# The Neuroprotective and Anti-Inflammatory Effects of Annona Muricata (Graviola) on Radiation-Induced Rat Sciatic Nerve Injury.

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**Research Article**

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# Abstract

**Background:** In radiotherapy (RT) exposure area, normal tissues are also affected that may cause serious complications in the patients. This study aimed to evaluate Annona muricata's radioprotective effects on sciatic nerve injury due to ionizing radiation (IR).

**Methods and Results:** 32 adult female Wistar albino rats separated into 4 equal groups; Control (C), Annona muricata leaf extracts (AME), radiation (RAD), radiation and Annona muricata leaf extracts (AME+RAD). In groups AME and AME+RAD, Annona muricata leaf extracts were administered at a dose of 300 mg/kg for the first day and 50 mg/kg everyday for following one week intraperitoneally. In RAD and AME+RAD, rats were exposed to a single dose of 20 Gray IR to their right legs. All the subjects were sacrified at the end of the first month. Oxidative stress biochemical parameters (SOD, CAT and GPx) from blood samples were analyzed. Right sciatic nerves extracted and histomorphology evaluated. 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 CAT ($p<0,01$), SOD ($p<0,01$) and GPx ($p<0,05$) in AME+RAD group compared to RAD group were found to be statistically significant.

**Conclusion:** Our study unveiled that AM could have a potential of biochemically and histomorphology healing on sciatic nerve injury due to ionizing radiation.


# 1. Introduction

New treatment modalities for cancer cure or survival prolongation are widely used in clinics all over the world. Further, radiotherapy (RT) keeps itself as a beneficial treatment option in many types of cancer [2]. However, serious complications may develop in patients due to the effects of both chemotherapeutic agents and RT on healthy tissues other than tumor tissue [2, 7]. These complications may occur acutely, subacutely and chronically, depending on the tolerance of the tumor to which it is applied and the tolerance of the normal tissue to RT. 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 ionizing radiation (IR) [1, 9, 23, 27, 33, 37]. The fact that this neurological damage results in permanent and heavy losses may cause the patient to face an additional stress and a situation that impairs quality of life, in addition to the psychological destruction caused by cancer treatment.

The degree of neuron damage due to IR is closely related to early biochemical and histomorphological changes and late scar formation [16, 38]. The mechanism of all these changes occurs due to the process that results in cell death due to DNA damage. The main responsible of lethal damage to DNA is nitrogen (RNS) and reactive oxygen (ROS) species and free radicals in the acute period due to the ionization of water, with an indirect mechanism rather than the damage caused by direct ionization in DNA [3, 18]. Superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) are antioxidant enzymes

that protect cells against free radical damage [38]. Since the mid-19th century, many chemical compounds, including antioxidant agents, have been used to reduce these side effects of IR [11, 15, 25, 38].

*Annona muricata* (AM) (Graviola), This tropical tree from the *Annonaceae* family consists of evergreen dark green leaves and heart-shaped fruits [19]. It is very common to consume this fruit, which is called a 'cancer killer' by the local people in tropical regions, by boiling the leaves in the treatment of cancer [19, 30]. In addition, AM, which contains many flavonoids, alkaloids and acetogens in its leaves, is also used for the management of infections, diarrhea, dermatitis, diabetes, fever and cardiovascular problems [8]. In support of this traditional use, various studies have shown the anticancer, antioxidant, antiviral, antihemolytic, sedative and neuroprotective effects of AM [4].

Radiation-induced peripheral nerve damage (RIPND) is permanent since nerve cells do not have the regeneration ability [5, 24]. Although necessary precautions are taken to prevent the development of RIPND, there is no effective treatment in case it does occur, and the treatment options to restore the patients' standarts of living are limited [40]. The incidence of RIPND keeps increasing in correlation with the use of aggressive RT programs. In this study, we evaluated AM's radioprotective effect on the changes in the acute period of damage to the rat sciatic nerve due to IR.

# 2. Materials And Methods

### 2.1. 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 (decree no: 2020/06). A total of 32 *Wistar albino* female rats, weighing 350-450 g, were randomly seperated 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). Sample size was determined by using G Power. The animals were kept below 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.

Rats in group C were injected intraperitoneally (IP) with 2ml/kg of normal saline per day for 1 week. In the AME group, 300 mg/kg AME was administered IP on the first day and 50 mg/kg AME every day for 7 days. Rats in the RAD and AME+RAD group exposed only one single IR dose (20 Gy) to their right legs without any treatment. The rats in AME+RAD group were given 300 mg/kg AME ip 30 minutes before IR application and 50 mg/kg AME IP every day for 7 days after the application. After 1 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) anesthesia for biochemical examination from the tail arteries. Blood samples were taken, after which all rats were sacrificed with high-dose anesthetic 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).

