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Original Article
Özgün Araştırma

Eucalyptol (1.8-cineole) attenuates Gentamicin-induced liver injury

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Abstract: Gentamicin (GM), which is in the aminoglycoside antibiotic class, is frequently preferred today in the treatment of diseases caused by gram-negative bacteria. However, its significant side effects on liver and kidney functions limit its clinical usefulness. The antioxidant and anti-inflammatory medical activities of eucalyptol (EUC, 1.8-cineole) have been reported in different studies. This study aimed to evaluate the effects of EUC on GM-induced hepatotoxicity. The study groups are consisted of control (C), EUC, GM and GM + EUC, and there were 7 rats in each group. At the end of the study, the rats were euthanized under appropriate conditions and samples were collected and biochemical, histopathological and immunohistochemical analyzes were performed. It was determined that there was a important increase in serum alanine aminotransferase (ALT), aspartate transferase (AST) and gamma-glutamyl transferase (GGT) enzymes in the GM group relative to the C group ($p<0.001$). GM application is determined that it caused histopathological damage in livers. Additionally, immunohistochemically, it caused an important increase in the expressions of 4-hydroxynonenal (4-HNE), 8-hydroxy-2′-deoxyguanosine (8-OHdG) and malondialdehyde (MDA) in rats in the GM group relative to the C group ($p<0.001$). Eucalyptol application (GM+EUC) regulated the increase in serum ALT, AST and GGT enzymes. It also attenuated 4-HNE, 8-OHdG and MDA expressions. It attenuated the histopathological damage caused by GM application. The results of this study revealed that EUC showed antioxidative, protective and curative efficacy in GM-induced liver damage.

Keywords: eucalyptol (1.8-cineole), gentamicin, hepatotoxicity, MDA, 8-OHdG

Okaliptol (1.8-sineol) Gentamisin kaynaklı karaciğer hasarını hafifletir

Özet: Aminoglikozid antibiyotik sınıfında yer alan gentamisin (GM), gram negatif bakterilere bağlı hastalıkların tedavisinde günümüzde sıklıkla tercih edilmektedir. Ancak karaciğer ve böbrek fonksiyonları üzerine belirgin yan etkilerinin olması klinik yararlılığını sınırlamaktadır. Okaliptolün (OKA, 1.8-sineol) antioksidan ve antiinflamatuar tıbbi aktiviteleri farklı çalışmalarda bildirilmiştir. Bu çalışmada GM kaynaklı hepatotoksisite üzerine OKA'nın etkilerinin değerlendirilmesi amaçlanmıştır. Çalışma grupları kontrol (K), OKA, GM ve GM + OKA'dan oluşmaktaydı ve her grupta 7 rat bulunmaktaydı. Çalışmanın sonunda ratlar uygun koşullar altında ötenazi edilerek örnekler toplandı ve biyokimyasal, histopatolojik ve immünohistokimyasal analizleri yapıldı. GM grubunda serum alanin aminotransferaz (ALT), aspartat aminotransferaz (AST) ve gama-glutamil transferaz (GGT) enzimlerinde K grubuna kıyasla anlamlı artış olduğu belirlendi ($p<0.001$). GM uygulamasının karaciğerlerde histopatolojik hasara neden olduğu belirlendi. Ayrıca immünohistokimyasal olarak GM grubundaki ratlarda K grubuna kıyasla 4-hidroksinonenal (4-HNE), 8-hidroksi-2′-deoksiguanozin (8-OHdG) ve malondialdehit (MDA) ekspresyonlarında anlamlı artışa neden oldu ($p<0.001$). Okaliptol uygulaması (GM+OKA) serum ALT, AST ve GGT enzimlerindeki artışı düzenledi. Ayrıca 4-HNE, 8-OHdG ve MDA ekspresyonlarını azalttı. GM uygulamasının neden olduğu histopatolojik hasarı azalttı. Bu çalışmanın sonuçları OKA'nın GM kaynaklı karaciğer hasarında antioksidan, koruyucu ve tedavi edici etkinlik gösterdiğini ortaya koydu.

