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High-level gamma radiation effects on radical-scavenging activity of black chokeberry (*Aronia melanocarpa*) ethanol extract

Y.D. Karamalakova[1]*, G.D. Nikolova[1], P.N. Denev[2], Sv. Momchilova[3], A.K. Slavova-Kazakova[3], V.D. Kancheva[3], A.M. Zheleva[1], V.G. Gadjeva[1]

[1]*DepartmentChemistry and Biochemistry, Medical Faculty, Trakia University, 11 Armeiska Str.,6000 Stara Zagora, Bulgaria;*

[2] *Laboratory of Biologically active Substances, Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, 139 Ruski blvd. 4000 Plovdive, Bulgaria*

[3] *Department of Lipid Chemistry, Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, Acad. G. Bonchev Str., 9, 1113 Sofia, Bulgaria.*

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Black chokeberry (*Aronia melanocarpa, A. melanocarpa*), belongs to the *Rosaceae* family, and originates from the North America eastern parts. In *A. melanocarpa* berries the polyphenolic compounds are characterized by stronger radical- scavenging activity than polyphenols, which are presented in other berries. In recent years, food irradiation has been used to protect against microorganisms, oxidative processes and radiation-induced toxicity, and this methodology is internationally recognized for effective long-term storage. The Electron Paramagnetic Resonance **(**EPR) is the most promising methods for identification of gamma (γ) -irradiated food.

In the current study by using *in vitro* EPR spectroscopy and spectrofotometrical methods were evaluated and compared free radical-scavenging abilities and radio-protective properties of Black Chokeberry (*A. melanocarpa*) ethanol extract before and after exposure to 10 kGy and 25 kGy irradiation. It was found that when irradiation dose was increased scavenging abilities of the *A. melanocarpa* extract against DPPH significantly decreased (68.612 ± 4.18 %, $p<0.005$ for 10 kGy and 35.09 ± 3.87 %, $p<0.005$, for 25 kGy). 10 kGy irradiation exhibited 4 times higher SOD-like activity than L-ascorbic acid, while 25 kGy radiation extract showed slightly decrease versus standard. Chain-breaking antioxidant activity of lipid soluble components decreases with increasing the irradiation dose. Based on the EPR singlet signals with equal values of g = 2.00455 after alkalization were assume that the semiquinone radical originates from the polyphenol substances presented in high concentration in *A. melanocarpa* extract.

Keywords: *A. melanocarpa*, polyphenols, scavenging activity, irradiation.

INTRODUCTION

Aronia (Michx.) Elliott (*Aronia melanocarpa, A. melanocarpa*), with the common name black chokeberry, belongs to the *Rosaceae* family, originates from the North America eastern parts. The ripe black berries fruits were used to produce jams, juices and wine, because of the high content of sorbitol, sugars (*glucose and fructose*), minerals (*potassium*) and high source of natural food colorants [1, 2]. Several studies indicated that *A. melanocarpa* juice or fruits contain vitamins [3], polyphenolic acids [4, 5] anthocyanins (cyanidin-3-arabinoside, cyanidin-3-galactoside, cyanidin3-glucoside, and cyanidin-3-xyloside), proanthocyanidins, and hydro-xycinnamic acids [6, 7]. It is reported that polymeric proanthocyanins, predominantly of (−) epicatechin, are the major class of polyphenolic compounds in chokeberry, and represent 66% of fruits polyphenols, while anthocyanins are the second phenolic compound group in *A. melanocarpa* and are about 25% of total polyphenols [5]. *A. melanocarpa* berries polyphenolic compounds are characterized by stronger radical-scavenging activity than polyphenols, which are presented in other berries [8]. Anthocyanins from chokeberries are characterized by higher antioxidative activity against lipid oxidation induced by UV radiation in the liposome membranes (made from egg yolklecithin) than anthocyanins from sloe and honeysuckle [7]. The strong *A. melanocarpa* antioxidant properties provide an opportunity to be effectively used in the prophylaxis and treatment of health disorders associated with oxidative stress, especially diabetes and cancer [7].