### 2.2. Irradiation

The rats in the AME+RAD and RAD groups were anesthetized 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; dose was calculated with Eclipse treatment planning system, version 8.9 (Varian Medical Systems, Palo Alto-CA, USA). After the simulation, other parts of their bodies were protected using lead and beam collimation. 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 [26]. No rat was excluded due to death.

### 2.3. Chemical

Air-dried AM (Graviola) leaves were obtained from a local supplier in the Mediterranean region in Turkey. They cut into small pieces after washing with distilled water. 25 g samples 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 lyophilized (Telstar- LyoQuest, Spain) overnight to prepare a dry extract, which was kept at -20 °C until use.

### 2.4. Biochemical analysis

#### Superoxide Dismutase (SOD, U/ml)

Superoxide dismutase accelerates the conversion to hydrogen peroxide and molecular oxygen in the elimination steps of reactive oxygen radicals produced in oxidative reactions. This method takes place by means of 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 Superoxide dismutase activity. (Relassay, Turkey)

#### Catalase (CAT, U/ml)

This colorimetric assay involves two steps. The sample is incubated with a certain hydrogen peroxide. hydrogen peroxide is converted to water and oxygen by this sample. catalase concentration is proportional to this ratio. Enzyme plant and a fixed incubation period are completed. The peroxide remaining after this is determined as a chromogen appearance. Absorbances at 405 nm are expressed in U/ml. (Relassay, Turkey)

#### Glutathione Peroxidase (GPx, U/ml)

This method is based on the Paglia and Valentine method. GPx catalyzes 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)

### 2.5. Histomorphologic examination

Automated tissue processing equipment (Leica ASP300S, Wetzlar, Germany) was used for 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. Tissue sections stained with Hematoxylin-Eosin (HE) were evaluated under the microscope. The same procedure was performed for all samples. The camera used for the observation of all study materials was 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. Scoring is scored between 0 and 3 for each criterion. (0=normal, 1=Light, 2=medium, 3=heavy).

### 2.6. Statistical analysis

SPSS version 22.0 was used to analyze 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. For categorical variables, the differences between the groups were evaluated using Chi-Square analysis. Based on the p value <0.05, the results were considered statistically significant.

## 3. Results

### 3.1. Biochemical evaluation

Plasma CAT, SOD and GPx values in all the experimental groups are presented in Table 1. 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 1, 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 1, Figure 3) increased significantly compared to the RAD group. Similarly, there was a significant difference between the CAT, SOD and GPx levels of the AME and RAD groups ($p<0.05$, Table 1, Figure 3).

### 3.2. Histomorphologic evaluation

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

Increase of vasculature and degenerative changes in the RAD group found to be statistically significant compared to the C and AME groups ($p<0,001$, Table 2, Figure 2C). In the AME+RAD group, there was statistically significant increase in these changes compared to the RAD group ($p<0.001$, Table 2, Figure 2D). While necrotic change was not observed at all 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 2, Figure 2D) Swelling in myelin sheath was observed in the rats in the most severe RAD group, while it was observed at the lowest level in the rats 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 2).

# 4. Discussion

Due to technical advances in radiation therapy, RT has made significant contributions to the recovery or prolongation of survival of cancer patients [13]. RT mainly targets tumor cells; however, when applying radiation to the tumoral area 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 [38]. 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 and adversely affects quality of life, especially in patients who have been successful in the fight 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. 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.

The pathology of RIPND is not clear; It is widely believed that there are two phases. The first of these is the phase consisting of bioelectrical, biochemical and histochemical changes, and the second is the phase consisting of late fibrosis [40]. Many experimental studies have shown that peripheral nerves are significantly resistant to radiation [40, 41]. We observed that there was no consensus on the sciatic nerve in the literature review we did before we begin our work on which will lead to the damage of the radiation intensity [32, 38]. Okuhara et al. observed that there was 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 [32]. They observed the desired electrophysiological and histological changes by performing their studies with the maximum dose of 90 Gy. Shabeeb et al. evaluated the radioprotective effects of melatonin in their sciatic nerve damage studies with 30 Gy-intensity radiation [38]. In experimental studies, histological changes are observed after 4,12 or 20 weeks in general; electrophysiological changes were observed only after 12 and 20 weeks [32, 38]. In this

context, we observed that there was no functional change in the sciatic nerve 1 month after using 20 Gy radiation in our study. However, similar to the literature, we observed statistically significant changes in our AME+RAD group compared to the RAD group, both histomorphologically and biochemically (Tables 1 and 2).