Anahtar kelimeler: gentamisin, hepatotoksisite, MDA, okaliptol (1.8-sineol), 8-OHdG

Introduction

One of the drug classes frequently used in the treatment of diseases today is antibiotics. Antibiotics prevent many problems caused by infection. However, antibiotics can damage various organs such as skin, kidney, liver, brain, mouth, etc. Amino-

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glycoside antibiotics have been used as antibacterial treatment for many years. Aminoglycosides are defined as a class of antibiotics that consists of at least 2 amino sugars and are used to treat gram (-) bacteria. Following treatment with aminoglycoside antibiotics, approximately 5-10% of patients may experience ototoxicity, nephrotoxicity, and hepatotoxicity side effects (Mahi-Birjand et al., 2020; Thy et al.2023; Lang et al.2023)

Gentamicin (GM), which is in the aminoglycoside antibiotic class, is frequently preferred today in the treatment of diseases caused by gram-negative bacteria. However, its notable side effects on liver and kidney functions limit its clinical usefulness (Yarijani et al., 2019; Babaeenezhad et al., 2021; Akcakavak et al., 2024). The liver and kidneys are particularly sensitive to drug toxicity because they play important roles in normal homeostasis in detoxification and excretion of drugs and their metabolites. Currently, the exact mechanism of GM hepato and nephrotoxicity have not been fully clarified. Diffe-rent hypotheses have been proposed in many stu-dies and oxidative stress is the most emphasized in GM hepato and nephrotoxicity (Galaly et al., 2014; Laaroussi et al., 2021). Because reactive oxygen species (ROS) and other free radicals are suggested to be one of the important mediators of GM toxicity (Banday et al., 2008). Different studies have shown that GM stimulates excessive production of ROS metabolites, affecting different processes including lipid peroxidation, protein oxidation and DNA damage, leading to necrosis and cellular damage. In addition, excessive production of free radicals causes activation of nitrosative tissue stress, modulation of the caspase family and upregulation of the inflammatory process (Morales et al., 2002; Sanchez-Gonzalez et al., 2011; Ali et al., 2020; Bulboacă et al., 2022).

Eucalyptol (EUC, 1.8-cineole), a saturated monoterpene, is obtained from botanical sources such as eucalyptus, camphor and rosemary. EUC is known as the main active ingredient of eucalyptus essential oils. EUC has a long history of use in traditional medicine. It is also frequently used in food, fragrance and cosmetics due to its taste and aroma (Galan et al., 2020; Hoch et al., 2023). EUC has a number of medical effects including antimicrobial, anti-inflammatory, antioxidant, analgesic and bronchodilator (Galan et al., 2020; Cai et al., 2021; Kazak, 2022; Akcakavak, et al., 2024, Kazak et al., 2024a; Kazak et al., 2024b). It has been reported in various studies that EUC shows highly effective free radical scavenging activity and has the ability to defend cells from oxidative damage by neutralizing ROS (Rašković et al., 2014; Ryu et al., 2014). Ryu et al. 2014 stated that EUC significantly reduced ROS overproduction in patients with ischemic stroke. In an experimental arthritis study in rats, EUC administration was reported to reduce the levels of proinflammatory cytokines (Iqbal et al. 2024). In a lead acetate-induced liver injury study, it was stated that EUC could prevent damage by reducing inflammation and oxidative stress (Abdollahi et al.2024). It is also reported that EUC has an inhibitory effect against lipid peroxidation (Moon et al., 2014; Hsouna et al., 2019).

