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PROTECTIVE EFFECTS OF ALUMINOSILICATES ON LEAD-ACETATE TOXICITY IN BROILER CHICKENS

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ABSTRACT. The study examined the effect of dietary supplements of lead acetate (PbA) and/or aluminosilicates (zeolite and montmorillonite) on growth traits, relative organ weights, activity of liver enzymes and activity of enzymes of antioxidant protection and lipid peroxidation in liver, erythrocytes, pancreas and spleen of chickens. Weight gain, feed conversion ratio, relative weights of liver, pancreas and spleen and activity of antioxidative enzymes in pancreas and spleen were not influenced with dietary treatment. Dietary intake of PbA induces oxidative stress and promotes lipid peroxidation in liver and erythrocytes. Activities of liver enzymes (alkaline phosphatase, γ-glutamyltransferase and α-amylase) were influenced by PbA also. Aluminosilicates alone did not provoke any adverse effect and did not disturb normal biochemical and physiological homeostasis in broilers. The combined data showed that chickens fed aluminosilicates received significant protection against the effects of the PbA for most parameters measured.

Keywords: *aluminosilicates; antioxidative enzymes; bentonite; chickens; erythrocytes; lead acetate; liver; montmorillonite*

INTRODUCTION

Lead is one of the well-known ubiquitous non-essential metal poisons in the environment, particularly widespread in industrial areas. Thus, it is obvious that exposure to lead is implicated a broad range of physiological,

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biochemical and behavioral dysfunctions in animals and humans [1,2]. The main sources of exposure are contamination of feed and soil by industrial pollution, agricultural and food processing [3]. Its toxicity is closely related to its accumulation in certain tissues and its interference with the bioelements, whose role is critical for several physiological processes. The most deleterious effect of lead is on erythropoiesis, soft tissues, kidney function, and the central nervous system [4,5]. The extent of which orally administrated lead is absorbed is small. However, due to its slow rate of elimination, harmful levels of lead can accumulate in tissues after prolonged exposure to low quantities [1,6,7]. One of the most important mechanisms suggested for lead toxicity is a disruption in the prooxidant/antioxidant balance and inducing oxidative stress in cells with the generation of highly reactive oxygen species (ROS), such as hydroxyl radical, hydrogen peroxide, superoxide anion and lipid peroxides [3,8].

Aluminosilicates have been the subject of tremendous interest of both scientific and industrial world. They are used on a large industrial and scientific scale for a great variety of processes [9]. Clays and zeolites are hydrated and composed mostly of aluminium and silica and belong to the group of aluminosilicates. Phyllosilicate clays are hydrated, crystalline aluminosilicates containing alkali and alkaline earth cations and have layered structure. Montmorillonite, main constituent of phyllosilicate ore bentonite is trimorphic phyllosilicate formed by a 2:1 condensation of layers with aluminium sandwiched between two layers of silica. Montmorillonite possesses exchangeable sodium or calcium cations and has expandable sheets [10]. Because of accessibility and functional properties, bentonite and similar materials are widely used as a feed additives [11]. Natural zeolites are hydrated aluminosilicate minerals characterized by cage-like structures, with high internal and external surface area, and high cation-exchance capacities. The basic building blocks of natural zeolites are electrostatically charged tetrahedra of silica and aluminium, with the negative charge balanced by alkaline or alkaline earth cations. The stacking of these tetrahedral gives rise to various three-dimensional honeycomb structures containing tunnels or channels of uniform diameter [12]. Zeolites, due to their high ion-exchange capacity, have been used effectively for the prevention of heavy metal toxicity in animals [13].

The goal of present study is to throw the light on the possibilities of feed additive based on natural occurring hydrated aluminosilicates (Antitoxic nutrient-ATN) in preventing or minimizing the oxidative stress induced by chronic administration of lead acetate in chickens.