AM is a member of the Annonaceae family, also known as 'graviola', 'soursop' or 'corossol'. Considering that it is good 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 [8]. Apart from this traditional use, it has been shown that the acetogenins contained in the AM leaf have selective toxic effects against cancer cells [35, 36]. Similarly, there are studies on the content of alkaloids and flvanoids with strong antioxidant effects [20, 39]. In this study, we investigated the radioprotective effect of AME as an agent shown to have anticarcinogenic, anticonvulsant, anti-inflammatory, antioxidant, radioprotective (against Gamma-ray) and neuroprotective properties [10, 22, 28]. There are only a few studies on the radioprotective effect of AM in the literature [10, 17, 21]. However, these studies were not done with X-ray; The radioprotective effect of AME against gamma-ray damage was examined. Mansour et al. showed that AME 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 [17]. We performed our study by applying 20 Gy X-ray to the right sciatic nerves of rats. This is the first study to look at the radioprotective effects of AM using dose and X-ray. In the results of our study, we observed that in addition to the reduction of oxidative stress caused by radiation, necrotic and degenerative changes were less in the group treated with AME (Figures 2 and 3).

In the presence of a suitable agent with a radioprotective effect, surrounding tissues due to necrotic and/or apoptotic processes occurring in cancer cells after RT can be protected [31]. For this reason, AM, a tropical plant, can have a radioprotective effect without any toxic effect with its antioxidant and anti-inflammatory effects. 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. High intracellular ROS levels can cause nerve cell death via apoptosis and/or necrosis [14, 42]. The first defense mechanism against these harmful effects due to oxidative stress is provided by SOD. SOD neutralizes ROS to the less harmful compound hydrogen peroxide ($H_2O_2$) [22]. However, excessive cellular $H_2O_2$ accumulation increases oxidative damage by causing the formation of reactive free hydroxyl (OH-) radical [34, 43]. The CAT enzyme reduces this damage by converting $H_2O_2$ to water and oxygen [12]. Another important antioxidant enzyme, GPx, reduces lipid peroxides to hydroxyl lipids and waters through the conversion of glutathione to glutathione disulfide. There are studies in the literature on the antioxidant and anti-inflammatory effects of both crude extracts and phytochemical compounds of AM. In one study, AM was more effective in increasing antioxidants (SOD, CAT, and GPx) than leaf and stem bark, fruit and root bark methanolic extracts [6]. 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 as well as increased CAT, SOD and GPx activities and reduced oxidative stress [28]. 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 [29]. Our results 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 1, Figure 3).

Histomorphological studies are the most widely used descriptive approach. In the literature, histomorphological results were normal in C and AME groups in our study. After irradiation, 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 2). However, histomorphologically, vascular, degenerative and necrotic changes and swelling in the myelin sheath were found to be statistically significantly reduced in the AME+RAD group compared to the RAD group (Table 2). Shabeeb et al. investigated the radioprotective effects of melatonin after radiation-induced sciatic nerve injury [38]. In their study, they showed that inflammatory changes increased in the radiation group 4 weeks after irradiation. Our histomorphological evaluations are consistent with the results of this study. In conclusion, in this study, the histomorphological results of the RAD group showed partial sciatic nerve damage; however, AME has been shown to improve these effects.

### Limitations and further studies

Histomorphological changes in RIPND are not only in the 1st month, as in our study; There are studies in which it was evaluated at 12, 20 and 24 weeks. These periods are of great importance especially in terms of the evaluation of late fibrosis seen in the second phase of RIPND. However, we did not observe any functional changes due to sciatic nerve damage in our study, due to reasons that we think are related to the termination time of our study, the intensity of the applied radiation and the single dose. However, unlike our study in the literature, functional losses were observed in the sciatic nerve in studies in which higher radiation intensity was applied and follow-up periods of up to 24 weeks. In this context, it will be possible to evaluate the findings related to different histomorphological changes and functional losses by extending the period of termination of our study to at least 6 months. However, at this point, it was not possible to carry out our study in more than 1 month because the available resources were not sufficient for the rats to live longer. Despite all these shortcomings, with the experience we gained from this study, it will be possible to expand it to investigate RIPND with different histomorphological and electrophysiological protocols and radiation doses of different intensities.