Medicinal plants have been widely used to treat many diseases since ancient times. Today, the use of herbal supplements has a special importance in the treatment of liver diseases due to their numerous properties and low side effects. EUC, a saturated monoterpene, is obtained from botanical sources such as eucalyptus, camphor and rosemary (Galan et al., 2020, Cai et al., 2021). Recently, many experimental researches are being conducted to prevent and/or treat GM-induced hepatoxicity using different agents (Babaeenezhad et al., 2021; Mirazi et al., 2021; Ogundipe et al., 2021). The main purpose of these studies is to find new, effective and safer antioxidant compounds that can prevent and/or treat GM-induced hepatotoxicity. EUC may be an important candidate due to a number of medicinal effects such as anti-inflammatory, antioxidant, antimicrobial, analgesic. Although there are studies in the literature evaluating the effects of EUC on hepatotoxicity induced by different agents, no study evaluating its effectiveness on GM-induced hepatotoxicity was found. The aim of the present study was to evaluate the effect of EUC on GM-induced hepatotoxicity by determining histopathological damage, hepatic functional enzymes, and lipid peroxidation markers in the tissue.

Materials and methods

Animals

The material for the study consisted of 28 female Wistar Albino rats weighing 300-400 g. The rats were housed in standard plastic cages, at room temperature of 20-22°C, in 12 hours of light and 12 hours of darkness, and were fed *ad libitum*.

Experimental procedure

Rats were randomly divided into 4 groups, 7 in each group; control (C), EUC, GM, GM+EUC. GM was implemented as a single dose of 100 mg/kg intra-

peritoneal (i.p.) for 10 consecutive days of the study. EUC solution was implemented by oral gavage at 100 mg/kg for 10 consecutive days. EUC solution was prepared relative to the previously stated study (Akcakavak et al., 2024). In addition, the vehicle solution was administered to C and GM groups by oral gavage for 10 consecutive days throughout the experimental period. On the 11th day of the study, intracardiac blood was taken from the experimental animals under xylazine and ketamine (10-90 mg/kg) anesthesia, and the animals were sacrificed. Afterwards, the liver tissues of the necropsied rats were placed in 10% neutral formaldehyde solution for histopathological and immunohistochemical examinations.

Determination of serum biochemical parameters

Blood samples taken under anesthesia were centrifuged at 5000 rpm for 15 minutes at 4°C. The obtained sera were transferred to cryotubes and stored at -80°C. Liver function was evaluated via measurement of ALT, AST and GGT activities in serum using autoanalyzer (AU5800, Beckman Coulter, Japan) and commercial kits.

Histopathological examination

Liver tissues taken following necropsy were fixed in 10% neutral formaldehyde solution. Then, formaldehyde was removed by washing and passed through graded alcohols (70º, 80º, 90º, 100º) and xylene steps, respectively. Afterwards, the relevant liver tissues were embedded in paraffin. Sections were taken from paraffin blocks onto ground slides and stained with Hematoxylin-Eosin (H-E). Histopathological evaluation was performed semi-quantitatively in 10 different fields at x20 magnification (0; none, 1; mild, 2; moderate, 3; severe).

Immunohistochemical examination

4-5 µm sections were taken from paraffin blocks onto adhesive slides. The sections were subjected to paraffin extraction and rehydration processes. Immunohistochemical staining was performed using a commercial kit according to the previously stated method (Akcakavak et al., 2023). 8-OHdG (8-hydroxy-2′-deoxyguanosine, Santa Cruz Biotechnology, sc-393871, 1/200 dilution, 1 hour incubation), 4-HNE (4-Hydroxynonenal, Abcam, ab46545, 1/200 dilution, 1 hour incubation), MDA (Malondialdehyde, Abcam, ab6463 1/1000 dilution, 1 hour incubation) antibodies were used as primers. 3. 3′-diaminobenzidine (DAB) was used as a chromogen and was examined under light microscopy after counterstaining with Mayers hematoxylin. Immunohistochemical scoring was performed semi-quantitatively (0; none, 1; mild, 2; moderate, 3; severe)(Akcakavak et al., 2024).