In recent years, food irradiation has been used to protect against microorganisms, oxidative processes and radiation-induced toxicity, and this methodology is internationally recognized for effective long-term storage. The Directive 1999/3/EUN of the EP established a *Community list of food and food ingredients* [9] that maybe treated with ionizing radiation and maximum overall average absorbed dose could be 10 kGy for food containing cellulose (*nuts, berries, dry herbs and spices, vegetable seasonings*), meat and fish bones

* To whom all correspondence should be sent:
E-mail: ykaramalakova@gmail.com

and food containing crystalline sugars (*dried figs, mangoes, papayas and raisins)*.

Electron paramagnetic resonance (EPR) identified different organic, inorganic, and transition metal radical species, changes in chemical composition and different spectrum shapes as a function of time and temperature or radiation type [10, 11]. EPR is the most promising methods for identification of γ-irradiated food. The stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) is widely used for *in vitro* determination of antioxidant and free radicals-scavenging activity of plant/dried fruit extracts by direct EPR spectroscopy [10,11].

In the current study using *in vitro* EPR spectroscopy and spectrofotometrical methods were evaluated and compared free radical-scavenging abilities and radio-modulatory properties of Black Chokeberry (*A. melanocarpa*) ethanol extract before and after exposure to 10 kGy and 25 kGy irradiation.

MATERIALS AND METHODS

Black chokeberry fruits

Black chokeberry fruits were supplied from Vitanea Ltd (Plovdiv, Bulgaria) in the stage of full maturity, in August 2017. Fresh fruits were put in polyethylene bags and frozen at - 18˚C. After that, frozen fruits were freezed dried (Christ Alpha 1-4 LDplus, Martin Christ GmbH, Germany) [6].

Chemicals

The xanthine/xanthine oxidase systems, nitroblue tetrazolium (NBT), L-ascorbic acid and DPPH was purchased from Sigma Chemicals, USA. Other chemicals used were analytical or HPLC grade.

γ- irradiation of dried black chokeberry (A. melanocarpa)

Freeze dried berries were irradiated at a ^{60}Co source with 8 200 Ci activity. The Gamma-ray facility has a mobile irradiation chamber with 4.0 L volume and dimensions: 13.5 cm diameter and 22 cm height. During the irradiation, the chamber rotates on its vertical axe. For the study of the absorbed dose distribution Alanine dosimeters (Kodak BioMax) were used, measured by ESR spectrometer E^{-scan} Bruker and calibrated in units of absorbed dose in water. In each point, three dosimeters were placed. 10 kGy and 25 kGy irradiated fruits were used in the experiment. Controls and irradiated samples were kept in polyethylene bags. Before extraction and analysis, samples were milled on a laboratory mill to fine powder [6].

Ethanol extraction of polyphenols

Briefly, 0.5 g of the fruit powder were weighted accurately, transferred to extraction tubes and mixed with 40 ml of 60% ethanol. Samples were extracted for 1h, at room temperature on a magnetic stirrer. After that, samples were centrifuged (6000 x g) and supernatants were further used for EPR analysis of total anthocyanins and total polyphenols [6].

Acetone extraction of polyphenols

Freeze dried fruits were milled to fine powder in a laboratory mill. 20 g of the milled dried fruits were transferred into extraction tubes and mixed with 200 ml acetone. Extractions were conducted on a magnetic stirrer, for 2 hours at room temperature. After that, samples were centrifuged (6000 x g), supernatants were collected and dried via rotary evaporation. Dry extracts were used for chain-breaking antioxidant activity determination.

