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Evaluating the radioprotective effect of arbutin on mice exposed to megavoltage X-rays based on hematological parameters and lymphocytes micronucleus assay

S. Nadi[1], A. Banaei[2,3], H. Mozdarani[4], A. Shabestani Monfared[5], G.R. Ataei[6], R. Abedi-Firouzjah[7*]

[1]Department of Medical Physics Radiobiology and Radiation Protection, School of Medicine, Babol University of Medical Sciences, Babol, Iran
[2]Medical Physics Department, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran
[3]Department of Radiology, Faculty of Paramedical Sciences, AJA University of Medical Sciences, Tehran, Iran
[4]Department of Medical Genetics, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran
[5]Cellular & Molecular Biology Research Center, Health Research Institute, Babol University of Medical Sciences, Babol, Iran
[6]Department of Radiology Technology, Faculty of Paramedical Sciences, Babol University of Medical Science, Babol, Iran
[7]Cellular and Molecular Research Center,Yasuj University of Medical Sciences, Yasuj, Iran

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Corresponding authors:
Razzagh Abedi-Firouzjah, Ph.D.,
E-mail:
razzaghabedi@gmail.com

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ABSTRACT

Background: X-irradiations induce damages to the hematopoietic system by reducing the production of blood cells in bone marrow. In this study, the radioprotective effect of arbutin was investigated in megavoltage x-irradiated mice by measuring changes in hematological parameters and lymphocyte cells with micronucleus assay. ***Material and Methods:*** Sixty NMRI mice were irradiated with 6 MV photon beam (2 and 4 Gy in one fraction). Various concentrations of arbutin (50, 100, and 200 mg/kg) were injected intra-peritoneal, 2 hours before whole body X-irradiation. Samples of peripheral blood cells were collected from the left ventricle. The frequency of micronuclei in 1000 cells were measured for each sample and level of peripheral blood cells were analyzed. The data were statistically evaluated using one-way ANOVA, and Tukey HSD test. ***Results:*** X-radiations significantly decreased the hematological parameters such as white blood cells (WBC), lymphocyte (LYM), red blood cells (RBC) counts, and hemoglobin (HGB) levels compared to the control group ($P < 0.001$). The frequency of micronuclei in "2 and 4 Gy X-irradiation + distilled water" groups was significantly higher than "2 and 4 Gy irradiation + 50 mg/kg arbutin", "2 and 4 Gy irradiation + 100 mg/kg arbutin", and "2 and 4 Gy irradiation + 200 mg/kg arbutin" groups, followed by the above-mentioned blood cell parameters were dropped remarkably. ***Conclusion:*** The present investigation showed that arbutin is a strong radioprotector. There were not any significant differences between the various concentrations of arbutin, however, the concentration of 50 mg/kg showed higher radioprotective effect.

Keywords: *Radiation, arbutin, hematopoietic, radioprotector, micronucleus assay.*

INTRODUCTION

Ionizing radiations such as X-rays, nuclear medicine, and radiotherapy are used for medical diagnosis and treatment [1, 2]. But their use is limited because of the undesirable and side

effects such as normal tissue injury [3, 4].

Radiotherapy is used for the treatment of around half of patients due to the long-term advantages [2, 5]. On the other side, this kind of treatment can induce primary lesions in the biomolecular structures and carcinogenic effects on normal tissues [6], as well as damage to the hematopoietic, gastrointestinal, and central nervous systems [7].

The effect can be due to the production of free radicals that destroy the vital macromolecules such as proteins, lipids, RNA, and DNA damage/chromosomal mutation, and after exposure, a hematologic crisis occurs, characterized by hypoplasia/aplasia of the bone marrow [8, 9].

Two of the main strategies, non-pharmacological and pharmaceutical, are able to reduce the radiation damages with repairing and recovery enhancers and modifiers of immune system [10]. The non-pharmacological strategy can reduce radiation injuries, however, the probability of secondary cancers is still considerably high [11]. Therefore, focus on radiation protection effects of pharmaceutical products can be helpful against damage when given prior to radiation exposure [12, 13].