Statistical analysis

The statistical program SPSS (Inc., Chicago, USA 25.0) was used to analyse the obtained data. The biochemical, histopathological and immunohistochemical findings obtained in the study were evaluated using One-way ANOVA and Duncan's test as post-hoc test. $p<0.05$ was used as the significance limit.

Results

Serum biochemical findings

Serum biochemical findings of the groups regarding liver function tests are presented in Table 1. The activities of the ALT, AST and GGT enzymes in the GM group were 2.1, 1.7 and 2.2. fold respectively higher than those of the C group ($p<0.001$). The activities of the ALT, AST and GGT enzymes in the GM+EUC group significantly decreased (0.7, 0.8 and 0.7 fold, respectively) compared with the GM group, although the activities of the ALT, AST and GGT enzymes in the GM+EUC group significantly increased (1.4, 1.3 and 1.6 fold, respectively) relative to C group ($p<0.001$).

Table 1. The impacts of EUC on the activities of ALT, AST, and GGT in rat liver, intact and with GM-induced liver injury (Mean±SE, n;7)

Liver function tests	C	EUC	GM	GM+EUC
ALT (ul/l)	35.83±1.67^{c}	39.62±2.94^{c}	75.83±1.83^{a}	52.34±2.09^{b}
AST (ul/l)	90.33±3.57^{c}	93.50±2.86^{c}	152.17±5.49^{a}	121.00±2.90^{b}
GGT (ul/l)	4.50±0.56^{c}	4.67±0.42^{c}	9.83±1.37^{a}	7.10±0.60^{b}

$^{a-c}$ Indicates statistical significance between groups in the same line ($p<0.001$). (**ALT**; alanine aminotransferase, **AST**; aspartate transferase, **GGT**; gamma-glutamyl transferase, **C**; Control, **EUC**; eucalyptol, **GM**; gentamicin, **GM+EUC**: gentamicin+eucalyptol)

Histopathological results

Histopathological statistical scores of the groups are give in Table 2. C and EUC groups showed normal histology (Figure 1.A-B). Degeneration and necrosis were detected in hepatocytes in GM and GM+EUC groups. Especially degenerative and necrotic changes were localized in the centrilobular region (Figure 1.C-E). Degeneration and necrosis scores were significantly reduced in the GM+EUC group relative to the GM group ($p<0.001$). In addition, bile duct proliferation, inflammatory cell infiltration and sinusoidal dilatation were seen in the GM and GM+EUC groups (Figure 1.C-E). The GM+EUC group significantly reduced the relevant disorders relative to the GM group ($p<0.001$). Moreover, hemorrhage foci were detected in places in the GM and GM+EUC groups.

Table 2. Histopathological scores between groups (Mean+SE, n;7)

Histopathological lesion	C	EUC	GM	GM+EUC
Degeneration of hepatocytes	$0.50+0.22^{c}$	$0.67+0.21^{c}$	$3.17+0.17^{a}$	$2.17+0.17^{b}$
Necrosis of hepatocytes	$0.33+0.21^{c}$	$0.50+0.22^{c}$	$2.83+0.17^{a}$	$1.83+0.30^{b}$
Inflammatory cell infiltration	$0.33+0.21^{c}$	$0.33+0.21^{c}$	$2.67+0.21^{a}$	$1.67+0.33^{b}$
Bile duct proliferation	$0.50+0.22^{c}$	$0.50+0.22^{c}$	$2.33+0.33^{a}$	$1.33+0.21^{b}$
Sinusoidal dilatation	$0.67+0.21^{c}$	$0.83+0.17^{c}$	$2.5+0.22^{a}$	$1.67+0.21^{b}$

a-c Indicates statistical significance between groups in the same line ($p<0.001$). (**C**; Control, **EUC**; eucalyptol, **GM**; gentamicin, **GM+EUC**: gentamicin+eucalyptol)

Immunohistochemical results

The immunohistochemical scores of the groups are given in Table 3. In the C and EUC groups, immunopositivity was mild or absent for the relevant primers (8-OHdG, 4-HNE and MDA). 8-OHdG, 4-HNE and MDA immunopositivity was significantly increased in the GM group compared to the C group ($p<0.001$), and immunopositivity was prevalent in the centrilobular regions (Figure 2). The GM+EUC group was found to have significantly reduced immunopositivity (8-OHdG, 4-HNE and MDA) relative to the GM group ($p<0.001$).

