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The neuroprotective and anti-inflammatory effects of Annona muricata (Graviola) on radiation-induced rat sciatic nerve injury

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ABSTRACT

Objective: This study aimed to evaluate Annona muricata's (AM) radioprotective effects on sciatic nerve injury due to ionising radiation (IR).

Materials and Methods: Thirty-two adult female Wistar albino rats were separated into four equal groups; Control (C), Annona muricata leaf extracts (AME), radiation (RAD), radiation and AME (AME+RAD). In groups AME and AME+RAD, AME was administered at a dose of 300 mg/kg for the first day and 50 mg/every day for the following one week intraperitoneally. In RAD and AME+RAD, rats were exposed to a single dose of 20 Gray (Gy) IR to their right legs. All the subjects were sacrificed at the end of the first month. Oxidative stress biochemical parameters from blood samples were analysed. In addition, right sciatic nerves were extracted and histomorphology was evaluated.

Results: Statistically significant vasculature, degenerative and necrotic changes were observed in RAD, compared to C and AME ($p<0.01$). Swelling in myelin sheath was predominantly seen in RAD. Alterations in the level of catalase ($p<0.01$), superoxide dismutase ($p<0.01$) and glutathione peroxidase ($p<0.05$) in the AME+RAD group compared to the RAD group were found to be statistically significant.

Conclusion: Our study unveiled that AM could potentially enhance biochemical and histomorphological healing in the acute period on sciatic nerve injury due to IR.

Keywords: Radiation, Sciatic nerve, Annona muricata, Neuroprotection, Oxidative stress

1. INTRODUCTION

New treatment modalities for cancer treatments or survival prolongation are widely used in clinics worldwide. Further, radiotherapy (RT) keeps itself a beneficial treatment option in many types of cancer [1]. However, serious complications may develop in patients due to the effects of both chemotherapeutic agents and RT on healthy tissues other than tumour tissues [1,2]. These complications may occur acutely, subacutely and chronically, depending on the tolerance of the tumour and the tolerance of the normal tissue to RT. Furthermore, in addition to complications such as acute radiation pneumonia, nephropathy and hepatitis, sensory and/or motor neurological damage may occur in the extremities due to the neurotoxic effect of ionising radiation (IR) [3-8]. This neurological damage may cause permanent loss in function of the extremities. Therefore, the patients face additional stress and it reduces the patients' quality of life. Due to IR, neuron damage is closely related to early biochemical and histomorphological changes and late scar formation [9,10]. The mechanism of all these changes occurs due to the process that results in cell death due to DNA damage. Nitrogen and reactive oxygen (ROS) species and free radicals that arise due to the ionization of water in the acute period are responsible for lethal DNA damage with an indirect

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mechanism rather than the damage caused by direct ionization in DNA. [11,12]. Superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) are antioxidant enzymes that protect cells against free radical damage [10]. Since the mid-19th century, many chemical compounds, including antioxidant agents, have been used to reduce these side effects of IR [10,14-16].

Annona muricata (AM) (Graviola), the tropical tree from the Annonaceae family, consists of evergreen dark green leaves and heart-shaped fruits [13]. This fruit is widely consumed and is called a 'cancer killer' by the local people in tropical regions. They consume this fruit by boiling the leaves to treat cancer [13,17]. In addition, AM, which contains many flavonoids, alkaloids and acetogenins in its leaves, is also used to manage infections, diarrhoea, dermatitis, diabetes, fever and cardiovascular problems [18]. In support of this traditional use, various studies have shown the anticancer, antioxidant, antiviral, antihaemolytic, sedative and neuroprotective effects of AM [19,20].

Radiation-induced peripheral nerve damage (RIPND) is permanent since nerve cells do not have the regeneration ability [21,22]. Although, necessary precautions are taken to prevent the development of RIPND, there is no effective treatment, and the treatment options to restore the patients' living standards are limited [23]. The incidence of RIPND keeps increasing in correlation with aggressive RT programs. In this study, we evaluated radioprotective effect of AM on the changes in the acute period of damage to the rat sciatic nerve due to IR.

