ORIGINAL ARTICLE

THE PROTECTIVE EFFECT OF ORIGANUM MAJORANA ESSENTIAL OIL ON GENTAMICIN-INDUCED NEPHROTOXICITY IN RATS

Origanum Majorana Esansiyel Yağının Sıçanlarda Gentamisin Kaynaklı Nefrotoksisite Üzerindeki Koruyucu Etkisi

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Geliş tarihi/Received: 21.03.2021
Kabul tarihi/Accepted: 25.05.2021
DOI: 10.16919/bozoktip.900785

Bozok Tıp Derg 2022;12(2): 30-37
Bozok Med J 2022;12(2): 30-37

ABSTRACT

Objective: Gentamicin (GM) is an aminoglycoside antibiotic used in gram-negative bacterial infection. The use of gentamicin causes nephrotoxicity. Origanum majorana (OM) a plant, has powerful antioxidant and anti-inflammatory properties. The aim of this study is to determine the protective effects of OM on GM-induced nephrotoxicity in rats.

Material and Methods: Rats were divided into 6 groups (n=8): The Control group: was given olive oil, OM (0.32 ml/kg) group: was given by gavage OM (0.32 ml/kg) for 28 days, OM (0.64 ml/kg) group: was given by gavage OM (0.64 ml/kg) for 28 days, GM group: was given 100 mg/kg/day intraperitoneally GM from the 21st to the 28th day, OM (0.32 ml/kg) +GM group: was given by gavage OM (0.32 ml/kg) for 28 days and was given 100 mg/kg/day intraperitoneally GM from the 21st to the 28th day, OM (0.64 ml/kg)+GM group: was given by gavage OM (0.64 ml/kg) for 28 days and was given 100 mg/kg/day intraperitoneally GM from the 21st to the 28th day. Kidney tissues were evaluated histopathologically. The TUNEL method was used for evaluation of the apoptotic index. Superoxide dismutase (SOD), catalase (CAT), and malondialdehyde (MDA) levels in the kidney tissue were measured by ELISA assay.

Results: Histopathological damage, TUNEL positive cell count and MDA level increased in the GM group compared to the Control group. Moreover, SOD and CAT levels decreased in the GM group. There was a decrease TUNEL positive cell count in OM (0.32 ml/kg) +GM and OM (0.64ml/kg) +GM groups compared to GM group.

Conclusion: As a result, OM can be used as a protective agent to reduce kidney damage by suppressing apoptotic pathways and by increasing antioxidant capacity.

Keywords: *Gentamicin; Origanum Majorana; Oxidative Stress; Kidney Damage.*

ÖZET

Amaç: Gentamisin (GM), gram-negatif bakteriyel enfeksiyonda kullanılan bir aminoglikozid antibiyotiktir. Fakat GM kullanımı nefrotoksisiteye neden olmaktadır. Origanum majorana (OM), güçlü antioksidan ve anti-inflamatuvar özelliklere sahip bir bitkidir. Bu çalışmanın amacı, OM'nin sıçanlarda GM kaynaklı nefrotoksisite üzerindeki koruyucu etkilerini belirlenmesidir.

Gereç ve Yöntemler: Bu amaçla, sıçanlar 6 gruba ayrıldı (n = 8): Kontrol grubu: Sadece zeytinyağı verilen grup, OM (0,32 ml/kg) grubu: 28 gün boyunca OM (0,32 ml/kg/gavaj) verilen grup, OM (0,64 ml/kg) grubu: 28 gün boyunca OM (0,64 ml/kg/gavaj) verilen grup, GM grubu: 21-28. günler arasında 100 mg/kg/gün/i.p. GM verilen grup, OM (0,32 ml/kg) + GM grubu: 28 gün boyunca OM (0,32 ml/kg/gavaj) ile 21-28. günler arasında 100 mg/kg/gün/i.p. GM verilen grup, OM (0,64 ml/kg) + GM grubu: 28 gün boyunca OM (0,64 ml/kg) ve 21. günden 28. güne kadar 100 mg/kg/gün/i.p GM verilen grup. Böbrek dokuları histopatolojik olarak değerlendirildi. Apoptotik indeksin değerlendirilmesi için TUNEL yöntemi kullanıldı. Böbrek dokusundaki süperoksit dismutaz (SOD), katalaz (CAT) ve malondialdehit (MDA) seviyeleri ELISA testi ile belirlendi.

