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Gamma radiation effects on antioxidant activity of black chokeberry fruits (*Aronia Melanocarpa L.*) in mice models

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Food irradiation is a process of food exposing to ionizing radiation. The maximum dose absorbed by the food should not exceed 10 kGy unless it is necessary to achieve a legitimate technological purpose. The black chokeberry fruit *(Aronia Melanocarpa)* is a specific berry with high polyphenol content and possesses one of the highest *in vitro* antioxidant activities among other fruits. We aimed to investigate the *in vivo* effect on the plasmatic and hepatic oxidative status of experimental mice after treatment with non-irradiated and 10 kGy γ-irradiated samples of *A. Melanocarpa* (samples were pulverized to powder, 0.5 g of which was mixed with 40 ml of extract (30% ethanol solution). For this purpose, we studied the oxidative stress biomarkers levels -ascorbate radicals (·Asc), reactive oxygen species (ROS) products and nitric oxide NO· radicals by EPR spectroscopy. The obtained results show that the antioxidant defense system in the plasma of treated mice fails to overcome the induced oxidative stress after treatment of *A. Melanocarpa* extract with 10 kGy.

Keywords: oxidative stress, *Aronia Melanocarpa*, gamma radiation, ROS products, NO radicals, Ascorbate radicals

INTRODUCTION

Spices and herbs are always contaminated with microorganisms. Therefore, irradiation is currently used as the main method of decontamination of dry plant materials such as spices and herbs [1]. General standards for food irradiation state that the minimum absorbed dose should be sufficient to achieve the technological objective and the maximum absorbed dose should be less than that which would compose consumer safety. The maximum dose absorbed by the food should not exceed 10 kGy unless it is necessary to achieve a legitimate technological purpose [2].

Black chokeberry fruits (*Aronia Melanocarpa L.; A. Melanocarpa*; Michx Elliott) have a high content of B vitamins and trace elements as manganese and iron. *A. Melanocarpa* fruits are extremely rich in polyphenol substances – their content varies between 40 and 70 mg/g dm, 50 % of which are anthocyanins [3]. Recently, particular attention is given to natural products which are characterized by high antioxidant capacity due to the radical scavenging effects of their substituents on reactive oxygen species (ROS) and reactive nitrogen species (RNS) which occurs in the body due to metabolic reactions, as well as in the case of inflammation and neutralization of xenobiotics. The elevation in temperature of plant tissue and UV irradiation cause changes in polyphenol content, the range of which depends on the power and, sometimes, on the operating time of abiotic stress factors [4, 5]. The abiotic stress factors as UV-radiation, electromagnetic field, microwave radiation, and ultrasound, at different operation times, caused differentiation in the contents of anthocyanins, phenolic acids, flavonols, and flavan-3-ols in chokeberry [6].

The study aim was the *in vivo* examination of the effect on the plasmatic and hepatic oxidative status of experimental mice after treatment with non-irradiated and 10 kGy γ-irradiated 30% *A. Melanocarpa* ethanol extract. To realize the aim of the current research the levels of the oxidative stress biomarkers such as ascorbate radicals (·Asc), ROS products and NO· radicals were studied by direct and spin-trapping EPR spectroscopy.

EXPERIMENTAL

Carboxy.PTIO.K potassium salt, dimethyl sulfoxide (DMSO), and other (HPLC grade) solvents and reagents were purchased from Sigma-Aldrich (Steinheim, Germany). Black chokeberry

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fruits were supplied from Vitanea Ltd. (Plovdiv, Bulgaria) in the stage of full maturity in August 2017.

Fresh fruits were frozen at -18˚C, lyophilized (Christ Alpha 1-4 LDplus, Martin Christ GmbH, Germany) and stored in a desiccator until use.

Gamma-irradiation of dried Aronia Melanocarpa fruits

Freeze dried berries were irradiated at a ^{60}Co source with 8200 Ci activity. The chosen absorbed dose was 10 kGy. All gamma irradiated samples and untreated controls were pulverized to powder, 0.5 g of which were mixed with 40 ml of extract - 30% ethanol solution acidified with 0.5% formic acid. Samples were extracted on a magnetic stirrer for 1 h at room temperature [7]. The total polyphenol content of the investigated samples varied between 6935 ± 79 mg/100 g DW and the total content of anthocyanins was in the range of 1192 ±mg/100 g DW, at 10 kGy, as reference [8].