Antioxidant activity of ethanol extracts

The DPPH scavenging ability was studied according to Santos *et al*. [12]. *A. melanocarpa* extract (30 μg/ ml) before and after γ-irradiation was added to 250 μL ethanol solution of DPPH (80 μmol/l). After incubation at 23°C for 10 min/dark the samples were transferred into the EPR cavity, and their EPR spectra were recorded. Extracts scavenging ability was calculated according to the following formula:

$$\textit{Scavenged DPPH radicals (\%)} = [(I_0 - I)/I_0]\times 100,$$

where $\mathbf{I_0}$ is the integral intensity of the DPPH signal of the control sample and **I** is the integral intensity of the DPPH signal after addition of the tested sample to the control sample. The control samples contained 250 μl of DPPH ethanol solution + 30 μl ethanol. The settings were as follows: center field 3516.00 G, sweep width 200.00 G, modulation amplitude 5.00 G.

Superoxide dismutase like activity study of ethanol extracts

The superoxide dismutase (SOD) like activity of *A. melanocarpa* extracts, non-rradiated and 10 kGy and 25 kGy irradiated was studied by Sun *et al*. [13]. The xanthine/xanthine oxidase system was used to generate the superoxide anion ($\cdot O_2^-$). Superoxide anion reduces NBT to formazan, monitored at 560 nm. One unit (U) of enzymatic activity is defined as the amount of enzyme causing 50% inhibition of the NBT reduction to formazan.

Direct EPR spectroscopy study on ethanol extracts before and after γ-irradiation

The 30% *A. melanocarpa* ethanol extract was divided into three parts. One part of the extract was

not irradiated while the other two were irradiated with 10 kGy and 25 kGy, respectively. Three months after irradiation storage, the two extracts as well as the non-irradiated extract were examined by direct EPR spectroscopy. The same measurement was done after their 6 months storage. EPR study was made on X-band- EMX ^{micro} spectrometer (Bruker, Germany) equipped with a standard resonator. Spectral processing (g-value calculation) was performed with *Bruker WIN-EPR* and *Sim-Fonia* software. The following EPR settings were used: center field 3513.50 G, microwave power 20.03 mW, modulation amplitude 10.00 G; gain $2x10^2$; time constant 327.68 ms; sweep time 61.44 s.

Direct EPR spectroscopy study on non-irradiated and γ-irradiated ethanol extracts after alkalization

Water solution of NaOH (10mM) was added to the corresponding extract in a ratio 1:1 (v/v) and after 5 min EPR spectra were recorded. EPR settings were the same as the above.

Chain-breaking antioxidant activity of acetone extraction

Lipid samples: Triacylglycerols of commercially available sunflower oil (TGSO) were cleaned from pro- and antioxidants by adsorption chromatography and stored under nitrogen at 20°C. Fatty acid composition of the lipid substrate was determined by GC analysis of the methyl esters: 10:0 (0.2%); 14:0 (0.2%); 16:0 (7.4%); 16:1 (0.3%); 18:0 (2.6%); 18:1 (29.1%); 18:2 (59.1%); 18:3 (0.7%); 20:0 (0.3%). The numbers x:y indicate, respectively, the number of carbon atoms and double bonds in the fatty acid. Lipid samples containing various inhibitors were prepared directly before use. Aliquots of the antioxidant solutions in purified acetone were added to the lipid sample. Solvents were removed under a nitrogen flow. For more experimental details see reference [14].

Lipid autoxidation: The process was carried out in a thermostatic bath at (80 ± 0.2) °C by blowing air through the samples in special vessels. The oxidation process was monitored by withdrawing samples at measured time intervals and subjecting them to iodometric determination of the primary products (lipid hydroxyperoxides, LOOH) concentration, i.e. the peroxide value (PV).

Determination of the main kinetic parameters of the studied compounds [14-18]: Protection factor (PF) is determined as the ratio between the induction period in the presence (IP_A) and in the absence (IP_C) of antioxidant, i.e. $PF = IP_A/IP_C$. It is a measure of antioxidant efficiency.

