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Article

Radioprotective profile of *Urtica dioica* L. seed extract on oxidative DNA-damage in liver tissue and whole blood of radiation-administered rats

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It was aimed to investigate the radioprotective activity of Urtica dioica L. seed extract (UDSE) in the whole blood and liver of radiation-administered rats, both biochemically and immunohistochemically. 32 rats were divided into 4 groups (n:8). Control group (C): no administration for 10 days. Radiation group (IR): fed pellets for 10 days after exposure to radiation. Radiation + UDSE (IR+UDSE) group: exposed to radiation and fed UDSE for 10 days. UDSE group (UDSE): fed UDSE for 10 days. Radiation (5Gy) was given as a single fraction. 8-hydroxy-2-deoxyguanosine (8-OHdG) and deoxyguanosine (dG) levels were analyzed by biochemical method and glutathione peroxidase 1 (GPx-1) analyses were performed by immunohistochemical method in the liver and blood tissues of the rats. The increased 8-OHdG rates and decreased GPx-1 immunoreactivity was observed in the IR group. Those parameters were ameliorated in the IR+UDSE group when compared to the IR group. UDSE is likely to be a valuable radioprotector against the harmful effects of radiation.

Keywords: Radioprotective. DNA damage. Antioxidant. Urtica.

INTRODUCTION

Ionizing radiation is commonly used for the treatment of the majority of cancer patients. The main target of radiotherapy is to minimize exposure to non-target normal tissue while maximizing the dose to tumor tissue. It is known that the damaging effects of irradiation play an important role in the pathogenesis of tissues and in the hereditary material of the cell (such as DNA) by both direct and indirect mechanisms (Facchinetti, Dawson, Dawson, 1998). The direct action produces disruption of sensitive DNA molecules (Canakçi *et al*., 2009), whereas the indirect effects result from its interaction with water molecules (constitutes about 70% of body weight), which results in the production of highly reactive free radicals such as $^{\cdot}OH$, and e_{aq}^{-} and their subsequent action on subcellular structures (Cikman *et al*., 2015). Therefore, radioprotector substances should be taken during the radiotherapy process in order to reduce the oxidative damage that occurs in the non-targeted normal tissues and organs of irradiated organisms (Halliwell, Gutteridge, 1989). An optimal radioprotector should contain ingredients that are non-toxic to normal cells, be easy to administer, and not degrade performance or compromise the therapeutic effects of the radiation treatment in patients receiving radiotherapy (Hensley *et al*., 1999, Landauer, Srinvasan, Seed, 2003). Radioprotective compounds have been developed over the years, and majority were designed to reduce the levels of radiation-induced free radicals within the cells (Weiss, Landauer, 2003). These radio protective substances can be classified into 3 major categories. The 1st includes chemical substances such as amifostine, which is among the most commonly used radioprotectors. In this category, sulfide-containing chemicals such as amino sulfides, thiol-containing substances, or their derivatives can be mentioned (Giambarresi, Jacobs, 1987). The 2nd is natural substances, which includes antioxidant vitamins such as vitamin A (Kumar *et al*., 2002) and molecules such as melatonin and Beta-glucan (Kuntic *et al*., 2013). The 3rd is substances obtained from plants.

In recent years, an increasing number of studies have reported that some plant leaves, fruits, and seeds contain antioxidant substances (Pratt, 1992; Katalinic, Milos, Kulisic, 2006). Therefore, herbal substances administered

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solely or in combination are generally considered as a well-known form of complementary therapy for radioprotectors. In a study aiming to find effective natural antioxidants, some plant-based substances have recently gained recognition as biological response modifiers (Jagetia, Baliga, 2002). The screening of plants to find some effective molecules is gaining importance so as to avoid unwanted effects from irradiation.

In the literature, it has been shown that *Urtica dioica* L. (UD) was one of the most widely used plants in terms of its therapeutic properties in plant-based treatments in Turkey (Kav, Hanoğlu, Algıer, 2008), because UD has anti-oxidant, anti-inflammatory, anti-bacterial, anti-viral, immunomodulatory and pharmacological effects (Yener *et al*., 2009; Gulcin *et al*., 2004; Celik, Tuluce, 2007).