For the experiment, IRC/w non-inbred albino male mice (25±1.5 g) were used. The mice were housed in polycarbonate cages in controlled conditions (12 h light/dark cycles), at a temperature of 18-23°C and humidity of 40-70%, with free access to tap water and standard laboratory chow at Suppliers of Laboratory Animals of the Medical Faculty, Trakia University. The animal study was approved with Directive 2010/63/EU/ Ethical Committee for Animals of BABH and Trakia University, Stara Zagora, Bulgaria (131/6000-0333/09.12.2016). The experimental animals were randomly assigned to three groups, each of 6 mice: control, 30% ethanol solution *A. Melanocarpa* extract, 10 kGy 30% ethanol solution *A. Melanocarpa* extract. The mice were treated orally in the acute experiment according to Eftimov *et al.* [9]. The control group was pretreated orally with saline (30 mL/kg) for 2 h. The *A. Melanocarpa* and 10 kGy *A. Melanocarpa* groups were pretreated with the respective extract at a dose of 30 mL/kg.

The mice were anesthetized and euthanized 2 h after the treatment. The fresh blood (1.3-2 ml) was collected directly from the heart in cold EDTA-containers. After centrifugation the plasma samples were immediately studied by EPR spectroscopy for their radical scavenging abilities. The freshly isolated liver was collected on ice and homogenized. The electron paramagnetic (EPR) measurements were performed on an X-Band, Emx^{micro} Spectrometer (Bruker, Germany). Spectral processing was performed using Bruker WIN-EPR and SimFonia software.

Levels of ascorbate radicals

The plasma and liver homogenates from the three groups were prepared according to Bailey *et al*. [10]. The levels of ascorbate radicals were calculated as double integrated plots of EPR spectra and results were expressed in arbitrary units.

Levels of ROS products

The levels of ROS products were determined according to Shi *et al.* [11] modified by us. The levels of ROS products were calculated as double integrated plots of EPR spectra and results were expressed in arbitrary units.

NO· radicals

Based on the methods published by Yoshioka *et al.* [12] and Yokoyama *et al*. [13] we developed and adapted the EPR method for estimation of the levels of NO· radicals. The levels of NO· radicals were calculated as double integrated plots of EPR spectra and results were expressed in arbitrary units.

Statistical analysis

Statistical analysis was performed with Statistica 7, StaSoft, inc. and the results were expressed as means ± S.E. $p < 0.05$ was considered statistically significant. To define which groups are different from each other we have used LSD post hoc test.

RESULTS AND DISCUSSION

Phenolic compounds have a perfect chemical structure to scavenge and neutralize free radicals because they have phenolic OH groups that are ready to donate H^+ or an electron to a free radical and an expanded conjugated aromatic system to unblock the unpaired electron [14]. To evaluate the extractability of phenolic compounds in methanol/water extract of *G. biloba*, Pereira and coauthors have studied non-irradiated and irradiated with 10 kGy extract samples. They found that irradiated with 10 kGy methanol/water extract and infusion preparation gave the highest content of phenolic compounds and concluded that this dose of irradiation ensures the disinfection and decontamination of the products from microbes and contributes to increasing the extractability of the phenolic compounds [15]. All mentioned studies led us to assess *ex vivo* the effect of 10kGy radiation on the radical scavenging capabilities of an extract isolated from *A. Melanocarpa* fruits. Results from ascorbic radicals' levels measured by direct EPR spectroscopy in the plasma of control mice and mice treated with non-irradiated and irradiated *A. Melanocarpa* extract are given in Fig. 1.