2. MATERIALS and METHODS

Animals and Experimental Protocol

All experimental procedures were carried out in the Animal Experiments Laboratory of Bülent Ecevit University Faculty of Medicine. Approval was obtained from the local ethics committee of the university (approval no: 2020/06). A total of 32 *Wistar albino* female rats, weighing 350-450 g, were randomly separated into four groups of equal numbers (n = 8, total: 32): C (Control + Vehicle), AME (Annona Muricata leaf extracts), RAD (Radiation + Vehicle), AME+RAD (Radiation + Annona Muricata leaf extracts). The sample size was determined by using G Power. The animals were kept at a constant temperature (18°C – 21°C), and adequate nutrition and photoperiod (12 hours light/dark cycle) were provided for the duration of the experiment.

For one week, rats in group C were injected intraperitoneally (IP) with 2ml/kg of normal saline per day. In the AME group, 300 mg/kg AME was administered IP on the first day and 50 mg/kg AME every day for seven days. Rats in the RAD and AME+RAD group were exposed only one single IR dose (20 Gy) to their right legs without any treatment. The rats in the AME+RAD group were given 300 mg/kg AME IP 30 minutes before IR application and 50 mg/kg AME IP every day for seven days after the application. After one month, rats in all groups were sedated with xylazine (10 mg/kg, IP; Bioveta, Ankara, Turkey) and ketamine hydrochloride (80 mg/kg, IP; Ketalar, Pfizer, Istanbul, Turkey) anaesthesia for biochemical examination was injected via artery in the ventral tail. After blood samples were taken, rats were sacrificed with high-dose anaesthetic drug (Pentobarbital 200 mg/kg, IP; Bioveta, Ankara, Turkey). Right after this procedure, the right sciatic nerves of all rats were carefully separated from the surrounding tissues by blunt dissection and removed for histomorphological examination (Figure 1).

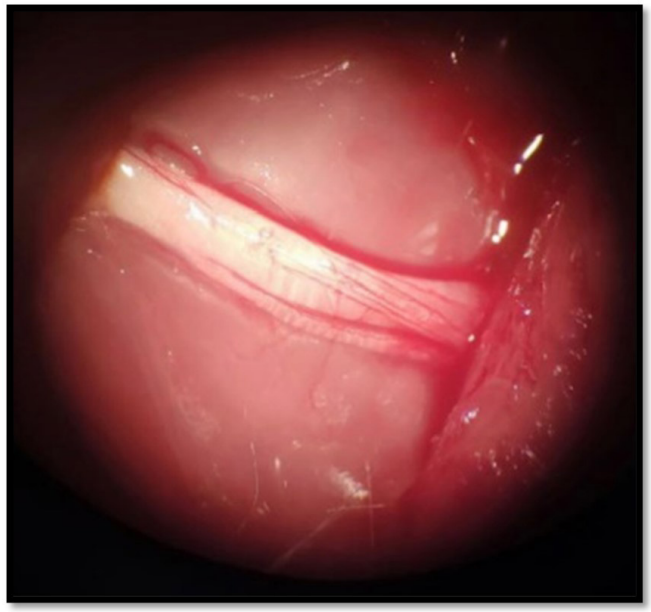

Figure 1. *Microscopically, right leg of the rat's sciatic nerve is seen (an original picture from our study).*

Irradiation

The rats in AME+RAD and RAD groups were anaesthetised using xylazine (10 mg/kg, IP; Bioveta, Ankara, Turkey) and ketamine hydrochloride (80 mg/kg, IP; Ketalar, Pfizer, Istanbul, Turkey) and placed in the prone position. Before the procedure, rat simulation was performed with a 1 mm section computed tomography scan; the dose was calculated with Eclipse treatment planning system, version 8.9 (Varian Medical Systems, Palo Alto-CA, USA) [24]. After the simulation, other parts of their bodies were protected using lead and beam collimation. A 6-mV linear accelerator (Clinac, Varian Medical Systems, Palo Alto CA, USA) was used to expose IR to the right legs of the rats in both groups with a 1.0 cm bolus source-skin (SSD) distance technique on the surface [24]. No rat was excluded due to death.