## 5. Conclusion

The results of this study showed that AME has the potential to ameliorate the histomorphological and biochemical changes of the irradiated sciatic nerve. However, despite these positive results, it should be able to go a long way in terms of its effects on radiation-induced peripheral nerve damage.

## Declarations

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## Conflicts of Interest/Competing Interests

All authors certify that they have no affliations with/or invasion in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membreship, employment, consltancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or Professional relationships, affliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

## Availability of Data

The authors confirm that the data supporting the findings of this study are available within the article [and/or] its supplementary materials.

## Code Availability

Not applicable

## 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 proedures performed in studies involving animals were in accordance with the ethical standarts of the institution or practice at which the studies were conducted. This article does not contain any studies with human participants performed by any of the authors.

## Consent to Participate

Not applicable

## Consent for Publication

Not applicable

## Authors' Contributions

**Author 1:** Emrah Keskin

- Concieved and designed the analysis
- Contributed data or analysis tools

**Author 2:** Özlem Elmas

- Contributed data or analysis tools
- Performed the analysis

**Author 3:** Havva Hande Keser Şahin

- Contributed data or analysis tools
- Performed the analysis

**Author 4:** Çağhan Tönge

- Collected data
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- Collected the data
- Contributed data or analysis tools

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# Tables

Table 1
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 RAD groups (P < 0.05). | | | | | |
| [b]Shows significant differences between RAD and AME groups (P < 0.05). | | | | | |
| [c]Shows significant differences between RAD and AME + RAD groups (P < 0.05). | | | | | |

Table 2
Comparison between all groups' histomorphologic scores.