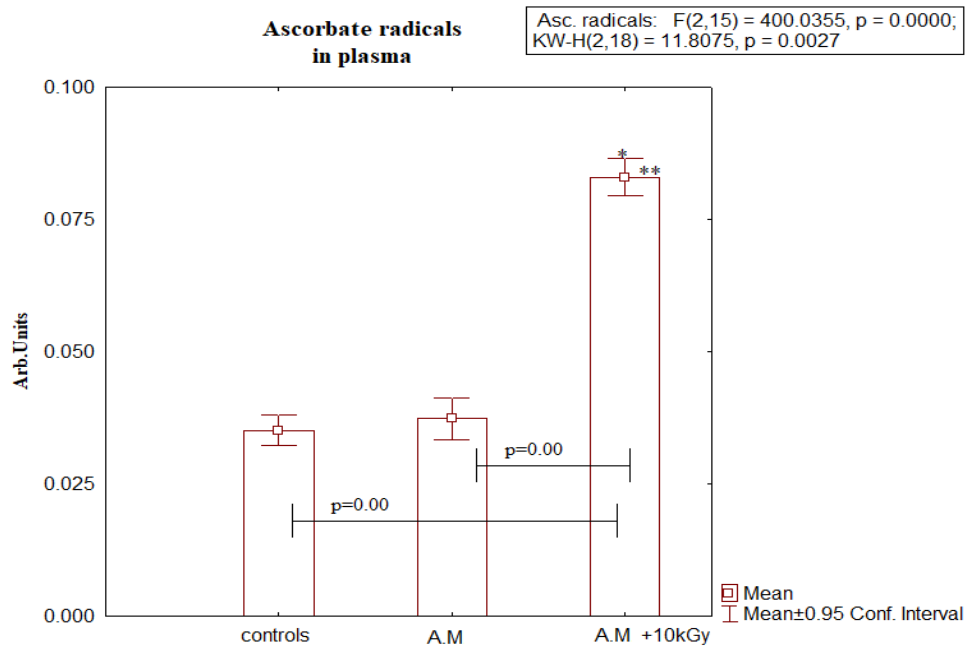

Fig. 1. ·Asc radicals levels measured in plasma of mice treated with non-irradiated and irradiated *A. Melanocarpa*, and compared with the controls. Significant difference *p < 0.05 vs controls. **p < 0.05 vs 10 kGy irradiated A. *Melanocarpa*. The LSD post hoc test was used to determine the groups differing from each other.

In the order of the standard one-electronic reduction potentials, the ascorbate ranks at the end of the series of oxidative free radicals such as hydroxyl (·OH); alkylperoxyl (ROO·); lipidperoxyl (LOO·); tocoperoxyl (·TO); $\cdot O_2^-/HO_2$.

All of these radicals have higher redox potential and can be reduced by ascorbic acid, and as a result, ascorbate radicals are generated [16]:

$$AscH^- + R\cdot = \cdot Asc^- + RH$$

Ascorbate radical has a relatively long half-life and can be directly confirmed by EPR spectroscopy [17]. This property turns the ascorbate radical into the best endogenous non-toxic biomarker for proving the generation of toxic reactive radicals in biological systems [18, 19].

The group treated with irradiated extract exhibits statistically significant higher levels of ·Asc radicals compared to controls (mean 0.083±0.001 *vs* mean 0.035±0.001, according to the LSD post hoc test p=0.00), and mice treated with non-irradiated *A. Melanocarpa* extract (mean 0.083±0.001 *vs* mean 0.037±0.001, according to the LSD post hoc test p=0.00). The group of mice with non-treated *A. Melanocarpa* extract shows values close to the controls (mean 0.037±0.001 *vs* mean 0.035±0.001, according to the LSD post hoc test p=0.03), while 10 kGy irradiation causes a 2.4- fold increase in plasma ·Asc levels comparing to controls and mice treated with the non-irradiated extract.

As is seen (Fig. 2) the levels of ROS products in plasma of mice treated with 10 kGy irradiated *A. Melanocarpa* extract were statistically significantly higher compared to healthy controls (mean 7.01±0.2 *vs* mean 2.05±0.1, according to the LSD post hoc test p=0.000) and the group treated with non-irradiated samples of *A. Melanocarpa* (mean 7.01±0.2 *vs* mean 2.42±0.1, according to the LSD post hoc test p=0.000). Moreover, ROS plasma values of mice treated with non-irradiated *A. Melanocarpa* were close to controls (mean 2.42±0.2 *vs* mean 2.05±0.1, according to the LSD post hoc test p=0.01).

As is shown (Figs. 1 and 2), the levels of the registered ·Asc and ROS products in the plasma of the mice treated with non-irradiated *A. Melanocarpa* extract were commensurable with the control group. This means that the non-irradiated extract does not induce ROS generation in the plasma of treated mice. The significant increase in ·Asc levels registered after treatment with 10 kGy irradiated extract shows that the antioxidant non-enzymatic defense system (in particular ascorbic acid) is involved in neutralizing the generated oxidative radicals to overcome the induced oxidative stress in mice plasma, but apparently it failed. This finding is additionally supported by the results obtained for the levels of ROS registered in mice plasmas after treatment with irradiated extract (see Fig. 2).