Bulgular: GM grubunda histopatolojik hasar, TUNEL pozitif hücre sayısı ve MDA düzeyi kontrol grubuna göre arttı. Ayrıca, GM grubunda SOD ve CAT seviyeleri azaldı. OM (0,32 ml / kg) + GM ve OM (0,64ml / kg) + GM gruplarında GM grubuna göre TUNEL pozitif hücre sayısında azalma oldu.

Sonuç: Sonuç olarak OM, apoptotik yolları baskılayarak ve antioksidan kapasiteyi artırarak böbrek hasarını azaltmak için koruyucu bir ajan olarak kullanılabilir.

Anahtar Kelimeler: *Gentamisin; Origanum Majorana; Oksidatif Stres; Böbrek Hasarı.*

INTRODUCTION

Aminoglycoside antibiotics are frequently used in serious infections of the abdomen and urinary tract. One of these antibiotics is Gentamicin (GM). GM is still considered an important aminoglycoside group antibiotic in the treatment of life-threatening gram-negative bacterial infections. On the other hand, besides being used effectively in the clinic for a long time, its nephrotoxic effect remains an important problem (1). The nephrotoxic effect of GM is caused by the proximal accumulation in the kidneys, and 'tubular necrosis' occurs, and then an increase in plasma creatinine and blood urea levels are observed (2). It has been demonstrated in experimental studies that kidney damage and renal dysfunction occurred in the long-term use of GM (3, 4). GM has been shown to cause apoptosis, inflammation and oxidative stress as well as renal tubular damage (5, 6). GM increases ROS and reduces antioxidant capacity (7).

Reactive oxygen species (ROS) cause the production of hydrogen peroxide (H2O2) and hydroxyl radicals (OH) that can attack DNA, oxidize it and finally trigger apoptotic pathways in both normal and tumoral tissue cells (8). Moreover, oxidative stress leads to lipid peroxidation (LPO) in the cell through the production of ROS. The endogenous antioxidant system (glutathione peroxidase, superoxide dismutase and catalase) aims to neutralize ROS. However, excessive formation of ROS consumes endogenous antioxidants and leads to peroxidation of membrane lipids and increases the oxidation of proteins (9). Formation of excessive ROS, the debilitation of the antioxidant defense system and lipid peroxidation of bio-membranes may cause the upregulation of inflammatory pathways and excessive releasing of pro- and anti-inflammatory cytokines.

Origanum majorana (OM), which is still used today for its therapeutic effects, has been widely used in Anatolia as a spice and to treat various diseases in ancient times. It is stated that carvacrol, which stands out as the main component, plays an important role in antibacterial, antifungal, anthelmintic, insecticidal, analgesic and antioxidant (10, 11). In some research on the protective effect of OM in rat and mouse kidneys; OM has been shown to reduce the damage to kidney tubules (12, 13). The literature review shows that not enough work on OM attracts attention.

In this study, we aimed to investigate the potential therapeutic effects of OM essential oil on GM-induced nephrotoxicity in rats by investigating oxidative stress and apoptosis. For this purpose, significant kidney damage was induced by GM, and OM administrations were performed to eliminate the hazardous effects of GM. Furthermore, histopathological, immunohistochemical, and biochemical changes were evaluated to determine the effects of GM and OM.

MATERIALS AND METHODS

Drugs

Gentamicin

Gentamicin was purchased from a pharmacy as GENTEKS (80 mg ampules), comprising gentamicin sulfate. Any preparation method was not performed for the preparation of the gentamicin because it is sold as a liquid form in ampules and it is ready for the intraperitoneal injection.