RESULTS AND DISCUSSION

Growth performance and relative organ weight

Results of body weight gain and feed efficiency in this experiment showed that lead acetate, at this level, alone or in combination with ATN did not manifest adverse effects (Table 1). Body weight was not found to change in any of the treated groups. Growth performance results from our experiment agreed with those of Tangpong and Satarug [14], Sainath et al. [8] and Flora et al. [15] performed on rats. Contrary to these results, studies of some other authors showed that adult treatment with different doses of lead acetate cause a significant decrease in pig's [16] and rat's [17] body weight. These studies suggested that the reduced growth was due to reduced food consumption via lead effects on the satiety set-point. Efficiency of feed utilization was not different among treatments. Also, Biesek et al. [18] and Banaszak et al. [19] found out that the use of halloysite and zeolite (0.5–2% in the feed) had positive effect on growth performance of broiler chickens in long-term experiment.

Table 1. Effect of chronic exposure to lead acetate (Pb) and ATN supplemantation on body weight gain and feed conversion ratio of broilers

	Experimental group			
Body weight gain [g/day]	**Control**	**ATN**	**Pb**	**Pb + ATN**
Day 1-7	13.73 ± 1.14	14.02 ± 0.92	14.22 ± 0.57	13.64 ± 1.08
Day 8-14	27.87 ± 0.87	29.02 ± 1.16	25.45 ± 1.65	29.74 ± 0.78
Day 14-21	49.26 ± 1.65	50.20 ± 2.00	50.24 ± 1.36	47.83 ± 2.16
Feed conversion ratio [kg/kg]	1.60	1.58	1.60	1.57
The data are mean values ± standard error				

The effects of lead acetate and ATN on relative organ weights of poultries are presented in Table 2. No significant changes in the relative weights of liver, pancreas and spleen were observed in control and experimental chickens although animals in ATN-treated groups showed a tendency to have increased weights of measured organs. In our previous experiment broilers fed with addition of ATN in 6-week trial had significantly increased relative weight of spleen and some organs of digestive tract [20]. It is also reported that supplemental clays and zeolites did not significantly affect the relative weights of the liver, pancreas and spleen in poultry [18, 21]. The animals treated with lead acetate alone, or in combination with ATN were comparable to the control.

Table 2. Effect of chronic exposure to lead acetate (Pb) and ATN supplemantation on relative organ weights of broilers

	Experimental group			
Organ	**Control**	**ATN**	**Pb**	**Pb + ATN**
Liver [g/kg]	24.00 ± 2.51	27.22 ± 2.24	25.56 ± 2.27	28.24 ± 3.09
Pancreas [g/kg]	3.31 ± 0.43	3.73 ± 0.40	3.44 ± 0.42	4.02 ± 0.70
Spleen [g/kg]	0.73 ± 0.19	1.00 ± 0.26	0.79 ± 0.20	0.92 ± 0.21
The data are mean values ± standard error				

Enzyme activity and lipid peroxidation in liver

In animal production, different aluminosilicates (clays and zeolites) are used as adsorbents of different toxic substances (mycotoxins, phytotoxins, enterotoxins, heavy metals etc.). Some aluminosilicates have potential to absorb and sequester different organic and inorganic ions and molecules in the lumen of the gastrointestinal tract of animals, favoring their expulsion from the body [22].

The liver plays a major role in lead's metabolism because after lead exposure liver is one of the major organs involved in the storage, biotransformation and detoxification [23]. Lead-induced hepatic damage with, cholestasis and is well-documented. Lead hepatotoxicity manifests itself in portal cellular infiltration, biliary hyperplasia, disorganization of the hepatic cords, cytoplasmic vacuolization and invading of infiltrative inflammatory cells. Chronic lead toxicity affects a range of cellular enzymes particularly those involved in energy production [24].