Results obtained for NO· in plasma are given in Fig 3. Plasma of mice treated with 10kGy irradiated *A. Melanocarpa* extract showed (Fig.3) statistically higher NO· values compared to controls (mean 6.84±0.1 *vs* mean 2.33±0.1, according to the LSD post hoc test p=0.00), as well as to those of mice treated with non-irradiated *A. Melanocarpa* (mean 6.84±0.1 *vs* mean 3.34±0.1, according to the LSD post hoc test p=0.00).

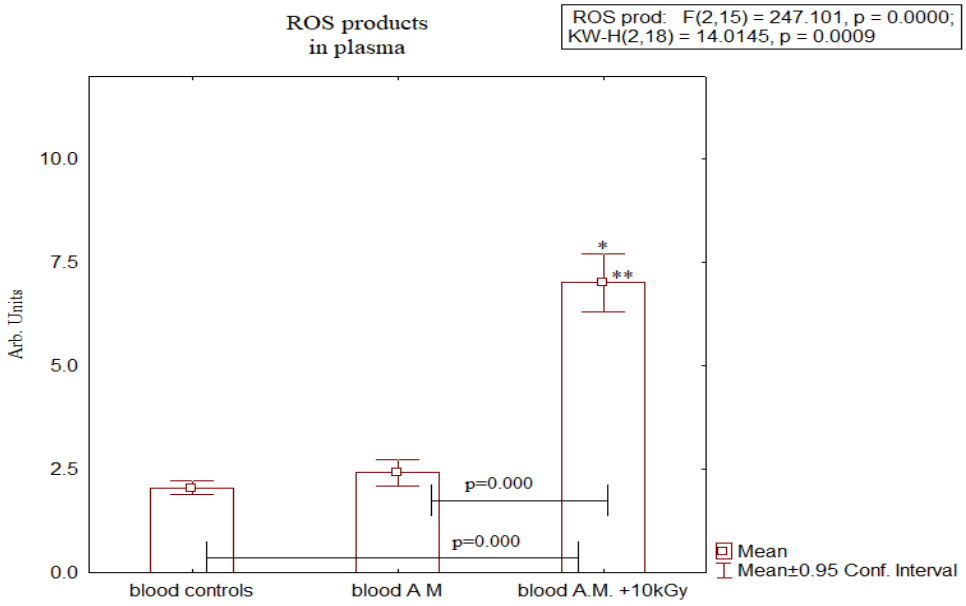

Fig. 2. Levels of ROS products measured in plasma of mice treated with non-irradiated and irradiated A. Melanocarpa extract, and compared to the controls. Significant difference *p < 0.05 vs controls. **p < 0.05 vs 10 kGy irradiated A. Melanocarpa. The LSD post hoc test was used to determine the groups differing from each other.

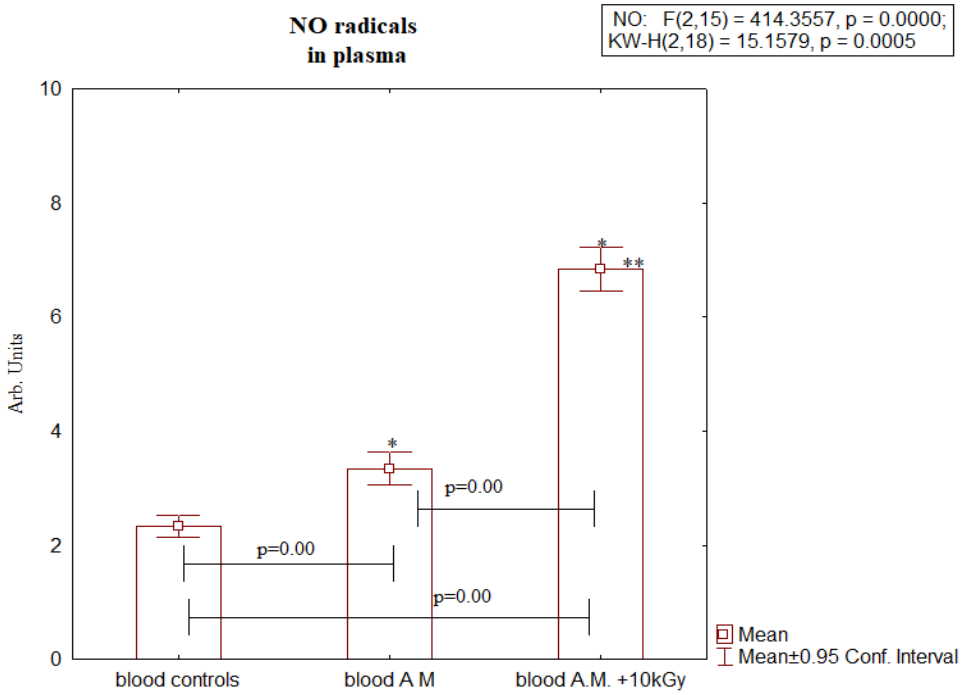

Fig. 3. Levels of NO· measured in plasma of mice treated wit*h non-irradiated and irradiated A. Melanocarpa, and compared to the control mice. Significant difference *p < 0.05 vs controls. **p < 0.05 vs 10 kGy irradiated A. Melanocarpa. The LSD post hoc test was used to determine the groups differing from each other.