Essential Oil Of Origanum Majorana

Origanum majorana essential oil was purchased from Botanika Agricultural Products Cosmetic Food, Oil Industry, and Marketing, which is a company providing agricultural products, along with a report of essential oil components. Report of essential oil components was issued by the Western Mediterranean Agricultural Research Institute Directorate of Food Medicinal and Aromatic Plants Research Laboratory, upon the request of the firm mentioned above dated 14.10.2015, with the number 34 dated 22.10.2015 and the components of Origanum majorana essential oil was given in Table 1.

Experimental Procedure

The experimental protocol of this study was accepted by the Erciyes University's Experimental Animal and Local Ethics' Committee with number 15/154/2015. In this study, all the animals received human care according to standard guidelines. In this study, 48 male Wistar albino rats (9 weeks old, weighing 200-250 gr) were obtained from Hakan Cetinsaya Experimental and Clinic Research Center, Erciyes University, Kayseri, Türkiye. Rats were kept at room temperature (20 - 24 ° C) for 12 hours light/12 hours dark cycle and ambient humidity for the duration of the experiment. Standard

chow and tap water were given to animals ad libitum. At the beginning of the experiment, the rats randomly divided into five groups as follows;

The Control group (n=8): was given olive oil. OM (0.32 ml/kg) group (n=8): was given by gavage OM (0.32 ml/kg) for 28 days. OM (0.64 ml/kg) group (n=8): was given by gavage OM (0.64 ml/kg) for 28 days.GM group (n=8): was given 100 mg/kg/day intraperitoneally GM from the 21st to the 28th day. OM (0.32 ml/kg) +GM group (n=8): was given by gavage OM (0.32 ml/kg) for 28 days and was given 100 mg/kg/day intraperitoneally GM from the 21st to the 28th day.OM (0.64 ml/kg) +GM group (n=8): was given by gavage OM (0.64 ml/kg) for 28 days and was given 100 mg/kg/day intraperitoneally GM from the 21st to the 28th day.

At the end of the experiment, animals were anesthetized with 30 mg/kg ketamine and 4 mg/kg xylazine and they were sacrificed. After sacrifice, kidney tissues were extracted from the animals for the histopathological and TUNEL examinations. For later biochemical assays, tissue samples were kept at -80 °C.

Histopathological Evaluation

Kidney sections of 5-6 µm from paraffin blocks were left in the oven for a certain period of time using histological methods, then paraffin was removed with xylene and passed through graduated alcohol series and diluted. Sections hematoxylin and eosin (H + E) was performed to see the general histological structure. Periodic acid schiff (PAS) staining was performed to investigate the microvilli and basal membrane in tubule epithelium. Sections were stained with hematoxylin and eosin (H&E) and periodic acid Schiff (PAS), photographs were taken with a light microscope (Olympus BX51, Center Valley, PA).

Apoptosis (TUNEL) Staining

Apoptotic cells in the sections taken from the subjects were determined using the Roche brand In Situ Cell Detection Apoptosis Fluorescein Kit. The staining was performed according to the kit procedure. Kidney sections taken at a thickness of 5 µm were deparaffinized and then rehydrated and washed twice with PBS for 5 minutes. Then, 270° C in a microwave oven in 0.01 M 5% sodium citrate buffer for antigen recovery was left for 5 minutes, then allowed to cool at room temperature for 10 minutes. Tissues washed with PBS for 3x5 minutes were then placed in the moisture chamber at 37.0^{C} with the TUNEL reaction mixture coming out of the kit and incubated in the oven for 75 minutes. Tissues washed 2 times for 5 minutes with PBS were contrasted with DAPI (4', 6-diamidino-2-phenylindole). Tissues sealed with glycerol closure solution were visualized on the Olympus BX51 fluorescent microscope at a wavelength of 450-500 nm. For the apoptotic index, apoptotic cells in ten different areas were counted in the 40x objective from each section.

Biochemical Analysis

The kidney tissue superoxide dismutase (SOD), catalase (CAT) and malondialdehyde (MDA) levels were measured by using Enzyme-linked Immunosorbent Assay (ELISA) method. For this purpose, kidney samples

Table 1. Gas Chromatography with Flame Ionization Detection-Mass Spectrometry(GC-MS/FID) analyze of the Origanum majorana essential oil.