Although UD seed extract (UDSE) has been shown to exert antioxidant effects in experimental studies, there has been no adequate study of whether it has a protective role against DNA damage due to irradiation. In a study by Shakeri-Boroujeni *et al*. (2016), a combination was tested as a radioprotective candidate in mice administered 2 Gy of radiation. In this herbal combination, UD was 1 among 3 other ingredients, and this combination was found to be effective in ameliorating the effects of ionizing radiation. However, there have been no studies conducted regarding the lone effectiveness of UD in an irradiation model. Determination of the inhibitory effect of UDSE on free radical formation in patients receiving radiotherapy will be important for future studies and it may contribute to treatments as an alternative natural antioxidant.

In this study, we aimed to determine the protective properties of UDSE against DNA damage and the tissue injury caused by irradiation. 8-hydroxydeoxyguanosine (8-OHdG) levels were investigated as a marker to evaluate DNA damage. In addition, the findings were compared with GPx-1 immunoreactivity by immunohistochemical staining of the liver tissues.

MATERIAL AND METHODS

Experiments and rat groups

A total of 32 male Wistar albino rats, 8 weeks old and weighing 190 ± 10 g at the time of irradiation, were bred at the Van Yuzuncu Yil University animal laboratory unit and used for the experiments. The rats were quarantined for at least 7 days prior to exposure to radiation. An illumination system was configured to provide a light/dark photoperiod of 12:12. All of the rats were provided daily standard pellet diet *ad libitum*. This commercial standard rat chow is commonly used pellets which are prepared to allow sufficient nutrients for rats. For UDSE groups those pellets were further processed with UDSE extract for administration of adequate amount of UDSE to each rat. Prior to initiation of experimental procedures, daily consumption of pellets by rats was recorded and given at adequate amount sufficient for number of animals in UDSE administered group cages. The temperature was fixed at 22 ± 1 °C. The study was approved by the Van Yuzuncu Yil University Ethical Committee (YUHADYEK/08, 25.06.2015).

The rats were divided into 4 groups (n = 8). Control group (C): fed pellets for 10 days. Radiation group (IR): fed pellets for 10 days after exposure to 5 Gy of radiation as a single fraction. Radiation + UDSE (IR+UDSE) group: exposed to 5 Gy of radiation as a single fraction and fed UDSE for 10 days. UDSE group: only fed UDSE for 10 days. UDSE dosage was chosen according to previous studies in the literature.

Plant materials and extraction procedure

The UD seeds (UDS) were purchased from a local herbal market in Van, Turkey. The UDS were powdered in a mixer, and their fixed oil was extracted with a rotary evaporator apparatus using ethanol as a solvent. The viscous extract was transferred to falcon tubes and freeze-dried under a vacuum at –51 °C to obtain a fine lyophilized powder. Finally, the resulting extract was mixed with 30 mL/kg of powder pellet meal and the obtained mixture was then pelletized again and dried.

Application of radiation

A single rat, which did not belonging to any of the study groups but was nearly the same weight and size, was used as simulation material in order to provide appropriate radiation dose distribution. This rat was processed using Siemens™ Somatom Sensation4 model computed tomography (CT)-simulator device and total body CT images (2.5 mm section thickness) were obtained in the prone position. These cross-sectional images were transferred to Prowess™ 3-dimensional radiotherapy treatment planning system, and all of the rat's tissues and organs were contoured. After the contouring process was complete, the rat's total body 3D dose plan was created using the 6-MV photon beams from two opposed anterior-posterior fields (AP-PA) of equal weight, to get a single fraction of 5 Gy of radiation in liver with total body irradiation. This dose schedule was designed to be the same for all of the rats and planned data for the rats were transferred to the Siemens™ Artiste (160 multi-leaf

collimator) model linear accelerator in an appropriate posture, and all of the groups were anesthetized with 50 mg/kg intraperitoneal (IP) ketamine for the total body irradiation procedure (Figures 1-2).

