-2-nitrobenzoic acid
Trx	thioredoxin
TOS	total oxidative status
TSH	total thiol groups
XOD	xanthine oxidase
Zn	zinc

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