There are some plants and herbs beside synthetic antioxidants which protect against radiation injuries [14], for example lettuce oil, aged garlic, and grape seed [15-17]. In a previous study, Nadi *et al.* [18] showed the protective effect of arbutin in mice bones as a radioprotector using erythrocytes micronucleus assay. Arbutin is one of the neutral radioprotectors and overcomes the toxic effect of radiation in cancer patients undergoing radiotherapy that has been approved in previous studies [18-20]. This radioprotector is a naturally occurring glucoside of hydroquinone family and has been traditionally used to treat pigmentary disorders [21]. It is found in a variety of plants including those from the Ericaceae and Compositae families [22]. The molecular weight and maximum absorbance spectrum of arbutin are 272.25 g/mol and 267 nm.

Since the hematopoietic system is a radiosensitive tissues, it could be considered as an appropriate indicator of radiation injuries. One of the best method for measuring chromosome loss and chromosome breakage is micronucleus assay [23]. Cytokinesis-block micronucleus (CBMN) assay, which blocked by cytochalasin-B, allows better precision for cell division assessment [24].

The radioprotective effect of arbutin on the hematological system have not been reported in previous studies, furthermore, regarding the high radiosensitivity of this system, it will be a good idea to evaluate the radioprotective effect of arbutin based on measurement of hematological parameters. Thus, the aim of this study was to evaluate the biocompatibility of the arbutin as a radioprotector agent in megavoltage therapeutic whole-body x-irradiated mice on hematological parameters at three different concentrations and also lymphocyte cells were assessed utilizing micronucleus assay.

MATERIALS AND METHODS

Animals

Our study was done following Animal Experimentation Ethics Committee and National Research Ethics Board approval with the registration number of "MUBABOL.REC.1391.4" at "22.12.97".

Sixty male NMRI mice, with average age (6-7 weeks) and body weight (25 ± 5 gr) were obtained from animal lab of Iran University of Medical Science (Tehran, Iran). The mice were kept at proper temperature, relative humidity, and light regime (12 hrs. light/12 hrs. dark). They were given fresh water and standard food of a commercial balanced diet.

Irradiation

The mice were placed in a Plexiglas collective cage for whole body exposure by a linear Elekta Compact accelerator (Elekta AB, Stockholm, Sweden) using 6 MV photons. The source-to-skin distance and field size were chosen 100 cm and 20 × 20 cm^2, in that order. Two doses of 2 Gy and 4 Gy were applied as single fraction at a dose rate of 200 cGy/min. All irradiations were performed 2 hours after the injection of arbutin. After irradiation the mice were brought back to

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the animal lab for the follow up tests.

Chemical

Pure (98%) arbutin powder (Sigma-Aldrich, USA) was injected intra-peritoneal at the concentrations of 50, 100, and 200 mg/kg, 2 hours before whole body x-irradiation. For each mouse, 0.5 cc distilled water was used as solvent.

Experimental design

Two weeks after acclimatization and conditioning, the animals were divided into twelve groups (five mice for each group) in separate Plexiglas cages as following:

I. "Sham irradiation + distilled water"
II. "2 Gy X irradiation + distilled water"
III. "4 Gy X irradiation + distilled water"
IV. "Sham irradiation + 50 mg/kg arbutin"
V. "Sham irradiation + 100 mg/kg arbutin"
VI. "Sham irradiation + 200 mg/kg arbutin"
VII. "2 Gy X irradiation + 50 mg/kg arbutin"
VIII. "2 Gy X irradiation + 100 mg/kg arbutin"
IX. "2 Gy X irradiation + 200 mg/kg arbutin"
X. "4 Gy X irradiation + 50 mg/kg arbutin"
XI. "4 Gy X irradiation + 100 mg/kg arbutin"
XII. "4 Gy X irradiation + 200 mg/kg arbutin"

Sample collection and hematological studies

After 36 hours of irradiation, blood samples were collected from the left ventricle and immediately placed in two tubes. One part was selected to show chromosomal damages of peripheral blood lymphocyte which measured by micronucleus assay and another part was chosen to measure the blood parameter levels.

Complete blood count (CBC) measurement

The CBC was done in differential analysis by veterinary department of Iran University of Medical Sciences. A hematological auto-analyzer (Orphee Mythic 22 Hematological Analyzer; Diamond Diagnostic; USA) was used for measuring CBC.