Chemical

Air-dried AM leaves were obtained from a local supplier in the Mediterranean region in Turkey. They cut the leaves into small pieces after washing with distilled water. 25 g samples

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were extracted in 70:70 ethanol for seven days with occasional shaking. The extract concentration procedure was done by removing ethanol. A rotary evaporator was used (Heidolph, Germany). After, the extract was lyophilised (Telstar – LyoQuest, Spain) overnight to prepare a dry extract, kept at – 20 °C until use.

Biochemical analysis

Superoxide Dismutase (SOD, U/ml)

Superoxide dismutase accelerates the conversion to hydrogen peroxide and molecular oxygen in the elimination steps of ROS radicals produced in oxidative reactions. This method takes place using xanthine and xanthine oxidase. Here, it is intended to generate superoxide radicals that react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to form a red formazan dye. The degree of inhibition of this reaction gives information about the SOD activity (Relassay, Turkey). 1.7 ml of reagent 1(R1a.+R1b.) was added to the cuvette. Then, 50 µl of sample was added to the pathway cuvette and mixed thoroughly. Then, 250 µl of reagent 2 (Xanthine Oxidase) was immediately added to the cuvette and mixed thoroughly. The first absorbance of A1(505 nm) after 30 seconds was read and at the same time the timer was started. After 3 minutes, the final absorbance of A2 (505 nm) was read.

Catalase (CAT, U/ml)

This colourimetric assay involved two steps. The sample was incubated with certain hydrogen peroxide. Hydrogen peroxide was converted to water and oxygen by this sample. CAT concentration was proportional to this ratio. Absorbances at 405 nm were expressed in U/ml (Relassay, Turkey). 300 µl of reagent 1 was added to the pathway cuvette. Then, 17 µl of sample was added to the pathway cuvette and mixed well. After 30 seconds, OD values were measured as 480 nm. Immediately after this, 250 µl of reagent 2 was added to the cuvette, the solution was mixed well. It was incubated at 37 °C for 5 minutes. OD for absorbance was measured at 700 nm per second.

Glutathione Peroxidase (GPx, U/ml)

This method is based on the Paglia and Valentine method. GPx catalyses the oxidation of glutathione by cumene hydroperoxide. In the presence of glutathione (GSSG), it is immediately converted to the reduced form by oxidation of NADPH to NADP. The decrease in absorbance at 340 nm is the measure (Relassay, Turkey). 250 µl of reagent 1 was added to the pathway cuvette. Then 10 µl of sample was added to the pathway cuvette and they were mixed thoroughly. Immediately after this, 40 µl of reagent 2 was added to the cuvette and they were mixed thoroughly. It was incubated at 37 °C for 5 minutes. OD for absorbance was measured at 340 nm.

Histomorphologic examination

Automated tissue processing equipment (Leica ASP300S, Wetzlar, Germany) was used for the routine processing of 32 sciatic nerve tissue samples fixed with 10% formalin. Afterwards, the tissues embedded in paraffin were cut using a Leica RM2255 rotary microtome (Wetzlar, Germany) to obtain 4 µm thick sections. Next, tissue sections stained with Hematoxylin-Eosin (HE) were evaluated under the microscope. The same procedure was performed for all samples. The camera used to observe all study materials were Nikon Digital DS-Ri2, and the microscope was Nikon Eclipse Ni-U equipped with the corresponding software. (Nikon, Tokyo, Japan).

In four separate groups, four parameters were examined for vasculature change, degenerative change, necrotic change, swelling in myelin sheath in sciatic nerve tissue. Histomorphological findings in tissues and cells were scored. Scores were between 0 and 3 for each criterion. (0=normal, 1=light, 2=medium, 3=heavy).

Statistical Analysis

SPSS version 22.0 was used to analyse the data. Descriptive statistics for quantitative variables were expressed as mean and standard deviation, and categorical variables were expressed as numbers and percentages. Normal distribution assumptions for continuous variables were checked with the help of Skewness and Kurtosis coefficients, Shapiro-Wilk test, and distributions in q-q graphs. As the data show a normal distribution, One-way analysis of variance with LSD post hoc was used to compare the differences of the groups. The differences between the groups were evaluated using Chi-Square analysis for categorical variables. Based on the p-value <0.05, the results were considered statistically significant.