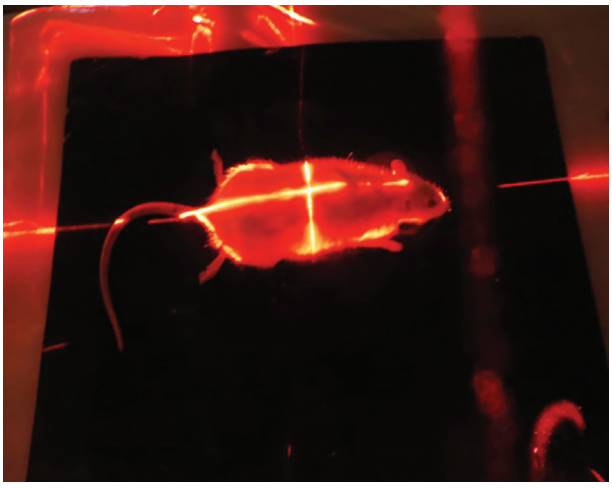

FIGURE 1 - Rat total body 3D dose plan a.

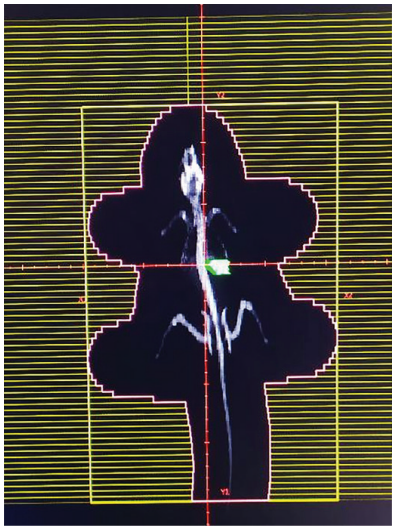

FIGURE 2 - Rat total body 3D dose plan b.

Preparation of the supernatant from liver tissue and collecting whole blood from subjects

At the end of 10 days, all of the rats were sacrificed (under 50 mg/kg IP ketamine anesthesia). Blood samples were taken from the rats via intra-cardiac route and transferred into tubes containing ethylene diaminetetraacetic acid (EDTA). In addition, liver tissue (1 g) was homogenized using a homogenizer device (Ultra Turrx-T25) in 1 mL of 20 mM Tris-HCl (pH 7.4) (Gumustekin *et al*. 2010). Thereafter, it was then centrifuged at 15,000 xg and 4 °C for 30 min. The upper supernatant was then transferred into another new tube. All of the samples were stored in by deep freezing at –80 °C until the biochemical measurements were taken.

Measurement of 8-OHdG and dG

A DNA isolation kit (GenAll DNA extraction kit, GenAll Biotechnology co LTD., Seoul, Korea) was used for DNA isolation from the whole blood and the DNA isolation was performed using a spin column according to the kit prospectus. DNA samples that were obtained for the 8-OHDG and dG analysis were hydrolyzed using formic acid at 150 °C according to method of Kaur and Halliwell (1996). The hydrolyzed DNA samples were dissolved in pure acetonitrile (final volume 1 mL). The 8-OHdG and dG levels were measured using an electron capture detector (ECD) and ultraviolet (UV) detector in the high-performance liquid chromatography (HPLC) device, respectively. A reverse phase C-18 (RP-C18) analytical column was used as the column (250 mm × 4.6 mm × 4.0 µm, Phenomenex, CA). The mobile phase was prepared as a mix of 0.05 M potassium phosphate buffer (pH: 5.5) and acetonitrile (97:3, v/v), and the flow rate was set to 1 mL/min. The amount of 8-OHdG and dG was determined using the ECD adjusted to 600 mV, and absorbance measurement at 245 nm with the UV detector, on the HPLC apparatus, respectively. For measurement of the 8-OHdG and dG, their standards were purchased from the Sigma Aldrich Company. The obtained 8-OHdG values were expressed as the number of 8-OHdG per 10^6dG (8-OHdG/10^6dG) (Tarng *et al*., 2000).

Immunohistochemical analysis

At the end of the experiment, a systemic necropsy was performed on all of the rats and liver tissue was taken for 72 h in a 10% buffered formalin solution. After a routine follow-up, the tissue samples were embedded into paraffin blocks. 4 µm-sections were placed onto poly-lysine slides using microtome (Leica RM 2135).