| 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

# Figures

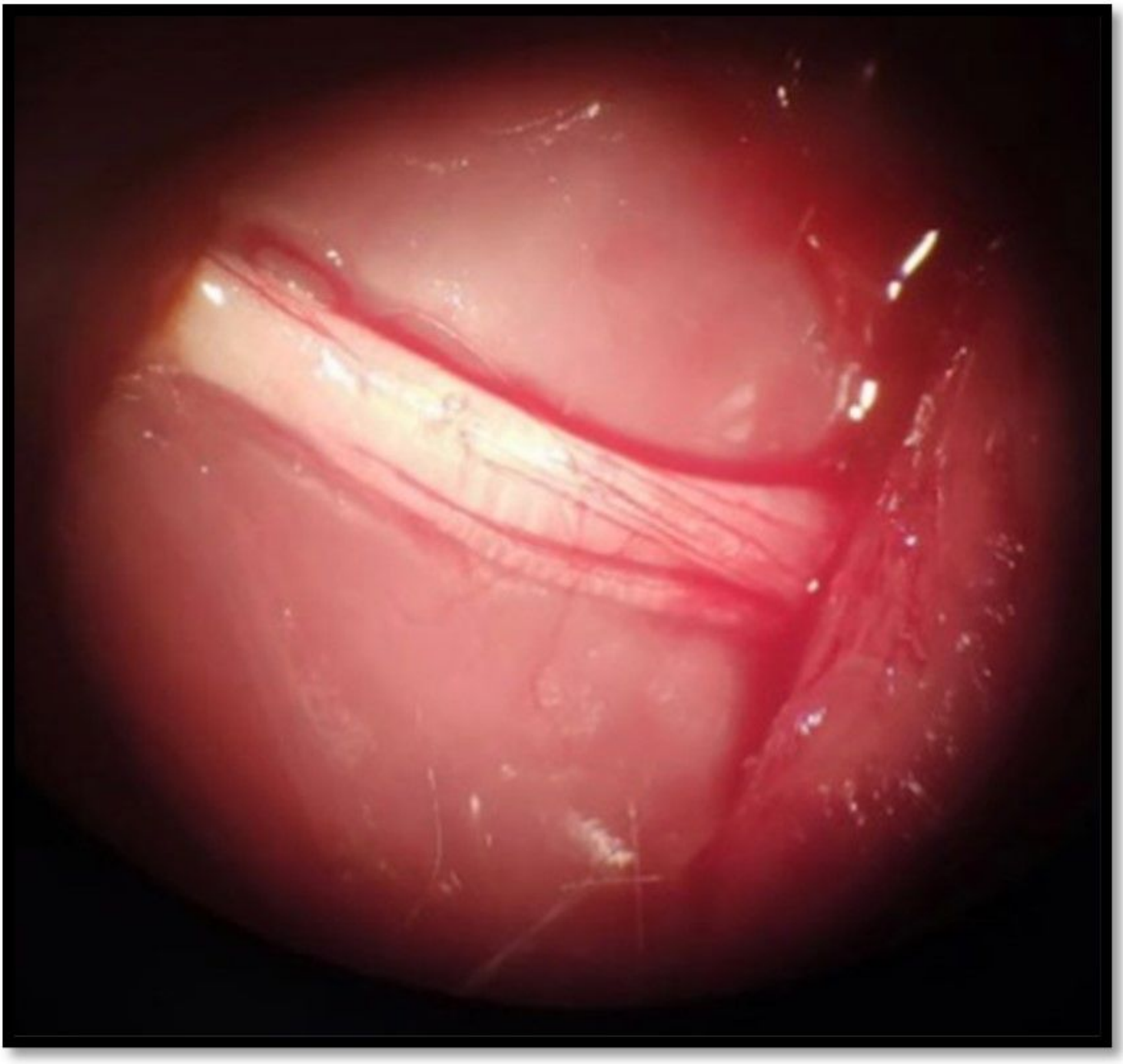


**Figure 1**

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