It should be mentioned that significantly higher NO· levels were also found for the group of mice treated with non-irradiated extract compared to the control group (mean 3.34±0.1 *vs* mean 2.33±0.1, according to the LSD post hoc test p=0.00).

It is of interest to note that in the plasma of mice treated with 10 kGy irradiated *A. Melanocarpa*

extract, about 2.3-fold increase in ·Asc levels was observed in comparison with both groups: mice treated with non-irradiated extract and control group. On the other hand, from 3 to 3.5-fold increase in the ROS levels was also found compared to the same groups. This result unambiguously suggests that the antioxidant system in mice blood plasma fails to protect from the oxidative stress induced by the treatment with 10 kGy irradiated extract. It might also be supposed that the dose of 10 kGy irradiation provokes changes in polyphenol structure responsible for *A. Melanocarpa* extract antioxidant activity.

It is a well-known fact that a critical target of $\cdot O_2^-$ may be NO· produced by endothelium macrophages, neutrophils, etc. Moreover, $\cdot O_2^-$ and NO· are known to rapidly react to form the stable peroxide anion (ONOO-). After protonation $ONOO^-$ can decompose and generate a strong oxidant such as HO·[20]. As can be seen from Figs. 2 and 3, after treating the mice with 10 kGy irradiated extract, the levels of ROS products and NO· in the plasma are 3.5 times and 3 times higher than the controls, respectively. We hypothesize that these increased amounts of NO· and ROS (in particular $\cdot O_2^-$) registered in mice plasma might produce $ONOO^-$, and ultimately generate the highly toxic HO·. We also accept that the probability of generating $ONOO^-$ and subsequently the highly toxic HO· is significantly lower in the plasma of mice treated with non-irradiated extract due to the fact that their ROS plasma levels were commensurate with controls and furthermore, their plasma NO· levels were about 2 times lower than those found in mice treated with 10 kGy irradiated extract.

The mice liver (Fig. 4) treated with 10 kGy irradiated *A. Melanocarpa* extract showed levels of ·Asc radicals, close to those in the group treated with non-irradiated *A. Melanocarpa* (mean 0.035±0.001 *vs* mean 0.033±0.001, according to the LSD post hoc test p=0.09). Both groups treated with *A. Melanocarpa* extract showed statistically significant increased levels compared to controls, namely mean 0.033±0.001 *vs* mean 0.029±0.001, according to the LSD post hoc test p=0.000, for mice treated with non-irradiated extract and mean 0.035±0.001 *vs* mean 0.029±0.001, according to the LSD post hoc test p=0.000, for mice treated with 10 kGy irradiated extract.

As shown in Fig. 5, the ROS products levels in the liver homogenates of mice treated with irradiated extract were significantly lower than those registered in the controls (mean 1.78±0.07 *vs* mean 2.05±0.02, according to the LSD post hoc test p=0.006) and the mice treated with non-irradiated *A. Melanocarpa* extract (mean 1.78±0.07 *vs* mean 2.01±0.02, according to the LSD post hoc test p=0.001). The values of ROS products in the group treated with non-irradiated *A. Melanocarpa* are close to those in the controls (mean 2.05±0.02 *vs* mean 2.01±0.02, according to the LSD post hoc test p=0.4).

NO· radicals registered in liver homogenates (Fig. 6) of mice treated with 10kGy *A. Melanocarpa* are statistically significantly higher than in controls (mean 8.53±0.2 *vs* mean 5.93 ±0.03, according to the LSD post hoc test p=0.000), and to mice treated with the non-irradiated extract (mean 8.53±0.2 *vs* mean 6.22±0.1, according to the LSD post hoc test p=0.000). No statistically significant increase was found in NO· levels in the liver homogenates of mice treated with non-irradiated *A. Melanocarpa* extract, compared to the controls (mean 6.22±0.1 *vs* mean 5.93±0.03, according to the LSD post hoc test p=0.09).