Table 3. Effect of chronic exposure of lead acetate (Pb) and ATN on liver enzyme activities of broilers

	Experimental group			
Parameter	**Control**	**ATN**	**Pb**	**Pb + ATN**
ALP [IU/mg protein]	$56.10^{a} \pm 5.97$	$51.14^{a} \pm 6.35$	$34.18^{b} \pm 2.02$	$42.08^{c} \pm 4.11$
ALT [IU/mg protein]	$35.72^{a} \pm 4.85$	$37.26^{a} \pm 3.77$	$36.14^{a} \pm 1.11$	$33.57^{a} \pm 1.09$
GGT [IU/mg protein]	$4.69^{a} \pm 0.07$	$3.73^{a,b} \pm 0.43$	$3.04^{b} \pm 0.28$	$3.86^{a,b} \pm 0.39$
AMY [IU/mg protein]	$9.37^{a} \pm 2.50$	$8.27^{a} \pm 1.32$	$11.85^{b} \pm 1.31$	$11.44^{b} \pm 1.56$
The data are mean values ± standard error ^{a,b,c} Values without the same superscripts within each row differ significantly ($P < 0.05$) ALP, alkaline phosphatase; ALT, alanine amino transferase; GGT, γ-glutamiletransferase; AMY, α-amylase				

During oxidative stress, which leads to cell damage or organ dysfunction, there is generally an increase in the activity of different enzymes: AST, ALT, ALP, LDH, GGT and others [25]. The serum levels of the enzymes ALT, GGT and ALP are considered as a biomarkers of hepatocyte function [26]. However, levels of this enzymes in serum could be results of metabolic disorders of other organs: heart [27], kidneys [28] or pancreas [29]. Many studies have demonstrated similar results that lead changes the liver function inducing liver fattening and increased activity of serum AST, ALT, ALP and GGT enzymes [23,30,31,32]. Our previous studies demonstrated that dietary inclusion of clinoptilolite or ATN do not affect activity of these serum enzymes in pigs [33,34,35] and broilers [20]. Treatment with lead acetate significantly (P<0.05) decreased hepatic ALP and GGT and increased activity of AMY compared with other experimental groups (Table 3). Activity of ALT in liver was not influenced by dietary treatment (P>0.05). These results are partially in accordance with those of Abdou and Newairy [32]. They hypothesized that the decrease in activity of hepatic AST, ALT and ALP could be expected to occur associating with the pathology involving necrosis of the liver. This decrement in enzyme activities is mainly due to the leakage of these enzymes from the liver cytosol into the blood stream which gives an indication on the hepatotoxic effect of lead.

The results of this study indicate that lead causes significant changes in oxidative stress in different organs, particularly in erythrocytes and liver which are found to be more vulnerable to lead-induced toxicity. SOD was found to play a major role in the first line of antioxidant defense by catalyzing the dismutation of superoxide anion radicals to form hydrogen peroxide and molecular oxygen. Oxidative stress created by the metal ions favors increased production of superoxide anions [36]. Under oxidative stress, SOD can behave in two different ways: initially and when stress is moderated, the cells act by increasing the SOD activity, but if the stress lasts a long time and favors increased production of ROS, the enzyme is exhausted and its activity falls [37]. In our case, decreased SOD-1 activity observed (P<0.05) could be explained by the massive production of superoxide anions, which override enzymatic activity and lead to the fall of its concentration in liver (Table 4). This could be due to a lead-induced copper deficiency. The low activity of SOD-1 could also be due to the inactivation of the enzyme by crosslinking or damage of DNA. Similar results were obtained by Ilesamni et al. [23]. Unlike Sainath et al. [8], Ilesamni et al. [23] and Mehana et al. [30], we did not observed any change in activity of other measured antioxidative enzymes in liver under lead intoxication. Our previous study also demonstrates that oral intake of ATN does not provoke inhibition or stimulation of liver GST in broilers [20, 38].

The oxidative stress has also been implicated to contribute to lead-associated tissue injury in the liver. Our study showed an increase in MDA content (P<0.05) in liver of broilers treated with the lead acetate, suggesting an increase in lipid peroxidation in hepatic cells. This result is in agreement with the studies undertaken by other authors [31,32] who recorded an increase in MDA content in the liver of rats subjected to subchronic exposure to lead for a period of several weeks.