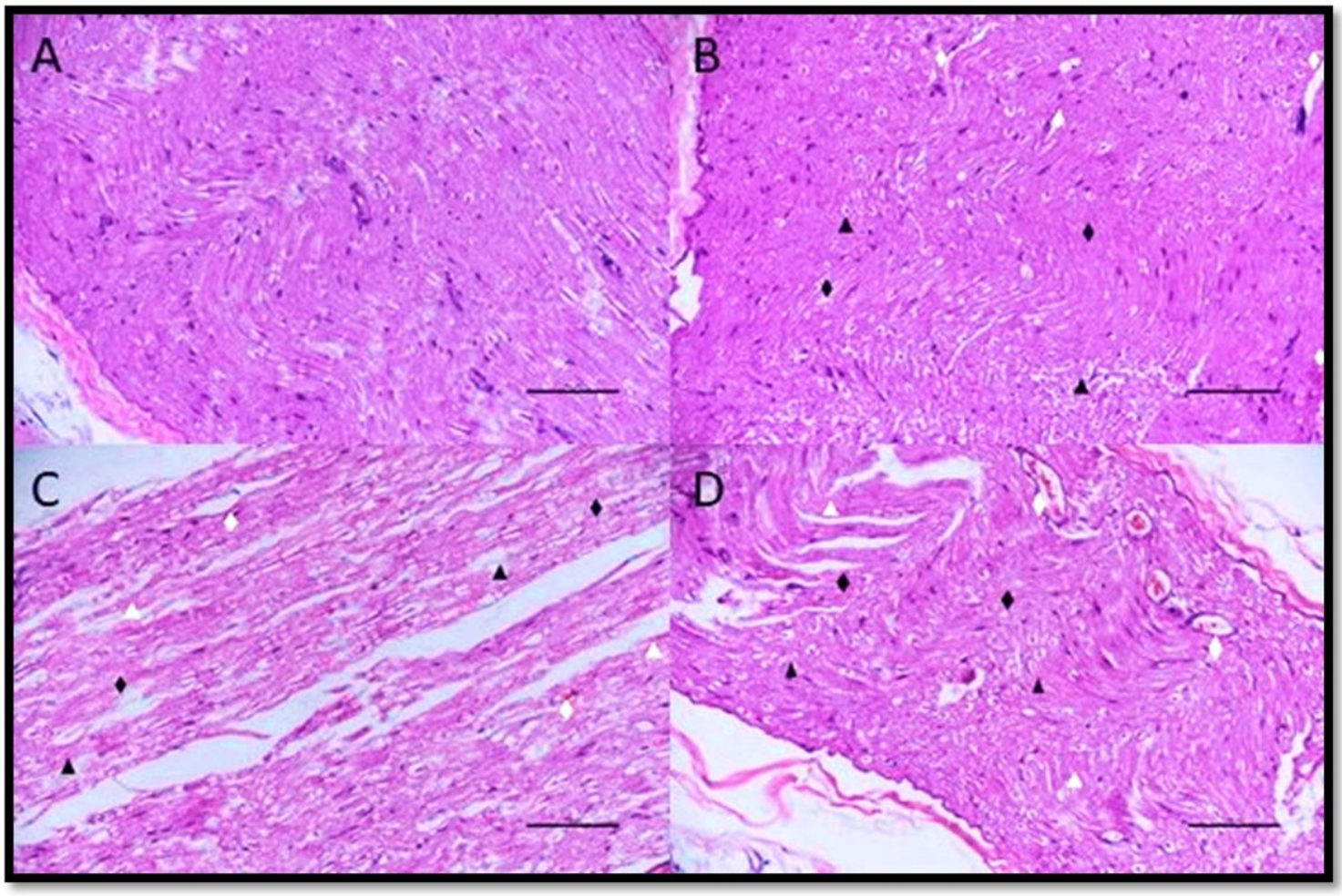
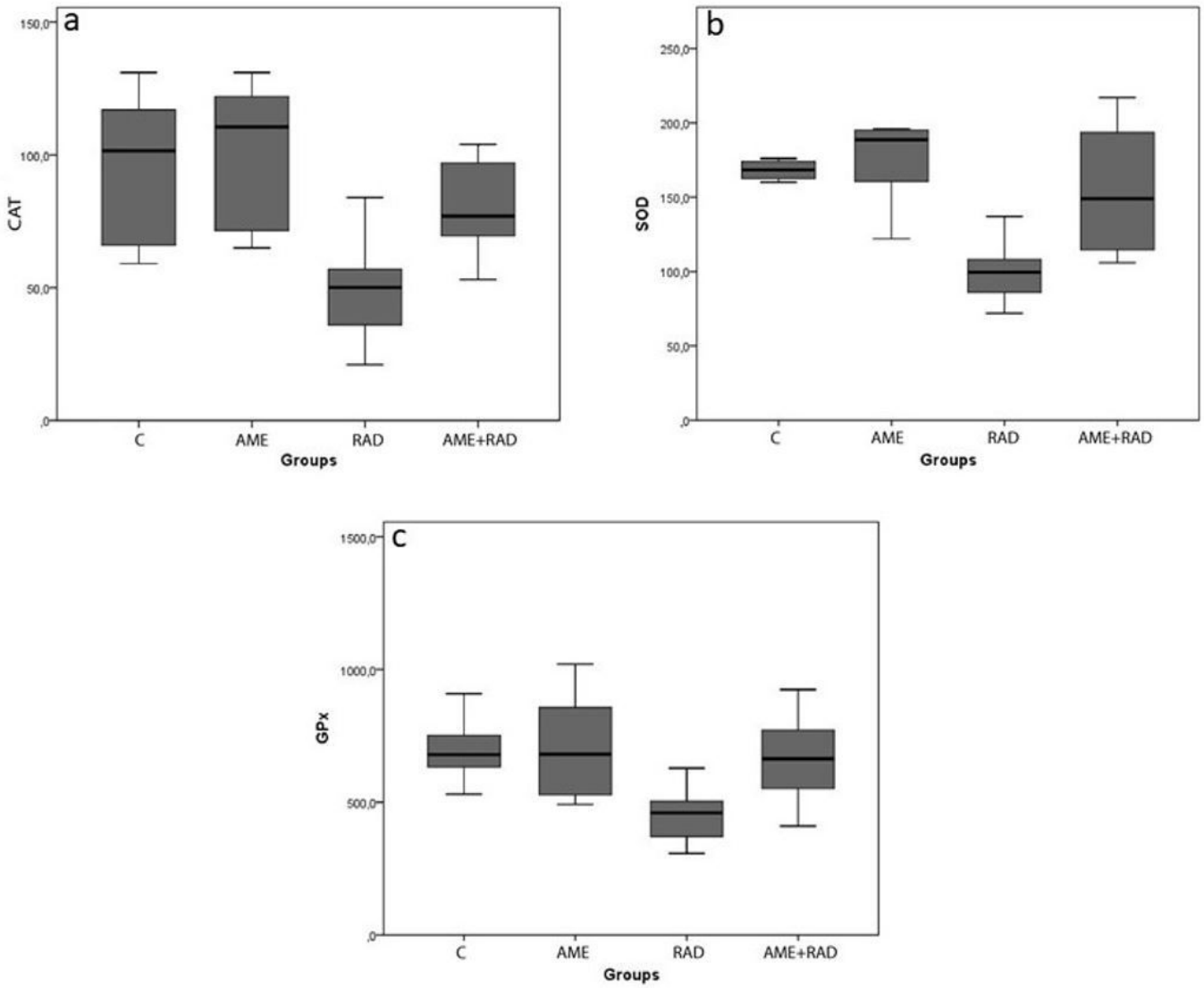



**Figure 2**

(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.