Inhibition degree (ID) is a measure of the antioxidant reactivity, e.g. how many times the antioxidant shortens the oxidation chain length, i.e. $ID = R_C/R_A$. The initial oxidation rates R_C in the absence and R_A in the presence of antioxidant were found from the tangents at the initial phase of the kinetic curves of hydroperoxides accumulation.

Statistical analysis

EPR spectral processing was performed using Bruker Win-EPR and Sim-fonia Software. Statistical analysis was performed with Statistica 8.0, Stasoft, Inc., one-way ANOVA, Student- t- test to determine significant difference among data groups. The results were expressed as means ± standard error (SE). A value of $p< 0.05$ was considered statistically.

All kinetic data are expressed as the average of two independent measurements which were processed using the computer programmes Origin 6.1 and Microsoft Excel 2010.

RESULTS AND DISCUSSION

DPPH scavenging activity of ethanol extracts before and after γ- irradiation.

In vitro DPPH scavenging capacity of *A. melanocarpa* extracts before and after irradiation was investigated by direct EPR method. The untreated extract (**Fig. 1**) showed maximum DPPH radical scavenging activity (87.433 ± 8.20 %). Our finding was in support of the Bräunlich et al. [19]. The same authors reported that 50% EtOH crude extract of *A. melanocarpa* enriched in procyanidins and anthocyanins possessed high radical-scavenging capacity towards DPPH.

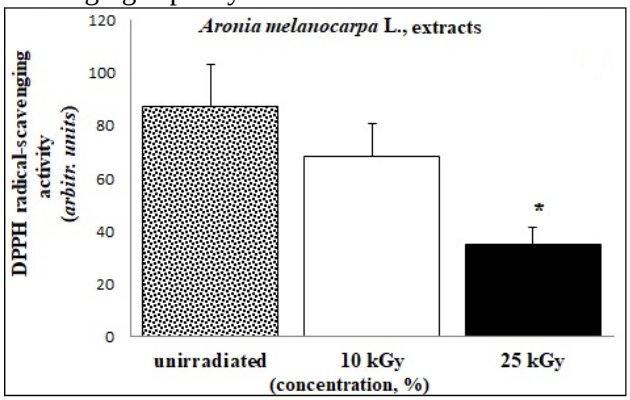

Fig. 1 DPPH scavenging capacity *of A. melanocarpa* extracts before and after irradiation. The experiments were made in triplicate. Values were expressed as mean ± SE. *$p < 0.05$ vs. the non-irradiated extract.

It was found that when irradiation dose was increased scavenging abilities of the *A. melanocarpa* extract against DPPH significantly decreased (68.612 ± 4.18 %, $p<0.005$ for 10 kGy and 35.09 ± 3.87 %, $p<0.005$, for 25 kGy). Almost

the same dependency was established after ultra-sonication (*150gg cycle/ 10sec*) and incubation in the dark (at 24^0C) of the both samples at different time intervals (*the results are not given*).

SOD-like activity of ethanol extracts

The SOD- like activity of 1ml of non-irradiated *A. melanocarpa* extract was more than 10 times higher than the standard antioxidant such as L-ascorbic acid (1mg) (**Fig. 2**). After, 10 kGy and 25 kGy irradiation extracts show decrease in SOD-like activity compared to non-irradiated sample.

It should be noted that 10 kGy irradiated sample exhibited 4 times higher SOD-like activity than L-ascorbic acid, while 25 kGy irradiated extract showed slightly decrease versus standard. It is known that phenolic compounds act not only as direct free radical scavengers [7], but can increase the activities of antioxidative enzymes [20]. Superoxide dismutase (SOD) is the antioxidant enzyme that converts the superoxide radical into hydrogen peroxide (H_2O_2). The applied SOD-like activity method is not enzymatic in nature [21]. The increase in H_2O_2 concentration of the non-irradiated and 10 kGy irradiated samples shows that probably polyphenols in our extracts are the compounds capable successfully to convert $\bullet O_2^-$ into H_2O_2.