3. RESULTS

Biochemical evaluation

Plasma CAT, SOD and GPx values in all the experimental groups are presented in Table I. There was a significant decrease in the activity of SOD, CAT and GPx antioxidants in the RAD group compared to the C group. ($p<0.01$, $p<0.01$ and $p<0.05$, respectively, Table I, Figure 3). It was found that the activity of CAT, SOD and GPx antioxidants in the AME+RAD group (respectively $p<0.01$, $p<0.01$ and $p<0.05$, Table I, Figure 3) increased significantly compared to the RAD group. Similarly, there was a significant difference between the AME and RAD groups CAT, SOD and GPx levels ($p<0.05$, Table I, Figure 3).

Histomorphological evaluation

Vascular system change, degenerative change, necrotic change, and swelling in the myelin sheath were evaluated for all groups. C and AME groups had a typical histomorphological structure in the sciatic nerve tissues (Figure 2A and Figure 2B). Findings were most severe in the RAD group (Figure 2C). In the AME+RAD group, the results were mild to moderate. (Figure 2D).

An increase of vasculature and degenerative changes in the RAD group were statistically significant compared to the C and AME groups ($p<0.001$, Table II, Figure 2C). In the AME+RAD group, there was a statistically significant increase

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in these changes compared to the RAD group (p<0.001, Table II, Figure 2D). While a necrotic change was not observed in the C and AME groups, it was moderate in 25% and severe in 75% of the rats in the RAD group. This change was observed less in the AME+RAD group than in the RAD group, and this difference was also statistically significant (25% mild, 75% moderate, p<0.001, Table II, Figure 2D). While swelling in the myelin sheath was severe in the RAD group, it was mild in group C. Moderate changes were observed in only 25% of the rats in the AME+RAD group, unlike the RAD group (p<0.001, Table II).

Table I. *Values of CAT, SOD and GPX in plasma*

	C	AME	RAD	AME+RAD	p
CAT	94.88 ± 28.13	100.50±26.90	48.88 ± 19.28	80.50±17.48	7.809 (< 0.01) [abc]
SOD	173 ± 17.43	175.75±29.15	99.50±19.66	154.63±45.13	11.219 (< 0.01) [abc]
GPx	696.60±115.07	750.63±304.84	450.38±102.11	663.63±165.94	3.840 (< 0.05) [abc]

[a] *Shows significant differences between Control and Radiation groups (P < 0.05).*
[b] *Shows significant differences between Radiation and Annona muricata leaf extracts groups (P < 0.05).*
[c] *Shows significant differences between Radiation and Annona muricata leaf extracts groups + Radiation groups (P < 0.05).*
C (Control), AME (Annona muricata leaf extracts), RAD (Radiation), AME+RAD (Radiation and AME)

Table II. *Comparison of histomorphologic scores between groups*

Parameters Groups	0	1	2	3	p
Vasculature change C	6 (75%)	2 (25%)	-	-	
AME	3(37.5%)	5(62.5%)	-	-	0.001*
RAD	-	-	3 (37.5%)	5 (62.5%)	
AME+RAD	-	2 (25%)	4 (50%)	2 (25%)	
Degenerative change C	6 (75%)	2 (25%)	-	-	
AME	3 (37.5%)	5 (62.5%)	-	-	0.001*
RAD	-	-	3 (37.5%)	5 (62.5%)	
AME+RAD	-	6 (75%)	2 (25%)	-	
Necrotic change C	8 (100%)	-	-	-	
AME	8 (100%)	-	-	-	0.001*
RAD	-	-	2 (25%)	6 (75%)	
AME+RAD	-	6 (75%)	2 (25%)	-	
Swelling in myelin sheath C	6 (75%)	2 (25%)	-	-	
AME	3 (37.5%)	5 (62.5%)	-	-	0.001*
RAD	-	–	2 (25%)	6 (75%)	
AME+RAD	-	6 (75%)	2 (25%)	-	