Table 3. Immunohistochemical scores between groups (Mean+SE, n;7)

Primers	C	EUC	GM	GM+EUC
4-HNE	$0.17+0.17^{c}$	$0.33+0.21^{c}$	$2.67+0.21^{a}$	$1.83+0.17^{b}$
8-OHdG	$0.00+0.00^{c}$	$0.17+0.17^{c}$	$2.83+0.17^{a}$	$2.00+0.25^{b}$
MDA	$0.17+0.17^{c}$	$0.33+0.21^{c}$	$2.67+0.21^{a}$	$1.83+0.30^{b}$

a-c Indicates statistical significance between groups in the same line ($p<0.001$). (**MDA**; malondialdehyde, **8-OHdG**; 8-hydroxy-2′-deoxyguanosine, **4-HNE**; 4-Hydroxynonenal, **C**; Control, **EUC**; eucalyptol, **GM**; gentamicin, **GM+EUC**: gentamicin+eucalyptol)

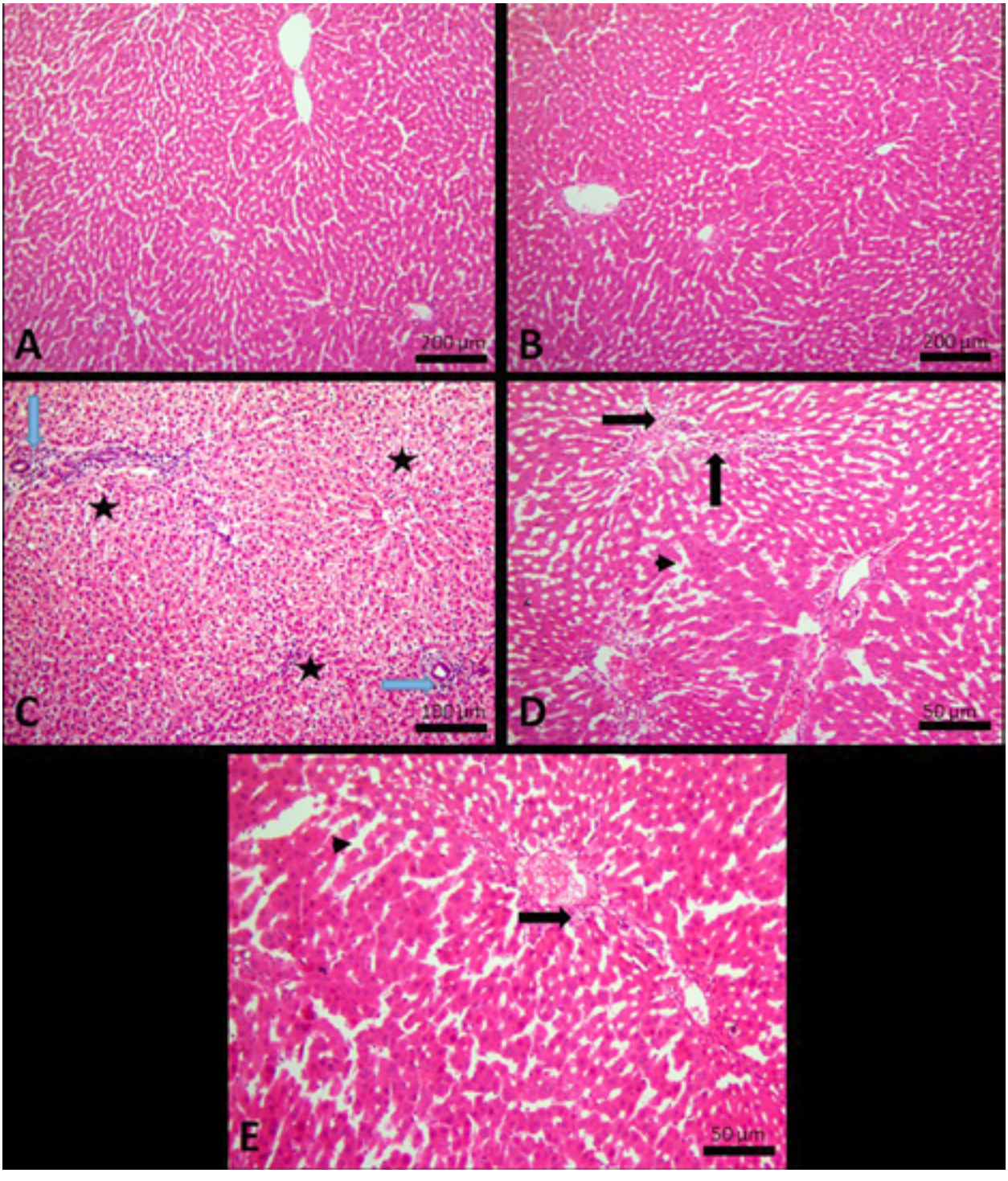

Figure 1. Histopathological examination between groups, Hematoxylin-Eosin, **A**; C group, **B**; EUC group, **C-D**; GM group, **E**; GM+EUC group. (Degeneration in hepatocytes (stars), necrosis (black arrows), inflammatory cell infiltration (blue arrows), sinuosidal dilatation (arrowheads), C; Control, EUC; eucalyptol, GM; gentamicin, GM+EUC; gentamicin+eucalyptol)