Analyze Results (%)					
No	**Name of component**	**Component quantity (%)**	**No**	**Name of component**	**Component quantity (%)**
1	α-Pinene	0.65	**9**	Linalool	6.60
2	α-Thujene	0.86	**10**	Terpinen-4-ol	0.69
3	β-Myrcene	1.41	**11**	βCaryophyllene	1.05
4	α-Terpinene	1.12	**12**	Aromadendrene	0.33
5	β-Phellandrene	0.47	**13**	α-Terpineol	0.65
6	γ–Terpinene	3.64	**14**	Borneol	0.48
7	Cymene	4.38	**15**	Thymol	1.22
8	Trans-Sabinene hydrate	0.28	**16**	**Carvacrol**	**76.17**

stored -80 degrees were used. The samples were homogenized on ice and then centrifuged to remove supernatants. 150 microliters of the supernatant were taken into eppendorf tubes separately for each group. For analysis, Superoxide dismutase (SOD) (Cat. No: 201-11-0169, Sun Red Biological Technology), Catalase (CAT) (Cat. No: 201-11-5106, Sun Red Biological Technology) and Malondialdehyde (MDA) (Cat. No: Cat. No: 201-11-0157, Sun Red Biological Technology) ELISA kits were used. In standard preparation, standard diluent was added to 5 tubes. Then 120 microliters of standard solution were added to the first tube and mixed. After then, 120 microliters from the previous tube were added to the other 4 tubes respectively. 40 microliters of 150 microliters from the supernatant were added to the samples section. Then, we added 10 microliters of antibody to the samples. We added 50 microliters of streptavidin HRP to both samples and standard section. then, incubated 60 minutes at 37 degrees Celsius oven. We washed plate five times and added chromogen solution A, B. We put it in a 37-degree oven for 10 minutes. Afterwards, we added stop solution and measured the optical density (OD) under 450 nm wavelength within 10 minutes.

Statistical Analysis

All statistical analyses were carried out by using GraphPad Prism version 7.00 for Mac, GraphPad Software, La Jolla, California, USA. D'Agostino Pearson omnibus test was used to identify the normal distribution of the data. In the case of normal distribution, quantitative variables were compared using one-way analysis of variance (ANOVA) and Tukey's post-hoc test. Kruskal Wallis test and Tukey's post-hoc test were used for comparing the quantitative with the abnormal distribution. The data were expressed as the mean of normalized data±standard deviation of the mean. $p<0.05$ was considered as statistically significant.

RESULTS

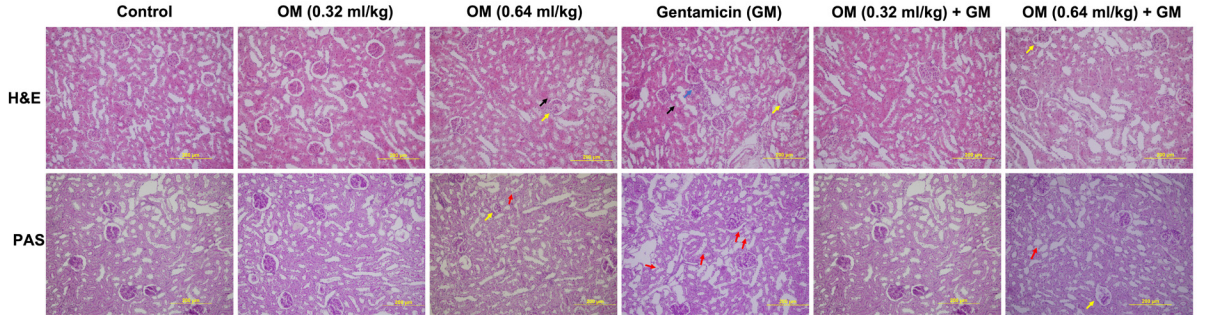
Histopathological Results

Kidney H&E and PAS staining histopathology images are shown in Figure 1. Normal histological structures were observed in the kidney tissue of the control group. In OM (0.32 ml/kg) and OM (0.64 ml/kg) groups, there was no significant difference in kidney tissue from the control group. Glomerular degeneration, proximal tubule dilatation, and mononuclear cell infiltration were seen in the GM group. In addition, when examined with PAS staining, it was observed that basal membrane degeneration in the GM group increased significantly when compared to Control and OM (0.32 ml/kg) groups. Kidney damage decreases in OM (0.32 ml/kg) +GM group. There is also a relative decrease in OM (0.64 ml/kg) +GM group.