Micronucleus assay

The heparinized blood samples were added to 4.5 ml complete medium (RPMI 1640 medium, Sigma-Aldrich, USA) containing 20% fetal calf serum, 100 µl/ml phytohemagglutinin (Sigma-Aldrich, USA), 100 IU/ml penicillin (Sigma-Aldrich, USA), 100 µg/ml streptomycin (Sigma-Aldrich, USA) and 2 mM glutamine (Sigma-Aldrich, USA). All cultures were incubated in a proportion condition (37 ± 1˚C temperature and in a humidity atmosphere with 5% CO2 and 95% O2). At 21 h medium was replaced with fresh medium lacking mitogen but containing 4 µg/ml Cytochalasin-B (Sigma-Aldrich, USA) was added to the cultures, and cells were collected by centrifuging (Corning LSE compact centrifuge, CLS6758 model, Sigma-Aldrich, USA; 800 rpm, 5 minutes) at 52 h of incubation [25].

The collected cells were suspended in 0.075 M cold potassium chloride (Merck, Germany) and centrifuged (Corning LSE compact centrifuge, CLS6758 model, Sigma-Aldrich, USA; 800 rpm, 6 minutes) and immediately fixed in a fixative solution (methanol: acetic acid, 6:1; Sigma-Aldrich, USA) for 3 times. Then, the fixed cells were dripped onto clear microscopic slides, air-dried and stained with Giemsa solution (Merck, Germany). Measurement of slides was done (×1000 magnification with Radical-614 optical microscope model, Radical, USA) to delineate the numbers of micronuclei in cytokinesis-blocked binucleate cells with the cytoplasm remaining intact. Diameter ranging from 1/16 to 1/3 of the diameter of the main nuclei, being non-refractive, no link or overlap with the main nuclei were considered to score small nuclei. For each blood sampling group (irradiated, control, irradiation plus arbutin sample) 1000 cells were assessed to score the frequency of micronuclei.

Statistical analysis

The average counts of WBC, RBC, LYM, and HGB levels and the frequency of micronuclei were compared utilizing one-way analysis of variance (ANOVA), and Tukey's HSD test as the post hoc between the experimental groups to determine which groups were significantly different at 0.05 level. Statistical analysis was carried out through SPSS 16 (SPSS Inc., Chicago, Illinois, US).

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RESULTS

Blood cell parameters

The results of the "sham irradiation + distilled water" group did not show significant differences of blood constituents compared to the "sham irradiation + 50 mg/kg arbutin", "Sham irradiation + 100 mg/kg arbutin", and "sham irradiation + 200 mg/kg arbutin" groups ($P > 0.05$) (table 1).

Table 1 shows that a remarkable reduction in RBC, WBC, LYM, and HGB blood cell parameters was observed in the "2 and 4 Gy x irradiation + distilled water" groups in comparison with the "sham irradiation + distilled water" group ($P<0.001$). Furthermore, a significant variation was found in the "2 and 4 Gy irradiation + distilled water" groups compared to the "2 and 4 Gy irradiation + 50 mg/kg arbutin", "2 and 4 Gy irradiation + 100 mg/kg arbutin", and "2 and 4 Gy irradiation + 200 mg/kg arbutin" (figures 1 and 2).

Statistical analysis showed hematological factors in the "2 Gy irradiation + 50 mg/kg arbutin" group were considerably higher than the "2 Gy irradiation + 100 mg/kg arbutin" and "2 Gy irradiation + 200 mg/kg arbutin" groups. There was no significant difference in the "2 Gy irradiation + 100 mg/kg arbutin" compared to the "2 Gy irradiation + 200 mg/kg arbutin" group ($P>0.05$) (table 1).

According to table 1, no significant differences were observed in the levels of the blood cells in the "4 Gy irradiation + 50 mg/kg arbutin" and "4 Gy irradiation + 100 mg/kg arbutin" groups, but there was a remarkable difference in the "4 Gy irradiation + 200 mg/kg arbutin" compared to the "4 Gy irradiation + 50 mg/kg arbutin" and "4 Gy irradiation + 100 mg/kg arbutin" groups (hematological factors in the "4 Gy irradiation + 200 mg/kg arbutin" were low).