**Statistical analysis for comparison between groups with Chi-squared: P < 0.05, statistical significance. C: Control, AME Annona muricata leaf extracts, RAD: Radiation, AME+RAD: Radiation and AME*

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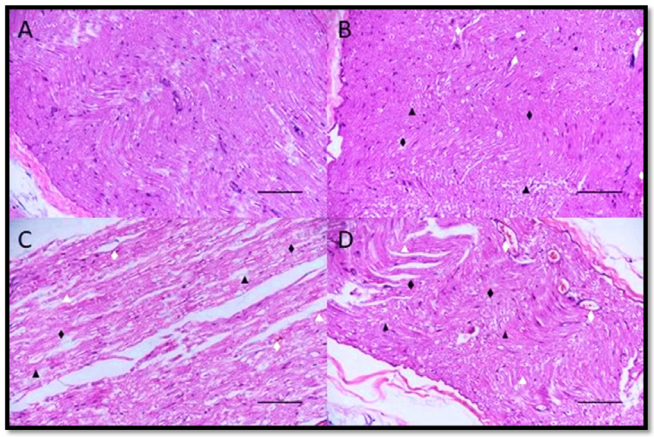

Figure 2. *(A) Histomorphologic findings of Group C. There are no vasculature changes, degenerative change, necrotic change, swelling in myelin sheath. H&E, Scale Bar: 100 µm. (B) Histopathological findings of Group AME. White diamond, light vasculature changes; black diamond light degenerative change; black triangle, light swelling in myelin sheath. H&E, Scale Bar: 100 µm. (C) Histopathological findings of Group RAD. White diamond, medium vasculature changes; black diamond heavy degenerative change; white triangle, heavy necrotic change; black triangle, heavy swelling in myelin sheath. H&E, Scale Bar: 100 µm. (D) Histopathological findings of Group AME+RAD. White diamond, medium vasculature changes; black diamond light degenerative change; white triangle, light necrotic change; black triangle, light swelling in myelin sheath. H&E, Scale Bar: 100 µm.*

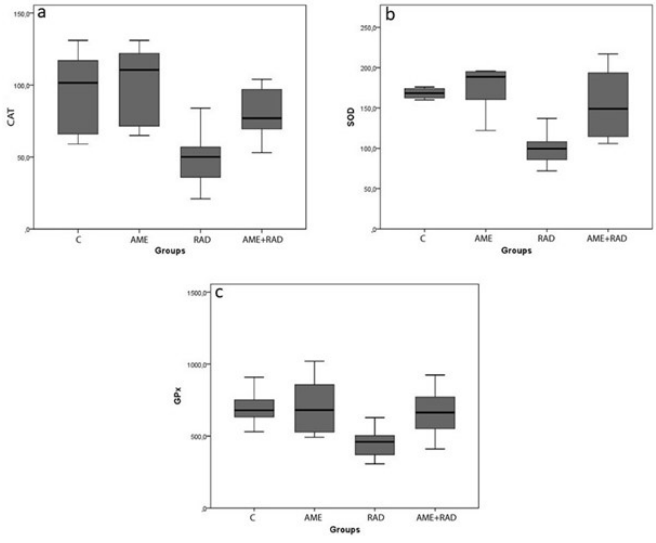

Figure 3. *Q1, Q3, median, minimum, and maximum values of CAT, SOD and GPx are resented with box plot. (a) Levels of plasma CAT (U/ml), (b) Levels of plasma SOD (U/ml), (c) Levels of plasma GPx (U/ml) in groups*