Table 4. Effect of lead acetate (Pb) exposure alone and in combination with ATN on the activity of endogenous antioxidant enzymes and lipid peroxidation in the liver of broilers

	Experimental group			
Parameter	**Control**	**ATN**	**Pb**	**Pb + ATN**
SOD-1 [IU/mg protein]	$18.46^{a} \pm 0.63$	$19.56^{a} \pm 0.86$	$15.14^{b} \pm 0.69$	$18.98^{a} \pm 0.54$
CAT [IU/mg protein]	$22.20^{a} \pm 1.75$	$24.05^{a} \pm 2.24$	$25.03^{a} \pm 0.94$	$22.53^{a} \pm 0.84$
GPx [IU/mg protein]	$2.08^{a} \pm 0.066$	$2.14^{a} \pm 0.062$	$2.18^{a} \pm 0.071$	$2.02^{a} \pm 0.070$
PPx [IU/mg protein]	$64.32^{a} \pm 3.22$	$58.19^{a} \pm 1.75$	$62.02^{a} \pm 2.20$	$60.01^{a} \pm 1.98$
GST [IU/mg protein]	$390.08^{a} \pm 10.24$	$376.21^{a} \pm 12.38$	$388.26^{a} \pm 11.63$	$367.14^{a} \pm 15.20$
LP [nmol MDA/mg protein]	$2.05^{a} \pm 0.14$	$1.92^{a} \pm 0.09$	$2.67^{b} \pm 0.04$	$2.07^{a} \pm 0.07$

The data are mean values ± standard error
^{a,b} Values without the same superscripts within each row differ significantly (P< 0.05)
SOD-1, superoxid dismutase; CAT, catalase; GPx, guaiacol peroxidase; PPx, pyrogallol peroxidase; GST, glutathion S-transferase; LP, lipid peroxidation

Enzyme activity and lipid peroxidation in red blood cells

The hematological system has been proposed as being important target for lead-induced toxicity. Lead may be rapidly absorbed and reached considerable amount in the blood. Once absorbed, blood lead is transported to the erythrocytes as lead diphosphate [4].

In the present study the decrease in the mean corpuscular hemoglobin concentration (P<0.05) of broilers treated with lead acetate may result from the direct and indirect toxic effects of lead acetate (Table 5). These results are in agreement with results of other authors mostly performed on rats [15], mice [39,40] and humans [41]. Lead is known to interfere with heme and hemoglobin synthesis and to affect erythrocyte morphology and survival. Anemia caused during lead poisoning is result of inhibition of heme synthesis and a decreased life span of circulating erythrocytes. This shortening of life span is probably due to the direct toxic effect of lead upon the cell membrane

integrity by inhibiting Na-K-ATPase. Lead, furthermore, interferes with iron utilization for heme formation in the mitochondria, and studies showed that lead competes with iron for the incorporation into red blood cells. Moreover, oxidative stress and free radicals produced in the presence of heavy metals cause changes in the structure and function of Hb, leading to anemia. Anemia observed in lead poisoning is caused also indirectly by the accumulation of δ-aminolevulinic acid [1,36].

The Table 5 shows the activity of antioxidant enzymes SOD-1, CAT, GPx, PPx, and GST in red blood cells. Significant decreases in SOD-1 activity of erythrocytes were recorded in Pb group as compared with control and ATN groups (P<0.05). Activities of all other measured antioxidant enzymes were significantly increased in Pg group of broilers when compared to values of control, ATN and Pb + ATN groups of broilers (P<0.05). The high level of antioxidant enzymes found in this study are consistent with the results of Wang et al. [39] and Tangpong and Satarug [14], who reported increased activity of glutathione peroxidase and decreased activity of SOD in red blood cells of mouses after Pb administration.

Table 5. Effect of lead acetate (Pb) exposure alone and in combination with ATN on the hemoglobin concentration, activity of endogenous antioxidant enzymes and lipid peroxidation in the erythrocytes of broilers