Bearing in mind that the ascorbate radical is a *real-time* biomarker for the oxidative processes, the about 1.2-fold increase in ascorbate radicals in the mice liver after treatment with either non-irradiated or 10 kGy irradiated *A. Melanocarpa* extract indicates that ascorbate is actively involved in neutralizing oxidative radicals generated in liver [21, 22]. This finding was also supported by the fact that the registered ROS level in mice liver treated with non-irradiated extract was the same as in the control group, while in the mice liver treated with 10 kGy irradiated extract, it was even lower than in controls. Given these results, we assume that hepatic antioxidant protection in mice is more effective in neutralizing oxidative toxic species than blood plasma. This assumption is additionally supported by the findings that: 1) NO· levels (see Figs. 3 and 6) measured in livers of both groups of mice treated with non–irradiated and 10 kGy irradiated *A. Melanocarpa* extracts were found to be lower than in the plasmas of the same groups; 2) NO· level in livers of mice treated with non-irradiated extract were close to the controls, while the NO· levels in the plasma of mice treated with non-irradiated extract were about 1.4 times higher than controls.

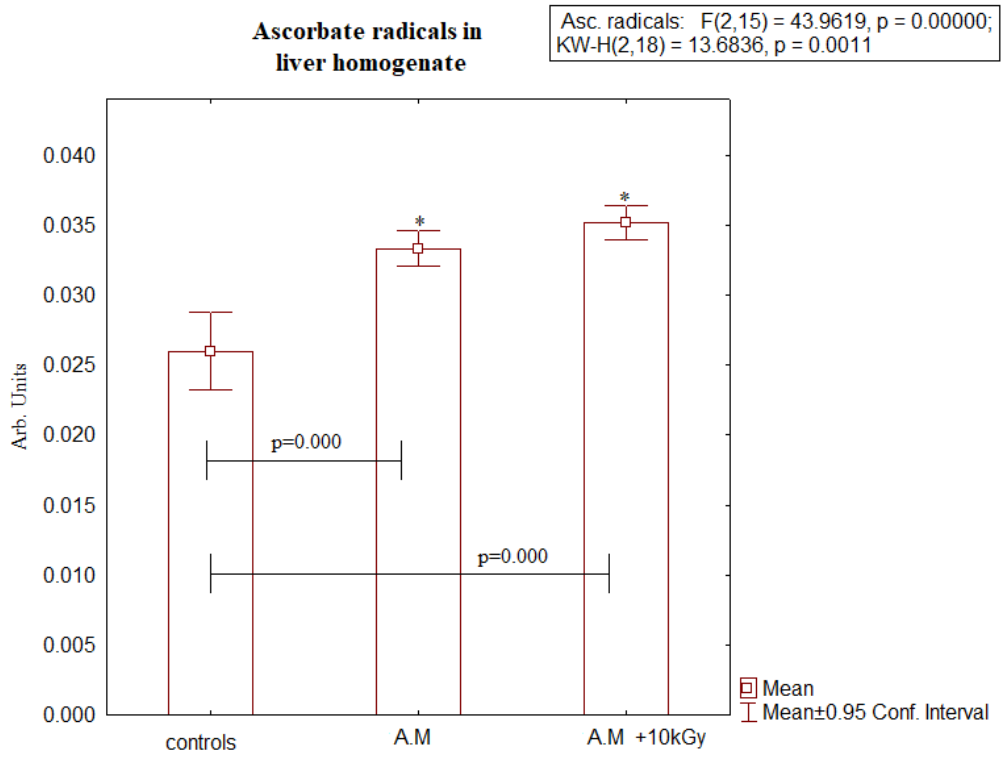

Fig. 4. ·Asc radicals levels measured in liver of mice treated with non-irradiated and 10 kGy irradiated *A. Melanocarpa*, and compared with the control mice. Significant difference *p < 0.05 vs controls. **p < 0.05 vs 10 kGy irradiated A. *Melanocarpa*. The LSD post hoc test was used to determine the groups differing from each other.