4. DISCUSSION

Due to technical advances in radiation therapy, RT has contributed significantly to cancer patients' recovery or prolonged survival [25]. RT mainly targets tumour cells; however, when irradiating the tumour with the appropriate dose, it is often impossible not to affect the surrounding tissues. RIPND is the least known and perhaps the most frightening of the late complications of RT [10]. This is because permanent damage (sensory and/or motor) may occur in the extremities both in the early stages of the disease and even years after its remission. Since, clinical results are generally irreversible in RIPND, it significantly increases morbidity. Therefore, it adversely affects the quality of life, especially in patients who have successfully fought against cancer. At this point, although, many experimental and clinical studies have been carried out from the past to the present, a radioprotective agent that can prevent RIPND has not been found. Studies on the neurotoxic effect of IR have mainly focused on the central nervous system (CNS). Egemen et al., compared the efficacy of dexamethasone and melatonin in IR induced brain and spinal cord injury [26]. As a result, they observed that the effects of melatonin to dexamethasone were equivalent and not superior to each other. Presman et al., showed that melatonin could increase sensitivity to glucocorticoids [27]. Based on the results of this study, Egemen et al. concluded that further studies are needed with the assumption that the two agents will be more effective when used together. However, these studies remained only in the experimental stage and have not been put into clinical practice. Based on our literature search, the radioprotective effects of post-radiation AM against sciatic nerve injury have not yet been evaluated in an experimental rat model before. In this study, we assessed the possible radioprotective efficacy of AM both biochemically and histopathologically.

The reason why the neurotoxic effect of IR is limited to the CNS; whether IR causes toxicity in peripheral neurons is not yet clear. It is widely believed that there are two phases. The first is the phase consisting of bioelectrical, biochemical and histochemical changes and the second consists of late fibrosis [23]. In this context, we evaluated biochemically SOD, CAT, GPx and histopathologically vascular, degenerative and necrotic changes and myelin hair changes. However, an important problem here is that the peripheral nerves are resistant to radiation [23,28]. We observed no consensus on the sciatic nerve in the literature review we did before we began our work on which will lead to the damage of the radiation intensity [10,29]. Okuhara et al., observed no significant change in sciatic nerve function for 24 weeks, even though different intensities such as 30, 50, 70 Gy were given in their preliminary evaluation before starting their studies on RIPND [29]. However, they observed no significant changes in sciatic nerve function at these doses despite histopathological changes [29]. For this reason, they increased the dose and carried out their studies with a dose of 90 Gy. They observed the desired electrophysiological and histological changes at this dose. Shabeeb et al., in their studies evaluating the radioprotective effects of melatonin, observed significant changes related to sciatic nerve damage with lower radiation (30 Gy), in contrast to this study [10]. Unlike these two studies, we observed significant changes related to nerve damage, both histomorphologically and biochemically, with 20 Gy radiation.

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AM is a member of the Annonaceae family, also known as 'Graviola', 'soursop' or 'corossol'. It is suitable for many diseases (such as fever, malaria, diabetes, rheumatism, various cancers) by local people in tropical regions; branches, bark, leaves, seeds and root parts are processed through various processes [18]. AM is known to have anticarcinogenic, anticonvulsant, anti-inflammatory, antioxidant, radioprotective (against gamma rays) and neuroprotective properties [20,30-32]. However, there are limited studies on the radioprotective effect of AM in the literature [30,33,34]. In addition, these studies were made with gamma-ray, not X-ray. El-Shahat et al., examined the biochemical changes in their studies with whole-body gamma irradiation at a dose of 2 Gy (8 Gy in total) every three days [30]. They observed that AM had an antioxidant effect against biochemical changes in the liver and kidneys. Mansour et al., showed that AM reduces radiation-induced toxicity by preventing oxidative stress and preserving antioxidant activities in lung and kidney tissues in experimental whole-body gamma irradiation (6 Gy) models [33]. We performed our study by applying a 20 Gy X-ray to the right sciatic nerves of rats. Our study is the first to evaluate the radioprotective effects of AM using X-ray. In our research, we observed that in addition to the reduction of oxidative stress caused by radiation, necrotic and degenerative changes were less in the group AME+ RAD (Figures 2 and 3).