	Experimental group			
Parameter	**Control**	**ATN**	**Pb**	**Pb + ATN**
Hb [g/l]	$146.42^{a} \pm 3.92$	$146.10^{a} \pm 3.11$	$105.22^{b} \pm 5.54$	$127.95^{a,b} \pm 6.65$
SOD-1 [IU/mg Hb]	$466.08^{a} \pm 12.33$	$489.75^{a} \pm 15.45$	$354.88^{b} \pm 12.76$	$485.45^{a} \pm 12.34$
CAT [IU/mg Hb]	$8.26^{a} \pm 0.48$	$8.12^{a} \pm 0.59$	$10.09^{b} \pm 0.29$	$7.50^{a} \pm 0.45$
GPx [IU/mg Hb]	$6.41^{a} \pm 0.24$	$6.83^{a} \pm 0.17$	$8.44^{b} \pm 0.09$	$6.78^{a} \pm 0.19$
PPx [IU/mg Hb]	$14.40^{a} \pm 0.50$	$15.00^{a} \pm 0.48$	$18.95^{b} \pm 0.57$	$14.80^{a} \pm 0.44$
GST [IU/mg Hb]	$120.05^{a} \pm 3.22$	$133.21^{a} \pm 3.46$	$162.44^{b} \pm 3.08$	$132.97^{a} \pm 3.00$
LP [nmol MDA/mg Hb]	$1.19^{a} \pm 0.089$	$1.41^{a} \pm 0.125$	$2.86^{b} \pm 0.088$	$1.45^{a} \pm 0.103$

The data are mean values ± standard error
^{a,b} Values without the same superscripts within each row differ significantly (P< 0.05)
Hb, hemoglobin; SOD-1, superoxid dismutase; CAT, catalase; GPx, guaiacol peroxidase; PPx, pyrogallol peroxidase; GST, glutathion S-transferase; LP, lipid peroxidation

There was no significant difference in red blood cells MDA concentration of the control and those of the animals treated with ATN or ATN along with Pb, whereas chickens treated with Pb only showed a significant increase in MDA concentration. This agrees with other reports on experimental lead intoxication in mice [14] and rats [42].

Enzyme activities and lipid peroxidation in pancreas and spleen

Literature data about oxidative process in pancreas and spleen of animals under lead toxicity are very limited. Lead is stored in almost all soft tissues, autopsy studies showed that liver is the largest repository of soft tissue lead, followed by the kidney cortex and medulla, and other soft tissues: pancreas, spleen, ovary, adrenal gland, prostate, testis, fat, brain, heart and skeletal muscle [4,40]. Muselin et al. [43] reported that chronic exposure to lead can induce severe lesions in spleen with small necrosis spots. Data presented in Table 6. and Table 7. demonstrate that oral administration of lead acetate or ATN did not provoke oxidative stress or induce activity of antioxidative enzymes in pancreas and spleen of broiler chickens (P>0.05).

Table 6. Effect of lead acetate (Pb) exposure alone and in combination with ATN on the activity of endogenous antioxidant enzymes and lipid peroxidation in the pancreas of broilers

	Experimental group			
Parameter	**Control**	**ATN**	**Pb**	**Pb + ATN**
SOD-1 [IU/mg protein]	$4.14^{a} \pm 0.12$	$4.29^{a} \pm 0.21$	$4.40^{a} \pm 0.22$	$4.31^{a} \pm 0.11$
CAT [IU/mg protein]	$7.55^{a} \pm 0.40$	$7.39^{a} \pm 0.21$	$8.42^{a} \pm 0.49$	$8.56^{a} \pm 0.25$
GPx [IU/mg protein]	$036^{a} \pm 0.011$	$0.39^{a} \pm 0.020$	$0.35^{a} \pm 0.017$	$0.40^{a} \pm 0.010$
PPx [IU/mg protein]	$2.66^{a} \pm 0.26$	$2.15^{a} \pm 0.13$	$2.95^{a} \pm 0.22$	$2.32^{a} \pm 0.14$
LP [nmol MDA mg^{-1} protein]	$3.26^{a} \pm 0.34$	$2.98^{a} \pm 0.06$	$2.84^{a} \pm 0.16$	$2.56^{a} \pm 0.34$

The data are mean values ± standard error
^{a,b} Values without the same superscripts within each row differ significantly (P< 0.05)
SOD-1, superoxid dismutase; CAT, catalase; GPx, guaiacol peroxidase; PPx, pyrogallol peroxidase; LP, lipid peroxidation