OM Ameliorates Gentamicin-Induced Apoptosis

TUNEL staining was performed to determine apoptotic cells in kidney tissue. In the GM group, the number of TUNEL-positive cells increased significantly compared to the Control group ($p<0.05$). There was a significant decrease in the number of TUNEL-positive cells in

Figure 1. H&E and PAS staining in the kidney tissues. In the Control, OM (0.32 ml/kg), OM (0.64 ml/kg), OM (0.32 ml/kg) +GM and OM (0.64 ml/kg) +GM groups is seen healthy kidney tissue. The following structures were seen pathologically in the GM group; Glomerular degeneration (yellow arrow), proximal tubule dilation (black arrow), mononuclear cell infiltration (blue arrow), and basal membrane degeneration (red arrow). Magnification: x200. H&E:Hematoxylin and eosin, PAS:Periodic acid Schiff, GM:gentamicin, OM:origanum majorana



the OM (0.32 ml/kg) +GM and OM (0.64 ml/kg) +GM groups compared to the GM group.

Biochemical Results

The biochemistry results are shown in Table 2 and Figure 3. CAT and SOD activity showed a relative decrease in the GM group compared to the control group. In contrast, in OM (0.32 ml/kg) +GM group, CAT and SOD activity increased relatively compared to the GM group, while OM (0.64 ml/kg) +GM group did not change significantly. MDA level increased in the GM group compared to the Control group. In the OM (0.32 ml/kg) +GM and OM (0.64 ml/kg) +GM groups, MDA level does not differ much compared to GM group.

DISCUSSION

Nephrotoxicity usually results from the use of chemotherapy agents (14). Nephrotoxicity caused by drugs generally develops due to their accumulation in the kidney cortex (15). Gentamicin (GM) is sugar and polycationic antibiotic that is attached to a 6-membered aminocyclitol ring by glycosidic bonds (16). Nephrotoxicity due to aminoglycoside occurs directly in the form of tubular damage and reduced glomerular filtrate ratio. In some studies, it has been shown that aminoglycoside can be directly involved in acute renal failure and membrane lipid peroxidation due to antibiotics (17). GM has been shown to induce apoptosis, oxidative stress and inflammation in kidney tubular epithelial cells (18, 19). Origanum majorana (OM) has a high antioxidant capacity due to its polyphenolic compounds. It has an important place in alternative medicine (20). In this study, we investigated

Table 2. Kidney tissue levels of SOD, CAT and MDA levels among experimental groups.

Groups	Control	OM (0.32 ml/kg)	OM (0.64 ml/kg)	Gentamicin (GM)	OM (0.32 ml/kg) + GM	OM (0.64 ml/kg) + GM	*p*
CAT (ng/mg)	27.78±4.95	30.05±3.81	29.53±7.95	26.73±2.41	30.15±4.34	30.2±8.64	**0.856**
SOD (ng/mg)	4.43±0.67	4.89±0.5	4.26±0.47	3.95±0.56	3.99±0.34	3.99±0.71	**0.046**
MDA (nmol/mg)	1.30±0.45	1.42±0.31	1.54±0.2	1.54±0.27	1.61±0.21	1.55±0.33	**0.567**

All data are expressed as the mean±SD. p <0.05 was considered as significant.
Abbreviations: GM, gentamicin; OM, Origanum majorana; SOD, superoxide dismutase; CAT, catalase; MDA, malondialdehyde.