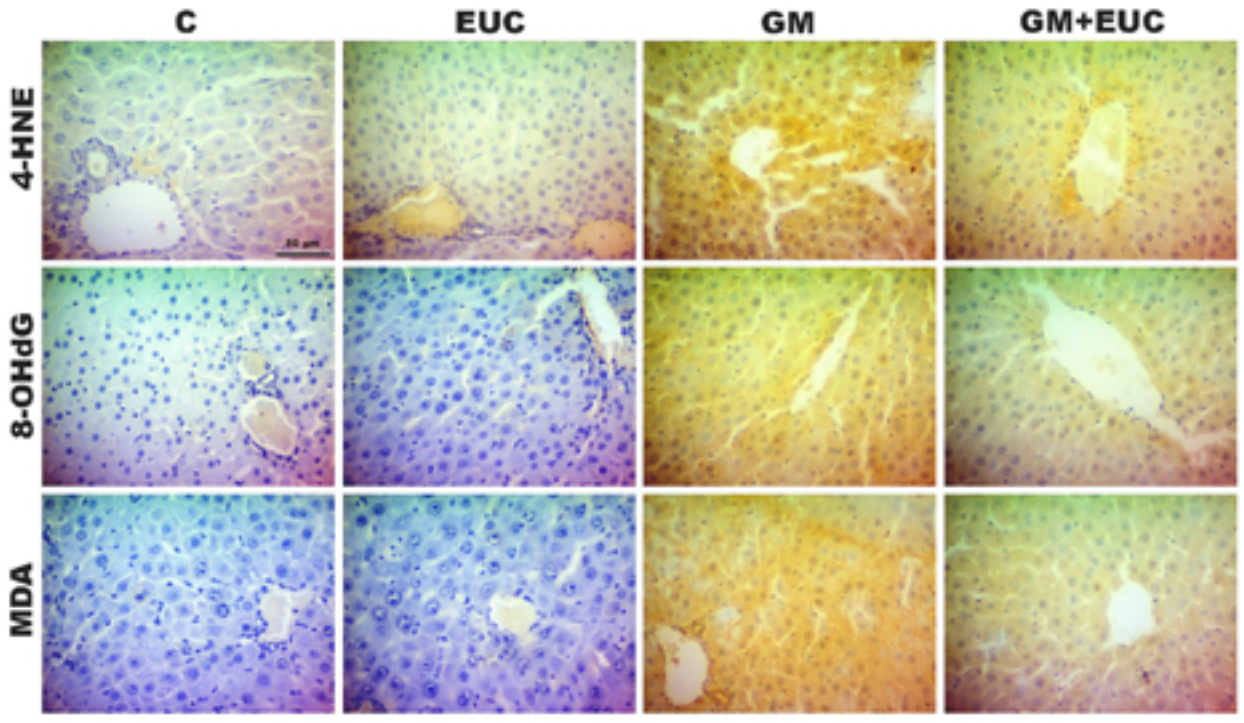

Figure 2. Immunohistochemical examination between groups (DAB), (**4-HNE**; 4-Hydroxynonenal, **8-OHdG**; 8-hydroxy-2'-deoxyguanosine, **MDA**; malondialdehyde, **C**; Control, **EUC**; eucalyptol, **GM**; gentamicin, **GM+EUC**; gentamicin+eucalyptol)

Discussion

Although the effects of many phytochemical components on GM-induced liver toxicity have been evaluated, no study evaluating the effect of EUC on hepatoxicity has been found. Present study, we aimed to evaluate the effects of EUC (100 mg/kg, 10 consecutive days) on liver damage induced by GM (100 mg/kg, 10 consecutive days) in rats. The current biochemical, histopathological and immunohistochemical findings showed that GM administration caused liver hepatoxicity and that the changes related to hepatoxicity could be prevented with simultaneous administration of EUC with GM.

Oxidative stress is a condition that occurs when the balance between free radicals and antioxidants in normal homeostasis is disrupted. Antioxidants are defined as protective systems that protect cells against the damaging effects of free radicals. It is known that suppression of the antioxidant system for various reasons can lead to a wide variety of pathological conditions, including liver damage. Gentamicin reduces the activity of antioxidant enzymes such as CAT, GPx and SOD in cells, causing the redox system to deteriorate and the formation of free radicals (Ďuračková, 2010; Randjelovic et al.2017; Laaroussi et al., 2021; Bulboacă et al., 2022).

AST is present in both hepatocyte cytosol and hepatocyte mitochondria while ALT exists prevalently in the hepatocyte cytosol. The release of these enzymes from hepatocytes is largely due to hepatocellular injury (Zoppini et al., 2016). The enzyme GGT is also associated with hepatitis and biliary tract obstruction (Ibraheem et al., 2021). Literature revealed that GM may directly lead to hepatotoxicity in rats through oxidative stress and apoptosis and finally cause the elevation of the serum activities of ALT, AST and GGT. It has been found that GM increased ALT, AST and GGT activities in rats (Yarijani et al., 2019; Babaeenezhad et al., 2021; Ibraheem et al., 2021; Bulboacă et al., 2022). In keeping with such findings, the present study demonstrated that GM caused liver damage as shown by important increases in serum ALT, AST and GGT activities. Thus, the elevation of ALT, AST and GGT in GM administered animals can imply the injury of liver cytoarchitecture and hepatic cell integrity, linked to microsomal membrane fluidity, mitochondrial dysfunction, and free radical generation. In addition, the activities of the ALT, AST and GGT enzymes in serum of the

groups that received GM and EUC importantly reduced, which indicates the protective effect of EUC against hepatotoxicity induced by GM.