Table 7. Effect of lead acetate (Pb) exposure alone and in combination with ATN on the activity of endogenous antioxidant enzymes and lipid peroxidation in the spleen of broilers

	Experimental group			
Parameter	**Control**	**ATN**	**Pb**	**Pb + ATN**
SOD-1 [IU/mg protein]	$3.88^{a} \pm 0.10$	$3.67^{a} \pm 0.14$	$3.66^{a} \pm 0.14$	$3.75^{a} \pm 0.13$
CAT [IU/mg protein]	$5.72^{a} \pm 0.12$	$5.12^{a} \pm 0.17$	$5.63^{a} \pm 0.17$	$4.85^{a} \pm 0.10$
GPx [IU/mg protein]	$0.59^{a} \pm 0.03$	$0.63^{a} \pm 0.03$	$0.64^{a} \pm 0.02$	$0.60^{a} \pm 0.02$
PPx [IU mg^{-1} protein]	$16.45^{a} \pm 1.13$	$14.25^{a} \pm 0.63$	$11.61^{b} \pm 0.83$	$13.31^{a,b} \pm 0.82$
LP [nmol MDA/mg protein]	$2.58^{a} \pm 0.05$	$2.60^{a} \pm 0.08$	$2.37^{a} \pm 0.17$	$2.24^{a} \pm 0.17$

The data are mean values ± standard error
^{a,b} Values without the same superscripts within each row differ significantly (P< 0.05)
SOD-1, superoxid dismutase; CAT, catalase; GPx, guaiacol peroxidase; PPx, pyrogallol peroxidase; LP, lipid peroxidation

CONCLUSIONS

Results of this study are similar to those from our previous study [44] where we demonstrate that application of ATN in broilers' feed can prevent biochemical alterations in kidneys, brain and duodenum induced by intoxication with lead acetate. Co-administration of natural occurring aluminosilicates (ATN) with lead acetate offers significant protection against lead-induced oxidative stress by inhibiting lipid peroxidation and activating antioxidant defense system in red blood cells and liver of chickens. It has ability to absorb lead in lumen of digestive tract and thus could be used as a supplementary agent in animal feeds.

EXPERIMENTAL SECTION

Chickens and diet

Eighty four 1-day-old, unvaccined broiler chicks of both sexes were obtained from a commercial hatchery. Individually weighted chicks were divided at random into four groups. There were seven replicates of three broiler chicks for each dietary treatment. The chicks were housed in electrically heated batteries under fluorescent lighting and received a commercial basal diet (maize and soybean meal diet 220 g protein, 13.00 MJ ME kg^{-1}) formulated to contain the National research Council (1994) requirements. Food and water were available *ad libitum* and lighting was continuous.

Experimental design

The experimental design consisted of four dietary treatments: 1. Control: basal diet; 2. ATN: basal diet plus 5.0 g ATN/kg diet; 3. Pb: basal diet plus 500 mg lead acetate/kg diet; 4. Pb + ATN: basal diet plus 500 mg lead acetate plus 5 g ATN/kg. ATN (Antitoxic nutrient) is a fine powder containing mostly zeolitic ore (with > 90% of clinoptilolite) and bentonite (with > 83% of montmorillonite), together with small amounts of activated charcoal (ratio 60:20:1/zeolite:bentonite:charcoal). The experimental protocol was approved by Ethics Committee on Animal Use of the University of Novi Sad.

Blood sampling and slaughter

When the chicks reached 3 weeks of age, the feeding trial was terminated and all broilers were bled by cardiac puncture. Heparin was used as an anticoagulant and non-coagulated blood was used for separation of

erythrocytes. Hemoglobin (Hb) concentration in red blood cells was determined by the cyanmethemoglobin procedure [45]. Blood samples (20 µL) were mixed with 5 mL Drabkin's solution (0.1% sodium bicarbonate, 0.005% potassium cyanide, and 0.02% potassium ferricyanide) for Hb determination. All 84 broilers were sacrificed by cervical dislocation and liver, spleen, and pancreas were removed and weighted. Homogenates of these organs with phosphate buffer (pH=7.0) were used for further biochemical analysis. Samples were homogenized with using a MiniBatch D-15 homogenizer (MICCRA GmbH, Müllheim, Germany).