Figure 2. TUNEL staining of the kidney tissue. Control (n = 8), OM (0.32 ml/kg) (n = 8), and OM (0.64 ml/kg) (n = 8), normal kidney tissue were observed compared to GM group; GM (n = 8) group, TUNEL-positive cells (yellow arrow) were mainly observed in the kidney tissue; OM (0.32 ml/kg) +GM (n=8) and OM (0.64 ml/kg) +GM (n=8) groups, decrease in TUNEL-positive cells was observed. Magnification: x400. GM:gentamicin, OM:origanum majorana

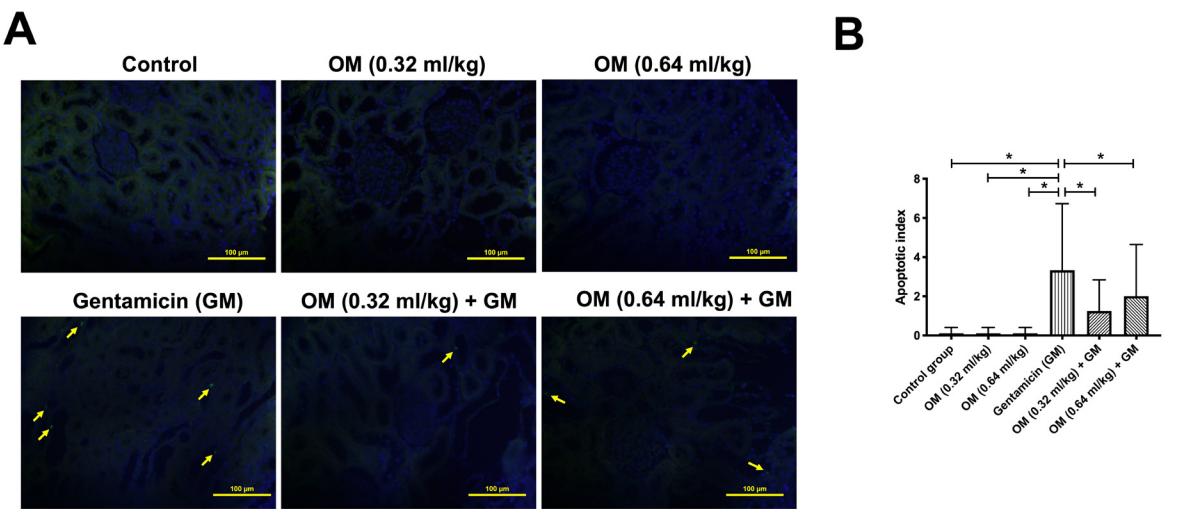


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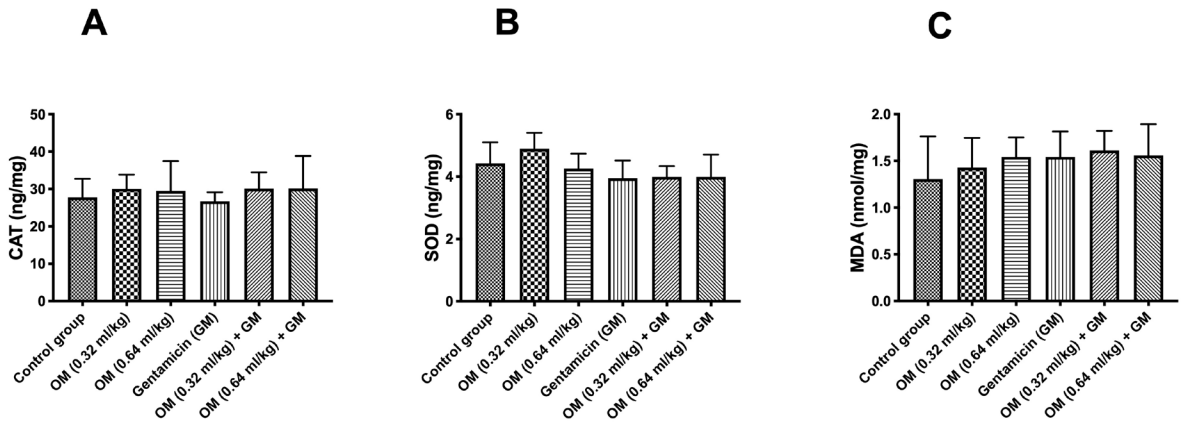
Figure 3. A-C. Measurements of the kidney tissue CAT (A), SOD (B) and MDA (C) levels obtained by ELISA assay and statistical analysis of them in experimental groups. A relative decrease in the kidney CAT and SOD level were determined in the GM group compared to the Control group. In the OM (0.32ml/kg) +GM group, an increase was detected in the kidney CAT and SOD level compared to GM group. However, OM (0.64ml/kg) +GM group did not show a different in terms of these parameters. Kidney tissue MDA level increased in the GM group compared to Control group. In the OM (0,32ml/kg) +GM and OM (0,64ml/kg) +GM groups, MDA level does not differ much compared to GM group. CAT:catalase, SOD:superoxide dismutase, MDA:malondialdehyde, GM:gentamisin, OM:origanum majorana