Micronuclei

According to figure 3, there was no side effects after injection arbutin 50, 100, and 200 mg/kg ($P>0.05$), but a significant increase was observed for the incidence of micronuclei in irradiated group (2 and 4 Gy) compared to the control group ($P<0.001$).

The frequency of micronuclei found in the "2 and 4 Gy x irradiation + distilled water" groups was significantly higher than the "2 and 4 Gy irradiation + 50 mg/kg arbutin", "2 and 4 Gy irradiation + 100 mg/kg arbutin", and "2 and 4 Gy irradiation + 200 mg/kg arbutin" groups ($P<0.001$).

The comparison into the groups indicated that the frequency of micronuclei in the "2 Gy irradiation + 50 mg/kg arbutin" group was considerably lower than the "2 Gy irradiation + 100 mg/kg arbutin", and "2 Gy irradiation + 200 mg/kg arbutin" groups, but there were not any differences between the "2 Gy irradiation + 100 mg/kg arbutin", and "2 Gy irradiation + 200 mg/kg arbutin" groups. No significant differences were observed among the "4 Gy irradiation + 50 mg/kg arbutin", "4 Gy irradiation + 100 mg/kg arbutin", and "4 Gy irradiation + 200 mg/kg arbutin" groups.

Table 1. Statistical significance between different groups using Tukey HSD test.

Blood count	Group I		Group I			Group VII		Group VIII	Group XII		Group X
	Group II	Group III	Group IV	Group V	Group VI	Group VIII	Group IX	Group IX	Group X	Group XI	Group XI
WBC (10^9/µl)	0	0	0.0	0.057	0.061	0.039	0.045	0.055	0	0.002	0.074
LYM (10^9/µl)	0	0	0.055	0.062	0.062	0.005	0	0.06	0.003	0.006	0.053
RBC (10^9/µl)	0	0	0.062	0.071	0.07	0.023	0.029	0.053	0.002	0.02	0.062
HGB (g/dl)	0	0	0.07	0.059	0.062	0.031	0.032	0.078	0.03	0.041	0.059

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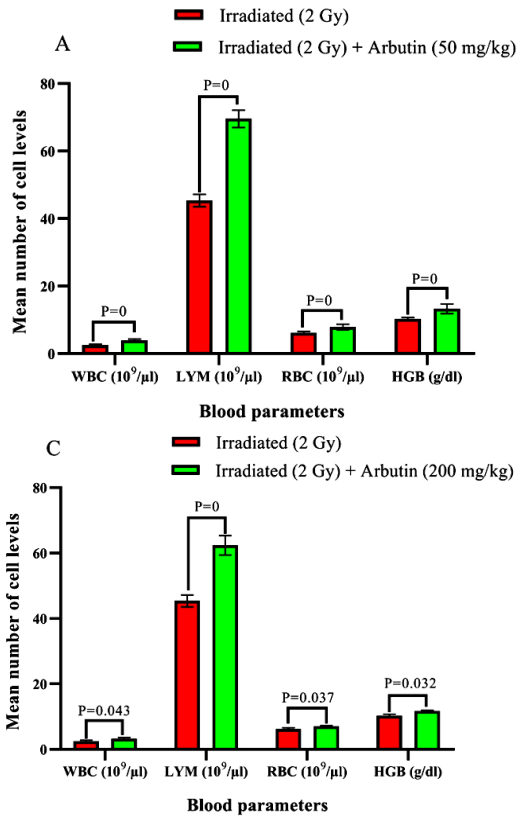

Figure 1. The effective of x-irradiation (2 Gy) and x-irradiation (2 Gy) along with various concentrations of arbutin (**A:** 50 mg/kg, **B:** 100 mg/kg, and **C:** 200 mg/kg) in the blood cell parameters.