On the other hand, higher gamma irradiation (25 kGy) is probably the abiotic stress factor responsible for significantly lower SOD-like activity compared to non-irradiated and 10 kGy irradiated extracts [7].

EPR spectrum before and after irradiation of ethanol extracts

A singlet almost symmetrical EPR signal with a g factor of 2.00456 was detected in non- irradiated *A. melanocarpa* extract (**Fig. 3 A).** EPR spectra with the same shapes were registered in both irradiated extracts (**Fig. 3B, 3C)**. 10 kGy irradiated extract show almost commensurate singlet intensity, but with slight change in the g value (g= 2.00431, **Fig. 3B**), as opposed to untreated extract. 25 kGy dose radiation decreased intensity signal and change the g=2.00412 value in *A. melanocarpa* samples (**Fig. 3C)**. As a rule scavenging properties of antioxidant compounds like flavonoids and phenolic acids are often associated with their abilities to form stable radicals. In many studies was shown that flavonoids and phenolic acids scavenging radicals effectively, could form semiquinone free radicals in alkaline solution stable enough to be detected by EPR spectroscopy [22, 23].

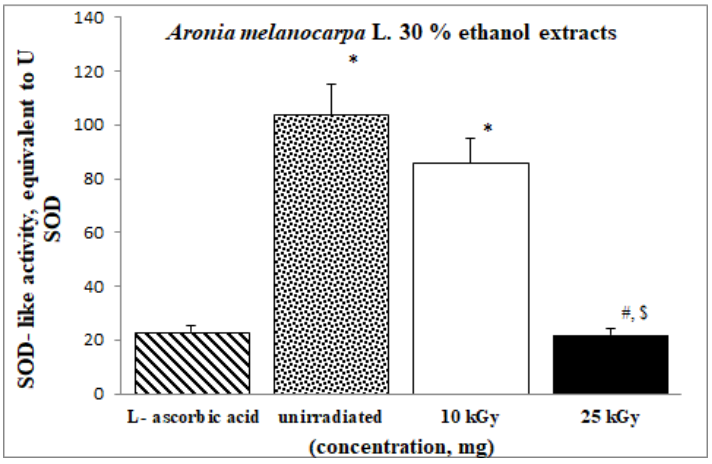

Fig. 2 SOD –like activity of the studied extracts of A. melanocarpa compared to L-Ascorbic acid. One unit (U) of enzymatic activity is defined as the amount of enzyme causing 50% inhibition. The SOD-like activity of *A. melanocarpa* was equal to 4.91 mg of L-ascorbic acid. Values were expressed as mean ± SE of 3 independent measurements. * $p < 0.05$ vs. the standard; $^{\#}$ $p < 0.05$ vs. the non-treated *A. melanocarpa* extract; $^{\$}p < 0.05$ vs. 10 kGy irradiated samples.