GM is often prescribed to treat bacterial conjunctivitis, sepsis, endocarditis, and infections caused by gram-negative bacteria (Chen et al., 2014). Although it has many beneficial effects, it increases ROS levels by inhibiting enzymatic and non-enzymatic antioxidants in the liver. Thus, in addition to increasing oxidative stress, it causes liver damage by causing damage to lipids, nucleic acids and cellular proteins in the membrane (Laaroussi et al., 2021; Ogundipe et al., 2021). Galan et al. (2014) detected histopathologically degeneration, steatosis, congestion, inflammatory cell infiltration and bile duct proliferation in liver injury induced by GM in rats. In another study examining liver damage due to GM, histopathological findings included steatosis, congestion, inflammatory cell infiltration, sinusoid dilatation, and an increase in the number of Kupffer cells (Al-Khamas et al. 2020). In another study, degeneration, necrosis and sinusoid dilatation were detected histopathologically in GM-induced liver damage (Wijayanti et al.,2023). When the histopathological findings of the current study were evaluated, it was seen that the histopathological changes observed in the GM group were consistent with the findings of previous studies. It is thought that the finding of degeneration and necrosis in the centrilobular region (Figure 1), especially in the GM group, may be because of the increase in ROS levels for GM application and the subsequent oxidative stress.

It was determined that GM-induced histopathological damage was reduced when EUC was administered together with GM. It has been reported in various studies that EUC shows highly effective free radical scavenging activity and has the ability to protect cells from oxidative damage by neutralizing ROS (Rašković et al., 2014; Galan et al., 2020; Akcakavak et al., 2024). EUC is suggested that it exhibits its antioxidant and anti-inflammatory medical activities through manipulations on Nrf2 and NF-kB pathways. Research in the literature reports that EUC causes upregulation of the Nrf2 transcription factor and downregulation of the NF-kB pathway, thus exhibiting strong antioxidant and anti-inflammatory bioactivities (Cai et al., 2021; Venkataraman et al., 2023; Akcakavak et al., 2024; Iqbal et al., 2024). The current study shows that EUC administration can prevent the upregulation of oxidative stress and inflammatory processes that occur with GM administration and thus reduce liver damage. In addition, the fact that EUC down-regulated pro-inflamatory cytokine levels in previous studies further strengthened our idea (Akcakavak et al., 2023; Akcakavak et al., 2024; Iqbal et al., 2024).

Excessive formation of ROS due to chemical toxicity causes oxidative damage to DNA. This situation contributes to the formation of 8-OHdG, which is the most crucial effect of DNA damage. 8-OHdG has been commonly assesed as a biomarker of oxidative DNA damage in recent years (Graille et al., 2020; Akcakavak et al., 2023). Another oxidative reaction induced by excessive ROS levels is lipid peroxidation. 4-HNE and MDA are known as the cytotoxic end products of lipid peroxidation (Yang et al., 2003; Ayala et al., 2014). In the literature, it is reported that causes upregulation of 4-HNE, 8-OHdG and MDA in different GM-induced toxicity studies (Aycan-Ustyol et al., 2017; Cui et al., 2019; Mohammed et al., 2019; Ince et al., 2020). In the present study, higher expressions of 4-HNE, 8-OHdG and MDA were detected in the GM group relative to the control groups and were consistent with the literature (Figure 2). Present findings showed that GM (100 mg/kg, 10 consecutive days) administration caused oxidative DNA damage and lipid peroxidation. GM and EUC administration provided protection against GM-induced hepatotoxicity by reducing the expressions of 4-HNE, 8-OHdG and MDA relative to the GM only group.

In conclusion, it has been determined that GM application causes complications characterized by degeneration and necrosis in hepatocytes, inflammatory cell infiltration, bile duct hyperplasia and sinusoidal dilatation, and this structural and cellular damage in the liver causes an increase in serum liver enzymes. The current study shows that EUC administered simultaneously with GM plays a protective/curative role in reducing GM-induced liver damage by suppressing liver function tests (AST, ALP, GGT), histopathological changes and 8-OHdG, 4-HNE and MDA expressions. These results suggest that EUC may be a promising candidate for clinical drug development in GM-induced liver damage.

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