the protective effect of OM in GM-induced nephrotoxicity through histopathology, apoptosis, and biochemical mechanisms.

Our histopathological findings show degenerations such as glomerular damage, necrosis, desquamation, hemorrhage, and dilatation, similar to GM-induced nephrotoxicity studies (21). In our study, it was observed that this damage decreased significantly in the groups treated with OM. Aminoglycosides from the proximal tubule are taken into the cell and play an important role in the formation of the damage (15). Necrosis and apoptosis occur as a result of the accumulation of aminoglycosides in the renal tubules (22). It has been shown in studies that a large number of apoptotic cells increased in renal tubular epithelial cells, especially in the cortex region, with GM-induced nephrotoxicity (23). In our study, the activation of the GM-induced apoptotic pathway significantly increased the number of apoptotic cells in tubular cells. With the application of OM (0.32 ml/kg) and OM (0.64 ml/kg), the number of renal apoptotic cells has decreased and can be effective in protecting against GM-induced renal tubular apoptosis. This feature is due to the anti-apoptotic and antioxidant properties of Origanum species in previous studies (24-26).

Oxidative stress leads to lipid peroxidation (LPO) in the cell through the production of ROS (27). The endogenous antioxidant system (glutathione peroxidase, superoxide dismutase, catalase) aims to scavenge the ROS in many cell types in the body. However, excess ROS produced consumes endogenous antioxidants and leads to peroxidation of membrane lipids and increases the oxidation of proteins (9). Although GM-induced nephrotoxicity cannot be fully explained, many researchers have reported that the formation of ROS is important (28, 29). Stimulation of oxidative stress has an important place in GM-induced kidney damage. Because with the increase of ROS, antioxidant capacity decreases and MDA level and renal damage increase as a result of increased lipid damage (30, 31). These studies show that antioxidant enzymes such as SOD and CAT decrease in GM-induced nephrotoxicity and increase the level of lipid peroxidation product MDA. In our study, it was observed that OM (0.32 ml/kg) reduced oxidative stress and decreased MDA damage. Likewise, OM (0.32 ml/kg) has played an important role in antioxidant capacity by maintaining SOD and CAT activity. Studies have shown that it is important in cellular protection by stimulating the antioxidant system in kidney damage

and other organ injuries (32, 33). Many studies have been done with Origanum vulgare and this species has been shown to reduce tissue damage due to its antioxidant and anti-apoptotic properties (34, 35). We used Origanum majorana as a different species in our study. It is important because there is not much work on tissue damage of Origanum majorana in GM-induced kidney damage and other damage models.

In the current study, we examined the effect of Origanum majorana (OM) on GM-induced nephrotoxicity using different doses. We observed that at the dose of OM applied as 0.32 ml/kg in kidney damage, both renal damages were reduced, and it was effective in antioxidant capacity. These results show that OM can be tried in various damage mechanisms and can be important in minimizing the damage.

Acknowledgment

No conflict of interest was declared by the authors. This project was supported by Erciyes University Scientific Research Projects Unit with number of TYL-2016-6433. This study was presented as abstract at the 15th International Congress of Histochemistry and Cytochemistry, Antalya, Türkiye.

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