The presence of a suitable agent with a radioprotective effect is essential to prevent the development of peripheral neuropathy and neoplasms that may occur due to IR [35]. However, neuropathic pain and motor losses due to possible nerve damage cause additional costs in terms of economy as well as negative patient psychology. Therefore, the main goal should be to protect the surrounding tissues from necrotic and/or apoptotic processes occurring in cancer cells in a patient receiving RT. Therefore, in vivo (melatonin, dexamethasone) and in vitro studies aimed to investigate whether neurotoxicity caused by ROS and DNA damage in IR-induced neurons can be prevented [26,36]. Antioxidant enzymes such as SOD, CAT and GPx play an active role in protecting against the harmful effects of oxidative stress-related ROS, hydrogen peroxide and lipid peroxidation [36]. High intracellular ROS levels can cause nerve cell death via apoptosis and/or necrosis [36,37]. The first defence mechanism against these harmful effects due to oxidative stress is provided by SOD. SOD neutralises ROS to the less toxic compound hydrogen peroxide (H_2O_2) [20]. However, excessive cellular H_2O_2 accumulation increases oxidative damage by causing the formation of reactive free hydroxyl (OH-) radicals [38-40]. The CAT enzyme reduces this damage by converting H_2O_2 to water and oxygen [41]. Another important antioxidant enzyme, GPx, reduces lipid peroxides to hydroxyl lipids and waters by converting glutathione to glutathione disulphide [41].

There are studies in the literature on the antioxidant, anti-inflammatory and neuroprotective effects of both crude extracts and phytochemical compounds of AM [30-32]. Moghadamtousi et al., examined the effects of AME on wound healing in an experimental injury model. They showed that it accelerated the stages of wound healing and increased CAT, SOD and GPx activities and reduced CO oxidative stress [31]. In addition, in another study by Moghadamtousi et al., they showed that AME also increased CAT, SOD and GPx activity in an experimental gastric injury model caused by ethanol [42]. The results of our study showed that 20 Gy radiation exposure of the sciatic nerves of rats (RAD group) caused an increase in oxidative stress in parallel with the decreased activity of antioxidant enzymes (SOD, CAT and GPx). However, there was a statistically significant increase in SOD, CAT and GPx activity in the AME+RAD group compared to the RAD group (Table I, Figure 3).

Histomorphological evaluations remain the most widely used descriptive option in studies. In this context, histomorphological evaluations in previous RIPND studies have shown that IR-induced neuronal damage followed by neuropathic changes secondary to fibrosis with necrosis [10,22,24]. Shabeeb et al., showed increased inflammatory changes in the radiation group 4 weeks after irradiation in radiation-induced sciatic nerve injury studies [10]. Normal histomorphological results were observed in the C and AME groups in our study. After IR, moderate and severe swelling of the myelin sheath and increased inflammatory changes were observed in the RAD group compared to group C (Figure 2C, Table II). However, histomorphological, vascular, degenerative and necrotic changes and swelling in the myelin sheath were significantly reduced in the AME+RAD group compared to the RAD group (Table II). In conclusion, in this study, the histomorphological results of the RAD group showed moderate sciatic nerve damage; however, AME has been observed to alleviate neurotoxic effects.

Limitations and further studies

In some studies histomorphological changes in RIPND were observed only in the 1st month, as in our study; but there are studies in which histomorphological changes were evaluated at 12, 20 weeks [10,22]. These periods are of great importance, especially in assessing the late fibrosis seen in the second phase of RIPND. In addition, unlike our study, there are studies with higher radiation intensity and follow-up periods of up to 24 weeks [29]. This provides a better assessment of sciatic nerve functional losses. In this context, evaluation of RIPND with different histomorphological and electrophysiological protocols, different intensities of radiation doses and longer sacrification times will strengthen the results obtained from the study.

Conclusion

This study showed that AME could ameliorate the histomorphological and biochemical changes of the irradiated sciatic nerve. However, despite these positive results, it should go a long way in terms of its effects on RIPND.

Compliance with the Ethical Standards

Ethics Approval: All experimental procedures were carried out in the Animal Experiments Laboratory of Bülent Ecevit University Faculty of Medicine. Approval was obtained from the local ethics committee of the university (decree no: 2020/06). All procedures performed in studies involving animals followed the institution's ethical standards or practice at which the studies

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were conducted. This article does not contain any studies with human participants performed by any authors.

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Author Contributions: EK: Concept, EK, HHKS and AG: Design, CT: Supervision, EK: Resources, EK, HHKS and AG: Data collection and/or processing, EK, CT: Analysis and/or interpretation, EK, CT and AG: Literature review, EK: Writing, CT and HHKS: Critical review.

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