Immunohistochemistry was performed to investigate Glutathione peroxidase 1 (GPx-1) expression by streptavidin-peroxidase method. Commercial antibody was visualized on 4-µm-thick sections from the paraffin block using an indirect streptavidin/biotin immunoperoxidase kit (Histostain Plus Bulk Kit, Zymed, South San Francisco, CA, USA). All steps were carried out following the standard method described by the manufacturer. Tissue sections were incubated with the GPx-1 (abcam-ab22604) (1:400) primary antibodys overnight at 4 °C. Finally, to visualize the reactions, the sections were reacted for 5-15 min with 3,3-diamino-benzidine (DAB) chromogens

for GPx-1 staining. After the development of the DAB reactions, the sections were counterstained with Mayer's hematoxylenes. The sections then were passed through alcohol and xylene and mounted directly with Entellan mounting medium. Negative controls used to verify staining. The slides were reacted with PBS instead of primer antibody as negative controls.

According to the glutathione peroxidase 1 (GPx1) immunoreactivity, the liver tissues of the groups were scored as: no staining (–), poor staining (+1), medium staining (+2), and strong staining (+3) (Table I). In order to specify whether the immunoreactivity in the tissues was specific to GPx1 or not, a negative control application was performed. The preparations were examined using a Nikon 80i-DS-RI2 research microscope (Figure 3).

Statistical analysis

All of the recorded data and analyses were performed using the Statistical Package for the Social Sciences software (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.). Categorical variables were summarized as frequencies (N) and valid percents (%), while continuous variables were expressed as median (minimum–maximum) and mean ± standard deviation (SD). The continuous variables were compared among the 3 groups using the Kruskal-Wallis test. To determine which group differed significantly from the others, post hoc were performed. The statistical significance level (P value) was considered as $P < 0.05$.

RESULTS

Biochemical results

The biochemical results of this study showed that the 8-OHdG/10^6dG rate, which was measured as a marker of DNA damage, was significantly augmented in the liver tissues of the IR group when compared to all of the other groups (3.535 ± 0.913). Concomitant administration in the IR and UDSE groups (1.835 ± 0.075) did not increase the 8-OHdG/10^6 dG levels when compared to levels in the C group (1.499 ± 0.135), and although this level was seen to have decreased in the UDSE group (1.403 ± 0.211) when compared to levels in the C group, this decrease did not reach statistical significance.

Whole blood 8-OHdG/10^6dG rates also showed a significant increase in the IR group (1.243 ± 0.079) compared to those in the C group (0.506 ± 0.062). Contrary to the liver results, 8-OHdG/10^6 dG levels of whole blood in the IR+UDSE group (0.670 ± 0.041) was significantly higher when compared to that in the C group, and 8-OHdG/10^6 dG levels of whole blood in the UDSE group (0.322 ± 0.094) was also significantly low than that in the C group.

Immunohistochemical results

Group-based distributions of GPx1 immunoreactivity in liver are summarized in Table I and Figure 3.

Staining (negative control) was performed in all of the groups, using phosphate buffered saline instead of the primer antibody, in order to determine whether GPx1 immunoreactivity was specific in the liver tissue sections, but no staining was observed (Figure 3A). GPx1 immunoreactivity was specifically detected in the liver tissue sections from all of the groups.

In the C group, there was the most intense staining. GPx1 reactivity was detected to be formed in the same intense in the centrilobular, midzonal and periasiner regions of the liver. While GPx1 immunoreactivity was found to be only in cytoplasm in a part of hepatocytes, both cytoplasmic and nuclear reactivity observed to be more intense. There weren't GPx1 immunoreactivity in vena sentralis and endothelium of vessels located in portal triad and Kupffer cells and sinusoidal vessel endothelium. On the other hand, moderate immunoreactivity found out in epithelial cells of bile duct (Figure 3B).