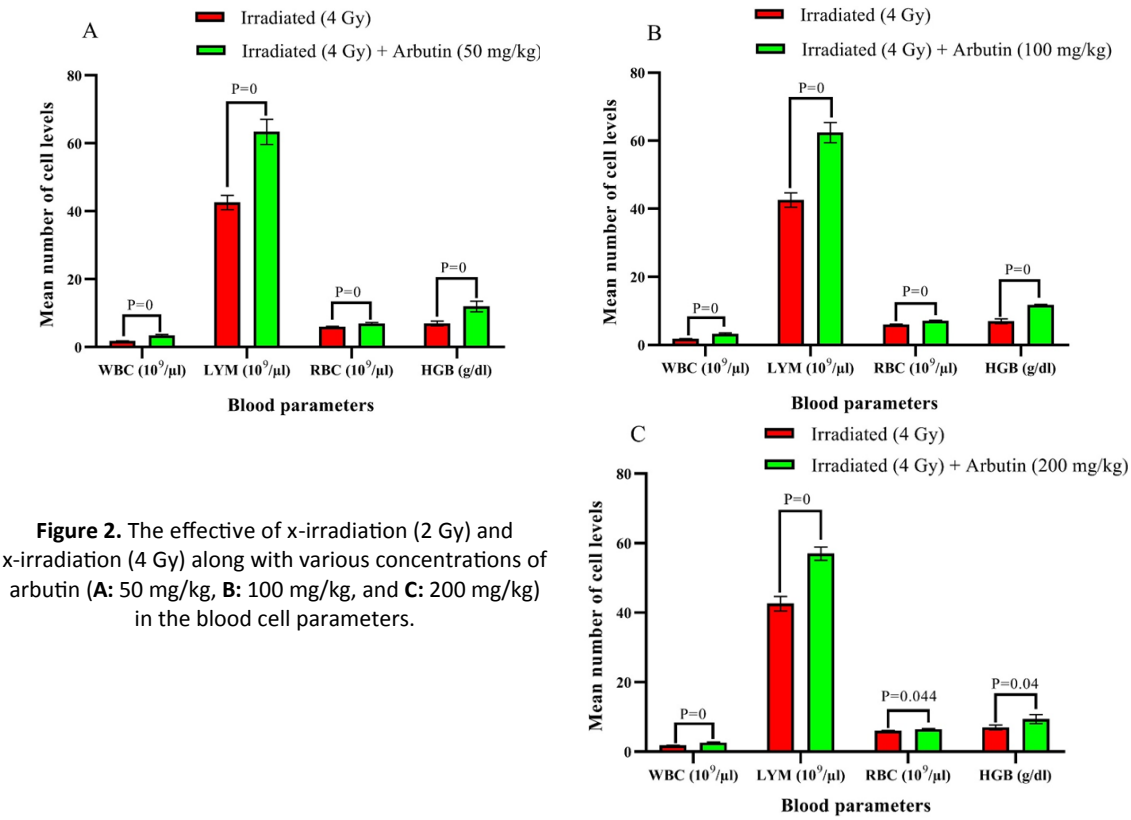

Figure 2. The effective of x-irradiation (2 Gy) and x-irradiation (4 Gy) along with various concentrations of arbutin (**A:** 50 mg/kg, **B:** 100 mg/kg, and **C:** 200 mg/kg) in the blood cell parameters.

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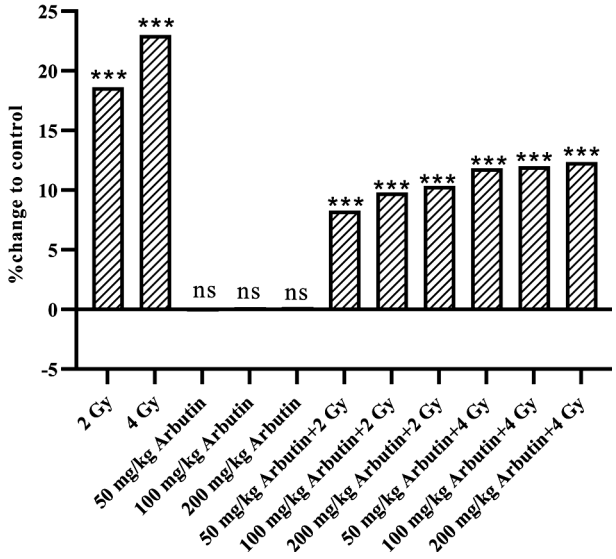

Figure 3. Effects of x-irradiation and different concentrations of arbutin in micronuclei level with respect to the control group. ***Significant difference from the groups compared to the control group at P < 0.001.