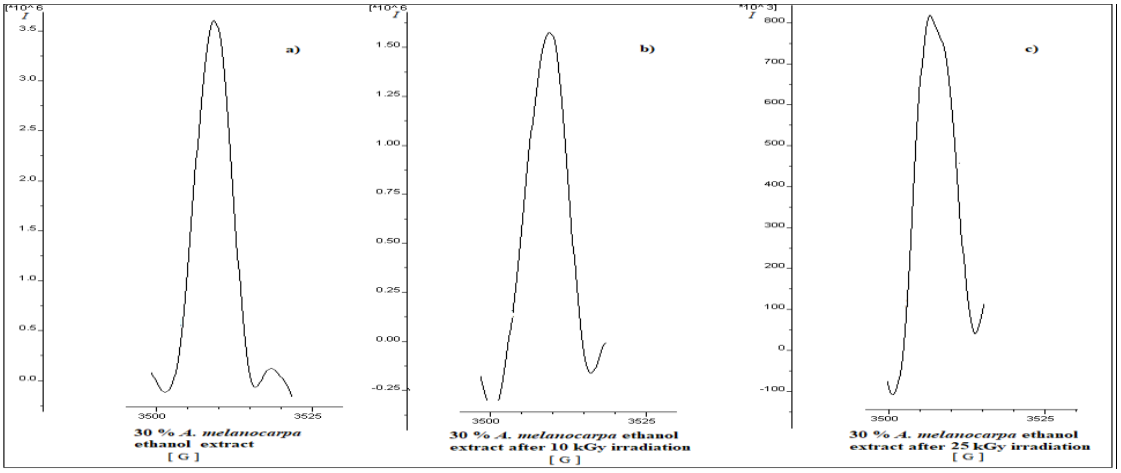

Fig. 3. Direct EPR spectra in non – irradiated (A) and gamma irradiated extracts samples (B, C). The experiments were made in triplicate and express in arbitrary units.

To verify possibility the radical structures registered in non-irradiated and irradiated extract samples belong to a semiquinone free radical, their EPR spectra were evaluated after alkalization (**Fig. 4 B**).

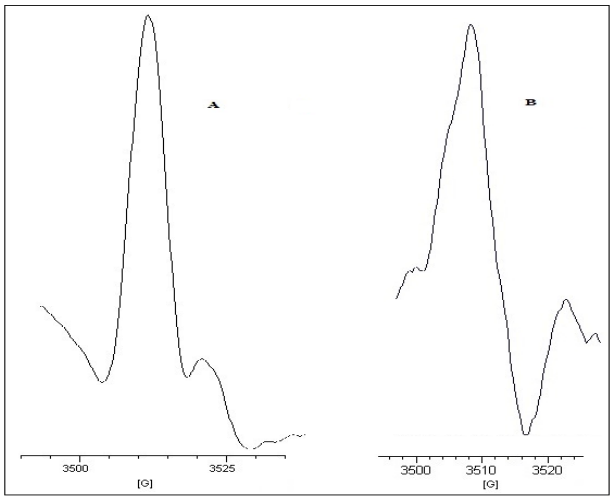

Fig. 4. EPR spectrum before (A) and after alkalization (B) of non-irradiated extracts of *A. melanocarpa*. The experiments were made in triplicate and express in arbitrary units.

After alkalization all studied samples give rise to EPR singlet signals with equal values of g=2.00455. Based on the above result and earlier studies [22, 23] we assume that the semiquinone radical originates from the polyphenol substances presented in high concentration in *A. melanocarpa* extract. Probably the polyphenol substances of *A. melanocarpa* that determine the DPPH scavenging activity undergo destruction by irradiation at doses higher than 10 kGy. Present findings are confirmed by both used methods -EPR and spectrophotometry.

Chain-breaking antioxidant activity.

New results were obtained concerning the extent of changes in the ability of the acetone extracts to inhibit effectively lipid oxidation process after irradiation. **Fig. 5** presents the experimental data of TGSO autoxidation kinetics in presence of dried extracts of *A. melanocarpa* (fruits) in concentration 0.13 % wt before and after applying doses of 10 kGy and 25 kGy. It is seen from the kinetic curves that the inhibiting capacity of the extracts decreases tendentiously after *gamma*-irradiation.

Furthermore, with increasing doses of irradiation the induction period becomes shorter (*the higher the radiation dose, the shorter the induction period*).

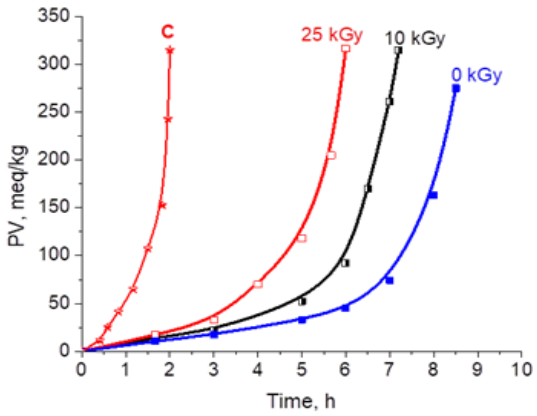

Fig 5. Kinetic curves of lipid peroxide accumulation during TGSO autoxidation at 80 ºC in absence (control, C) and in presence of 0.13 % wt acetone extract from A. melanocarpa (fruits).