In the IR group, the rat hepatocytes were stained with +1 diffuse cytoplasmic and nuclear type, while no staining was observed in a large number of hepatocytes. epithelial cells of bile duct were immunoreactivity similarly to those in the C group, whereas sinusoids and portal veins were not seen in the endothelial cells (Figure 3C)

TABLE I - Distribution of GPx1 immunoreactivity according to the groups in the protective efficacy of UDSE on liver tissues of experimentally-irradiated rats

Parameters (in the liver)	C	IR	IR+UDSE	UDSE
Cytoplasmic and nuclear in hepatocytes	+2 and +3	+1	+2	+2 and +3
Vascular endothelial cells in the sinusoidal and portal area	0	0	0	0
Bile duct epithelial cells	+2 and +3	+1	+2	+2 and +3

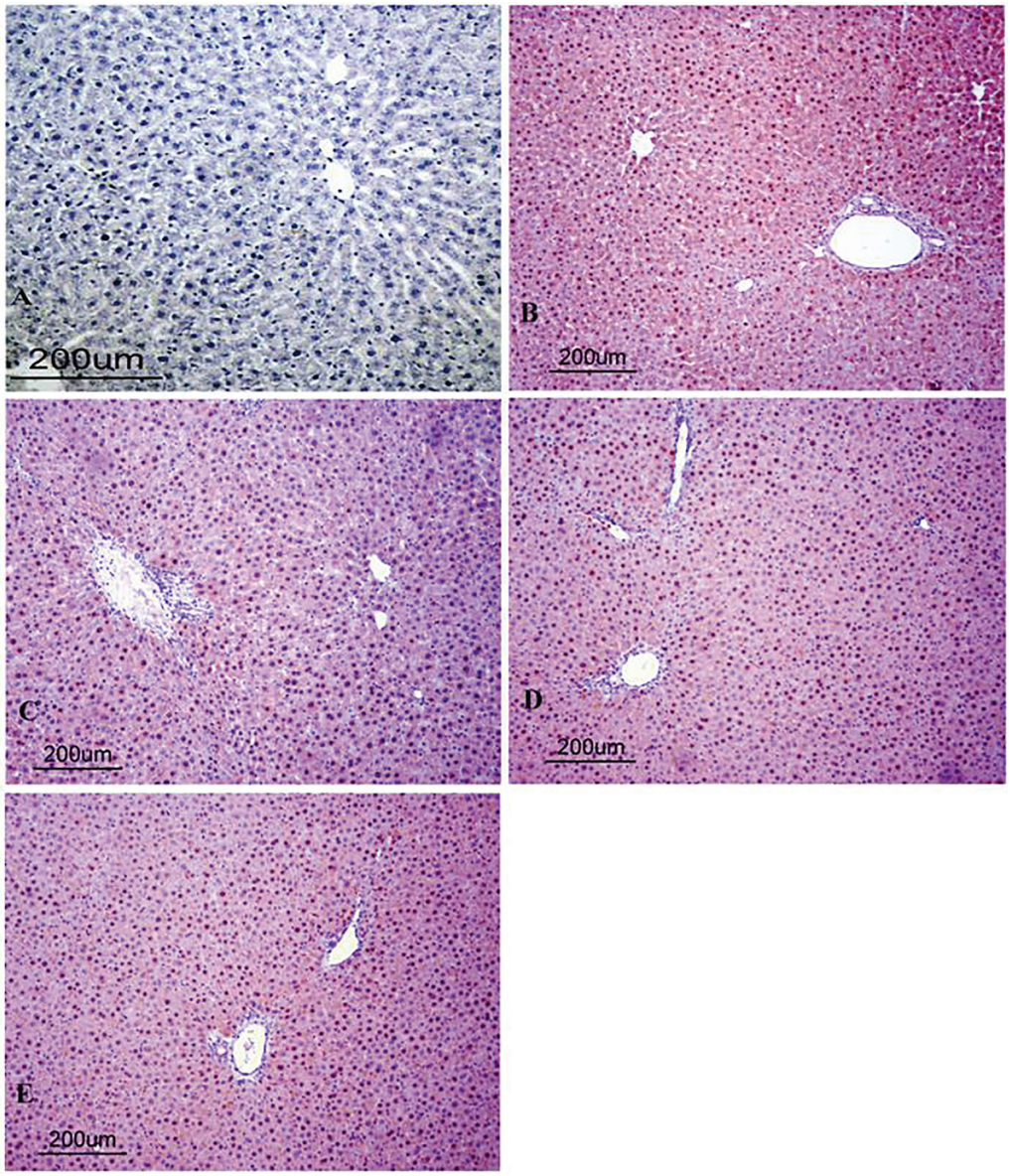

FIGURE 3 - Microscopic appearance of the degree of staining according to the immunoperoxidation method of GPx1 reactivity of the liver cells. A; negative control group, B; control group, C; IR group, D; IR+UDSE group, E; UDSE group.