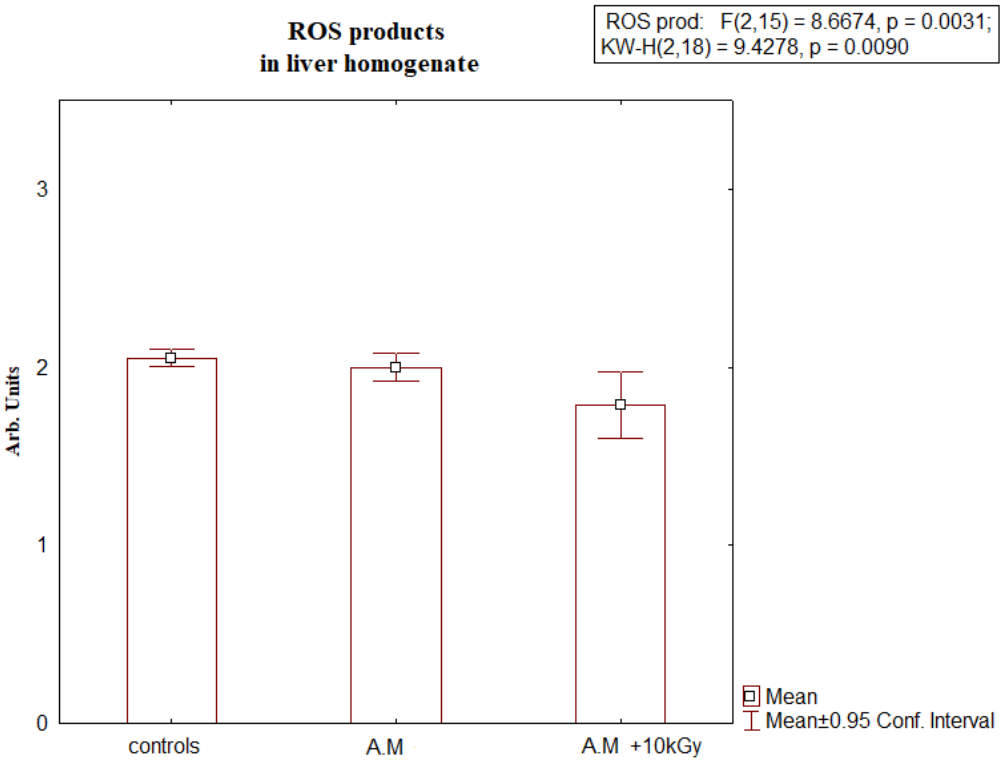

Fig. 5. ROS products levels measured in mice liver of treated with non-irradiated and 10kGy irradiated *A. Melanocarpa* and compared to the control mice. The LSD post hoc test was used to determine the groups differing from each other.