As it already has been mentioned, *A. melanocarpa* is extremely rich source of several classes polyphenols: anthocyanins, proanthocyanins and hydroxycinnamic acids. More research is needed to understand their mechanisms before and after irradiation. Unfortunately, anthocyanins are lipid insoluble and their role is not relevant in this model system. Together with the water-soluble antioxidants isolated from the fleshy part of the

fruits, there are seeds containing lipid-soluble components like tocopherols [24].

Table 1 presents the main kinetic parameters, characterizing the TGSO autoxidation at 80ºC in presence of studied extracts. Protection factor of non-irradiated acetone extracts (PF = 4.7) from *A. melanocarpa* is relatively high. For comparison, α-tocopherol in 0.1 mM concentration (0.005 $\%_{wt}$) ensures PF = 8.1 under the same experimental conditions. Irradiation doses of 10 kGy and 25 kGy lead to a significant decrease of oxidation stability of lipid substrate in presence of studied extracts and their PF value. No considerable changes in the initial rate of oxidation after irradiation of 10 kGy irradiated samples is observed but the decrease after applying the higher gamma irradiation (25 kGy) is not so negligible.

Table 1. Kinetic parameters of the extract from *A. melanocarpa*.

Extract from *A.melanocarpa*	IP_A h	PF	R_A, 10^{-6} M/s	ID	Activity
Non-irradiated	7.5 ± 0.4	4.7	1.0 ± 0.1	4.0	Moderate
Irradiated with 10 kGy	6.0 ± 0.4	3.8	1.1 ± 0.1	3.6	Moderate
Irradiated with 25 kGy	5.0 ± 0.3	3.1	1.5 ± 0.1	2.7	Weaker

*Kinetic parameters of the control sample: IP_C = (1.6±0.2) h; R_C= (4.0±0.5),10^{-6} Ms^{-1}

Fig. 6 presents graphically the main kinetic parameters, characterizing the TGSO autoxidation at room temperature in presence of studied extracts in concentration 0.2 % wt before and after irradiation. Here we observe the same tendency to decrease lipid oxidation stability when comparing the results obtained under the autoxidation at 80°C.

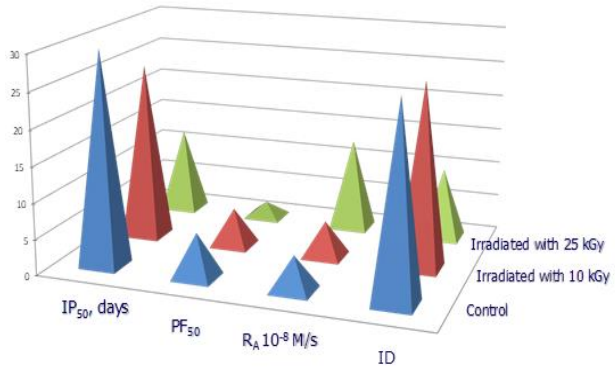

Fig 6. Determination of the kinetic parameters during autoxidation of triacylglicerols of sunflower oil (TGSO) at room temperature and concentration 0.2 % wt.

Comparable kinetic analysis was made on the basis of the time to reach a certain peroxide value according to Gordon *et al*. [16]. The reduction after applying the higher gamma irradiation (25 kGy) in this case is much more pronounced.

CONCLUSION

In conclusion current results show that used *A. melanocarpa* extract demonstrate well-expressed DPPH radical scavenging ability, SOD-like activity and radio-protective capabilities before and at 10 kGy irradiation. Chain-breaking antioxidant activity of lipid soluble components decreases with increasing the irradiation dose.

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

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