TABLE II - Comparison of the 8-Hydroxy-2'-Deoxyguanosine/10^6deoxyguanosine rates among the groups

Groups	Liver tissue 8-OHdG/10^6dG Mean + SD	Whole blood 8-OHdG/10^6dG Mean + SD
C	1.499 ± 0.135^{b}	0.506 ± 0.062^{c}
IR	3.535 ± 0.913^{a}	1.243 ± 0.079^{a}
IR+UDSE	1.835 ± 0.075^{b}	0.670 ± 0.041^{b}
UDSE	1.403 ± 0.211^{b}	0.322 ± 0.094^{d}

$^{a, b, c, d}$: Values with different letters in the same column are significantly different ($P < 0.001$).

In the IR+UDSE group, similar to the C group, diffuse nuclear and cytoplasmic staining was observed in level +2 and +3 hepatocytes (Figure 3D)

In the UDSE group, the GPx1 immunoreactivity was similar to that of the C group (Figure 3E).

No deaths were occurred during the experimental procedure. Animals gained weight and no statistically important difference was observed between groups.

DISCUSSION

The research on antioxidant substances has been increasing to remove free radicals that result from triggered indirect mechanisms such as irradiation. This

study aimed to evaluate the potential radioprotective effects of an aqueous extract of UDSE on 8-OHdG, which is a parameter of oxidative DNA damage and GPx1, which is an important indicator of the antioxidant defense system, by immunohistochemical and biochemical methods in liver and blood tissues in radiation-induced rats.

GPx1 immunoreactivity

GPx, which is an antioxidant and Seleno protein molecule, is found in all mammalian organs, but its expression level is known to vary according to the isoforms and tissues (De Haan *et al*., 2005). Cytosol and mitochondria were reported to be sites of GPx expression. It is known that it uses glutathione to reduce H_2O_2 and organic hydroperoxides (McClung *et al*., 2004). The studies on the localization of GPx have suggested that GPx1 is both a cytoplasmic and a mitochondrial enzyme, and GPx1 is a line of defense in most cells (Esposito *et al*., 2000), GPx1 was reported to be highly expressed in the cytoplasm and mitochondrion of rat liver (Cikryt, Feuerstein, Wendel, 1982).

In studies considering immunohistochemical techniques in rat liver, GPx was found in the nucleus (Asayama *et al*, 1996), cytoplasm (Yoshimura, Komatsu, Watanabe, 1980), and mitochondrion of hepatocytes more than in other organelles (Muse *et al*., 1994). Studies have shown that GPx provides a primary defense against the detoxification of H_2O_2 in intracellular media (Asayama *et al*., 1996). In a study by Deprem *et al*., (2009), the control and sham groups demonstrated similar immunolocalization of GPx1 in the liver, while GPx1 immunoreactivity in the diabetic group was somewhat weaker than in the sham group. In our study, in all of the groups, the diffuse type of GPx1 immunoreactivity was found in the cytoplasm and nucleus of hepatocytes. When the results of our study were compared, the results of the C, UDSE, and R+UDSE groups were similar, while the R group was the lowest. These results indicate the presence of antioxidant properties in UDSE. As a result, our work is in agreement with the reviewed literature.

8-OHdG/10^6dG for whole blood and tissue

This parameter of the study was chosen to evaluate impact of oxidative DNA damage via 8-OHdG and to assess any potential protective activity of Urtica extract. A study by Kim *et al*. (2016) investigated the effect of α-lipoic acid (ALA) on radiation-induced salivary gland injury in rats. Immunohistochemical staining of 8-OHdG, a reactive oxygen species (ROS)-induced DNA damage marker, was performed to investigate the effect of ALA on radiation-induced oxidative stress. The 8-OHdG-positive signals were detected in the nuclei of the acinar and ductal cells, which were both irradiated. IR can induce cellular damage and death through the ROS generated by radiolytic hydrolysis. In a study by Özyurt *et al*. (2014), rats were exposed to 8 Gy of whole-abdominal IR and given either vehicle or quercetin (20 mg/kg, IP). Radiation-induced inflammation was evaluated through tissue cytokine TNF-α levels. In order to examine the oxidative DNA damage, tissue 8-hydroxydeoxyguanosine (8-OHdG) and deoxyguanosine (dG) levels were measured. In the saline-treated irradiation groups, 8-OHdG was found to be increased in both tissues. In the quercetin-treated IR groups, all of these oxidant responses were prevented significantly. These data demonstrated that quercetin, through its free radical scavenging and antioxidant properties, attenuates irradiation-induced oxidative organ injury, suggesting that quercetin may be of benefit in radiotherapy by minimizing the adverse effects (Özyurt *et al*., 2014).