DISCUSSION

Ionizing radiation such as radiotherapy is one of the important techniques for medical treatment [2], but this technique causes undesirable physiological and biochemical changes in humans and animals [26, 27]. The DNA damage at the chromosome level is an essential part of genetic toxicology. Consequently, this effective of radiation can cause the inhibition in mitosis and decreasing the amount and life span of peripheral blood cells as the most sensitive system [15, 28, 29].

The objective of the current study was to detect what extent of arbutin as a radioprotector can ameliorate the levels of some cellular elements of blood during whole-body x-irradiation on mice. Furthermore, the lymphocyte cell levels were investigated by micronucleus assay.

Our findings showed that x-irradiations (2 and 4 Gy) induced a decrease in blood parameters such as WBC, RBC, LYM, and HGB compared with the control group. The current study corroborates the previous findings of Shaheen and Hassan [30], who declared that x-radiation caused a remarkable decrease in RBC [30]. Also, our results showed that the frequency of the micronuclei in the group which only received radiation (2 and 4Gy) was higher than the control group. Other studies by Hosseinimehr and Nemati [31], Shahidi and Mozdarani, [32] and Shokrzadeh *et al.* [33] confirmed the current results.

The increasing of micronuclei affected by chromosomal damages induced with radiation. Free radicals like hydroxyl and superoxide radicals produced by radiation can cause the RBC death and also an imbalance between its production and loss. In addition, after irradiation, the count of LYM may be reduced due to the regeneration of intracellular stores of reduced glutathione regarding the oxidative damage. The subsequent decline in the values of WBCs and LYM could be a result of the absence of infection and inflammation, furthermore, the generation of HGB can be reduced because of liver and bone marrow damage resulting from radiation exposure [15, 17, 34, 35].

The normal levels of hematological parameters usually measured can be modified by plants consumption or toxic substance [17, 36, 37]. Our results indicated that arbutin (all three concentrations) can increase blood parameters during megavoltage therapeutic x-irradiation on mice. In the groups which were only treated with arbutin there were not any remarkable changes in blood parameters, relative to the control group. Bertrand *et al.* [17] showed that aged garlic extract as a radioprotector can increase the hematological parameters such as WBC, RBC, LYM, HGB, mean corpuscular volume, and hematocrit value, when injected to rat's blood cells before ionizing radiation. In another study [15], they showed that lettuce oil could improve all of the above-mentioned blood parameters in rats induced by gamma irradiation. In this case they reported that lettuce oil may reduce the biological hazards of radiation. Also Dong *et al.* [38], expressed that ethanol extract from Ji-Xue-Teng is a strong radioprotetive agent due to the recovery from hematopoietic bone marrow damage and oxidative stress of the mice induced by whole body gamma radiation.

Arbutin is a considerable protector (dose

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reduction factor = 2.1) and has successfully ameliorated the hematological disturbances induced by radiations which is related to its antioxidant properties [18], hence, it can sweep free radical, inhibit lipid peroxidation in cell membrane, and stabilize plasma membrane [3].

The effects of the three concentrations of arbutin as a radioprotector were almost similar but a minor difference was observed between them in count of blood cells factors and frequency of micronuclei. The amelioration was more effective in the "2 Gy and 4 Gy x irradiation + 50 mg/kg arbutin" groups in comparison with the "2 Gy and 4 Gy x irradiation + 100 mg/kg arbutin" and "2 Gy and 4 Gy x irradiation + 200 mg/kg arbutin" groups.

As future research, it is suggested that other arbutin concentrations as a radioprotector along with x-radiation in different lengths and doses should be studied to find the precise optimized effect on blood parameters and micronuclei.

CONCLUSION

The micronucleus assay and the evaluation of some blood cell parameters (WBC, CBC, LYM, and HGB) demonstrated that arbutin has radioprotective effects on megavoltage therapeutic X-irradiated mice maybe due to antioxidant and free radical scavenging activities of arbutin. In addition, the study revealed that the radioprotective effect of arbutin was more pronounced with the lower dose (50 mg / kg) compared to the higher ones (100 and 200 mg / kg).

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***Conflicts of interest*: Declared none.**

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