Biochemical analysis

Activity of alkaline phosphatase (ALP), alanine aminotranspherase (ALT), γ-glutamyltransferase (GGT), and α-amylase (AMY) in liver homogenate were determined on a clinical chemistry analyzer (Microlab 200, Merck) according to the manufacturer's recommended procedure.

The antioxidant enzyme activity of superoxide dismutase (SOD-1), catalase (CAT), guaiacol peroxidase (GPx), pyrogallol peroxidase (PPx), and malondialdehyde (MDA) level was determined from erythrocytes, liver, spleen and pancreas. Glutathione S-transferase (GST) activity was evaluated in erythrocytes and liver homogenate. Protein content in homogenate of liver, spleen, and pancreas were determined according to the method of Bradford [46], using bovine serum albumin as the protein standard. SOD-1 activity was determined in samples according to the method of Mandal et al. [47] slightly modified by measuring its ability to inhibit photochemical reduction of nitro blue tetrazolium (NBT) chloride. The reaction mixture contained 50 mM phosphate buffer (pH 7.8), 13 mM L-methionine, 75 µM NBT, 0.1mM EDTA, 2 µM riboflavin and 20 µL of the enzyme extract. It was kept under a fluorescent lamp for 30 min, and the absorbance was read at 560 nm. One unit of the SOD-1 activity was defined as the amount of enzymes required to inhibit reduction of NBT by 50%. The activity of the enzyme was expressed in IU/mg of protein. The CAT activity was assayed by the method of Aebi [48]. The utilization of hydrogen peroxide by CAT in the samples was measured spectrophotometrically. The decomposition of H_2O_2 was followed as a decrease in absorbance at 240 nm. The enzyme extract (20-100 µL) was added to the assay mixture containing 1 mL of 50 mM potassium phosphate buffer (pH 7.0) and 10 mM H_2O_2. GPx activity was measured by following the H_2O_2 depend oxidation of guaiacol at 470 nm [49]. This method consists of an assay of tetraguaiacol - a colored product of guaiacol oxidation in the investigated sample. The enzyme extract (40 µL) was added to the assay mixture containing 3 mL of 20 µM guaiacol and 20 µL of 3 mM H_2O_2.

The absorbance was recorded at 436 nm. The activity of PPx was measured using pyrogallol as the substrate according to Murtic et al. [50]. This method is based on the measurement of the content of purpurogallin - a product of pyrogallol oxidation. The enzyme extract (20 µL) was added to the assay mixture containing 3 mL of 180 mM pyrogallol and 20 µL of 2 mM H_2O_2. The absorbance was recorded at 430 nm. GST activity in samples was evaluated using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate as previously described by Habig et al. [51]. The formation of adduct of GSH-CDNB (2,4-dinitrophenyl glutathione) was monitored by estimation of the increase in absorbance at 340 nm against a blank with a spectrophotometer. The reaction mixture consisted of 33 mM Hepes buffer (pH 7.5), 1.5 mM GSH, 1.5 mM CDNB, and water in a total volume of 1 mL. The activity of the enzymes was expressed in IU/mg of protein.

The MDA level (as a measure of lipid peroxidation (LP) intensity) was analyzed with 2-thiobarbituric acid, monitoring the change of absorbance at 532 nm with the spectrophotometer [47]. The enzyme extract (0.5 mL) was incubated with 2 mL of 20% trichloroacetic acid (TCA) containing 0.5% thiobarbituric acid for 40 min at 95° C. The reaction was stopped by cooling on ice for 10 min and the product was centrifuged at 10.000×*g* for 15 min. The total amount of TBA-reactive substances was expressed as nmol MDA/mg protein.

Statistical analysis

Results were expressed as mean of determinations ± standard error (SE). Statistical significance was tested by analysis of variance followed by comparison of means by Duncan's multiple range test ($P < 0.05$) calculated using STATISTICA for Windows version 13.0 (StatSoft, Tulsa, OK, USA). Stepwise multiple regression analyses were used to determine correlation among variables.

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