In a study by Inano and Onoda (2002), the radioprotective action of curcumin [1,7-*bis*(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] extracted from Curcuma longa LINN against the acute and chronic effects, and the mortality induced by exposure to radiation using female rats was evaluated. For the assay of 8-hydroxy-2'-deoxyguanosine (8-OHdG) in urine, a marker for acute effects, Wistar-MS virgin rats were fed a basal diet and exposed to 0 or 3 Gy of gamma-rays from a Co-60 source as the control was administered. To determine survival, the virgin rats received 9.6 Gy of whole-body irradiation and were fed a diet containing curcumin for 3 days before and/or 3 days after irradiation. After irradiation, all of the rats were assessed daily for survival for 30 days.

Acutely, in the virgin rats that received 3 Gy of irradiation, the creatinine-corrected concentration and total amount of 8-OHdG in the 24-h urine samples were higher (approximately 1.3-fold) than the corresponding values in the non-irradiated controls. Adding curcumin to the diet for 3 days before and/or 2 days after irradiation reduced the elevated 8-OHdG levels by 50%–70%. The evaluation of the protective action of curcumin against the long-term effects revealed that curcumin significantly decreased the incidence of mammary and pituitary tumors. However, the experiments on survival revealed that curcumin was not effective when administered for 3 days before and/or 3 days after irradiation (9.6 Gy). These findings demonstrated that curcumin can be used as an effective radioprotective agent to inhibit acute and chronic effects, but not mortality, after irradiation (Inano, Onoda, 2002).

In our study, the 8-OHdG/10^6 dG rate in the liver was significantly increased in the IR group when compared to all of the other groups, whereas IR+UDSE administration did not significantly increase the 8-OHdG/10^6 dG levels when compared to the C group. Since the liver and brain are more sensitive against oxidative stress and degenerative effects compared to other tissues, the radioprotective effect of UDSE on liver tissue was evaluated in this study. Similarly, the literature about UD presented it as potent radioprotector activity for liver tissue. Serum values also presented protective activity against a radiation-induced 8-OHdG/10^6dG increase. Since 8-OHdG was evaluated as a marker for sperm infertility (Hosen *et al*., 2015), UD extract can be assessed for protection against radiation-induced impact on sperm quality. 8-OHdG was also reported to increase due to electromagnetic fields, which are generated by power plants or conduction cables (Zhang *et al*., 2017). Therefore, UD extract can also be tested in such models.

Results of the study by Shakeri-Boroujeni (2016) also supported the protective effect of a herbal combination including UD against radiation-induced side effects. Our study suggests that this protection effect may be strongly related to its ingredient, UD. Plants constitute various metabolites that have an antioxidant nature. Those molecules may alleviate damage caused by ROS occurring during radiotherapy and may reduce tumor resistance against such therapies. Although we did not perform any analysis to elucidate molecular ingredients of this plant extract, various studies concerning Urtica gave detailed molecular composition of the plant. In addition we have chosen to use plant extract since plant based traditional therapies also administer this plant in its extract form rather than chemically fractioned compositions. Further studies could reveal the molecules responsible for the radioprotective effect of UD, which may help radiotherapy patients to reduce the side effects of the administered radiation while attaining adequate therapy.

CONCLUSION

A potential radioprotective substance should increase an organism's defense systems, such as antioxidant and detoxification enzymes, and molecules, as well as decrease harmful molecules, such as oxidizing substances or DNA damage. The current study states that aqueous UD extract significantly ameliorated an irradiation-induced increase in 8-OHdG/10^6 dG levels and decreased Gpx.

ACKNOWLEDGMENT

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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