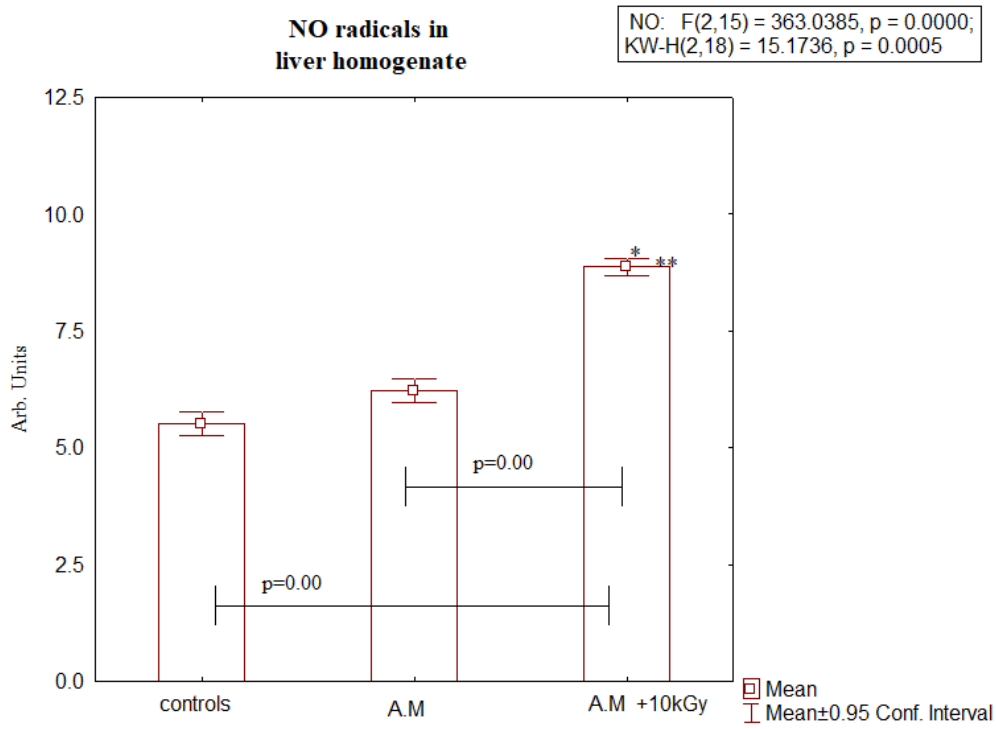

Fig. 6. NO· radical levels measured in liver of mice treated with non-irradiated and 10kGy irradiated *A. Melanocarpa*, and compared to the control mice. Significant difference *p < 0.05 vs controls; **p < 0.05 vs 10 kGy irradiated A. *Melanocarpa*. The LSD post hoc test was used to determine the groups differing from each other.

NO· radicals registered in liver homogenates (Fig. 6) of mice treated with 10kGy *A. Melanocarpa* are statistically significantly higher than in controls (mean 8.53±0.2 *vs* mean 5.93 ±0.03, according to the LSD post hoc test p=0.000), and to mice treated with the non-irradiated extract (mean 8.53±0.2 *vs* mean 6.22±0.1, according to the LSD post hoc test p=0.000). No statistically significant increase was found in NO· levels in the liver homogenates of mice treated with non-irradiated *A. Melanocarpa* extract, compared to the controls (mean 6.22±0.1 *vs* mean 5.93±0.03, according to the LSD post hoc test p=0.09).

Bearing in mind that the ascorbate radical is a *real-time* biomarker for the oxidative processes, the about 1.2-fold increase in ascorbate radicals in the mice liver after treatment with either non-irradiated or 10 kGy irradiated *A. Melanocarpa* extract indicates that ascorbate is actively involved in neutralizing oxidative radicals generated in liver [21, 22]. This finding was also supported by the fact that the registered ROS level in mice liver treated with non-irradiated extract was the same as in the control group, while in the mice liver treated with 10 kGy irradiated extract, it was even lower than in controls. Given these results, we assume that hepatic antioxidant protection in mice is more effective in neutralizing oxidative toxic species than blood plasma. This assumption is additionally supported by the findings that: 1) NO· levels (see Figs. 3 and 6) measured in livers of both groups of mice treated with non–irradiated and 10 kGy irradiated *A. Melanocarpa* extracts were found to be lower than in the plasmas of the same groups; 2) NO· level in livers of mice treated with non-irradiated extract were close to the controls, while the NO· levels in the plasma of mice treated with non-irradiated extract were about 1.4 times higher than controls.

Later studies have shown that elevated temperature and irradiation cause changes in the plant polyphenol content, the range of which depends on the power and, sometimes, on the operating time of abiotic stress factors [23]. The first EPR studies on the effect of 10 kGy irradiation of *A. Melanocarpa* extract in relation to the oxidative state in mice are somewhat in support of the research of Cebulak and collaborators [6]. The same authors established that among the abiotic stress factors employed, such as UV-C radiation, electromagnetic field, microwave radiation, and ultrasound, at different operation times, the statistically significant decrease in the content of all polyphenol compounds determined in chokeberry

fruits has been found only in the case after their exposure to 5-MFL (electromagnetic field) agent.

Based on the present EPR spectroscopy results it is very likely the observed decrease in antioxidant protection in the plasma of mice treated with *A. Melanocarpa* extract irradiated with a dose of 10 kGy to be indirectly caused by a reduction in the number of polyphenols in the extract after irradiation. It is not excluded this dose of irradiation to induce some changes in the polyphenols structures responsible for the *A. Melanocarpa* extract antioxidant activity.

CONCLUSION

For the first time, the effect of 10 kGy irradiated *A. Melanocarpa* extract on the level of oxidative stress induced in mice plasma and liver was assessed using direct and spin-trapping EPR spectroscopy. The results obtained show that the antioxidant defense system in the plasma of the treated mice fails to overcome the induced oxidative stress after treatment of *A. Melanocapra* extract with 10 kGy. In order to establish the reasons provoking oxidative stress in the blood plasma of experimental mice treated with a dose of 10 kGy we believe that detailed studies need to be made to clarify the changes occurring in the amount and structure of the constituents (in particular polyphenols) of the *A. Melanocarpa* extract responsible for the antioxidant activity of the latter. Therefore, further studies should be undertaken to broaden the current state of knowledge about the stimulating effect of various abiotic stress agents on the increased content of polyphenol compounds in the fruit.

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Conflict of Interest: *The authors declare that they have no conflict of interest.*

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