

Eumelanin protects the liver against diethylnitrosamine-induced liver injury

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ABSTRACT

This study aims to evaluate in vivo protective effects of eumelanin (EU) on diethylnitrosamine (DEN)-induced liver injury. Wistar albino male rats were divided into 6 groups (n = 6), Control, DMSO, DEN, DEN + EU10, DEN + EU15, and DEN + EU20. Animals in the DEN group were injected i.p a single dose of 200 mg/kg DEN, DEN + EU10 group was given 10 mg/kg EU, DEN + EU15 group was given 15 mg/kg, DEN + EU20 group was given 20 mg/kg EU for a week. The results showed that there was no significant difference in vessel volume density between the groups. Inflammatory cell infiltration, hydropic degeneration, and necrotic cells were observed in the DEN group, and these histopathological changes were significantly reduced in all treatment groups. Although there was a low intensity of PAS-positive staining in the DEN groups, moderate staining was observed in the treatment groups. While Caspase-3, PCNA, TNF- α , and IL-6 expressions increased in the DEN group, their expressions decreased in the EU-treated groups. DEN increased AST, ALT, and MDA levels and decreased CAT levels. In particular, the EU10 dose significantly improved these parameters. The present study revealed that eumelanin has protective effects against DEN-induced liver injury.

1. Introduction

Melanin is a pigment produced from L-tyrosine via the L-dihydroxyphenylalanine (L-dopa) by tyrosinase (Claus and Decker, 2006). Since melanin can disperse more than 99.9 % of UV radiation, it plays a very important role in protecting skin cells against the harmful effects of UV radiation and reducing the risk of cancer (Shalaby et al., 2019; Tan et al., 2019). Melanin is a common and important natural material found in everything from microorganisms to higher animals and plants. Based on chemical precursors used in their biosynthesis, melanin is divided into five types: eumelanin, pheomelanin, neuromelanin, allomelanin, and pyromelanin. Melanin pigments can be radioprotective, photoprotective, antioxidant, antimicrobial, heavy metal chelator, and so on (Solano, 2017; Martínez et al., 2019). The basic units of eumelanin are oligomers that are stacked and otherwise linked to form a substance with amorphous semiconductor properties (Kaxiras et al., 2006; Meredith and Sarna, 2006). The biosynthesis of this pigment begins with the catalysis of the tyrosine amino acid by the tyrosinase enzyme (Bayram et al., 2020; Bayram, 2021; Caldas et al., 2020). Previous in vitro studies have proven that eumelanin has an anticancer effect on Hep-G2 cells, a liver carcinoma cell line, and HCT-116, a colon carcinoma cell line (Shalaby

et al., 2019). Inflammation is the body's response to conditions such as tissue damage and infection. In the inflammatory response of the body, conditions such as increased endothelial permeability, increased migration of inflammatory cells to the interstitial space and the release of cytokines such as TNF- α , and IL-6 occur. In previous studies, it was documented that melanin has an anti-inflammatory effect by inhibiting COX expression (Kurian et al., 2015).

Nitrosamines are a large group of chemical compounds formed by the reaction of nitrite with primary, secondary, or tertiary amine precursors through external environmental conditions such as high temperature or an acidic environment (Yamashina et al., 2012; Tolba et al., 2015). For many years, they have been known that these chemical compounds are potentially carcinogenic (Uemura et al., 1980). They cause changes in DNA structure and initiation and progression of liver damage (Mansour et al., 2019). Diethylnitrosamine (DEN) is a hepatotoxic and hepatocarcinogenic substance belonging to the nitrosamine group (Weber et al., 2016). It is known that this chemical causes damage to the enzymes that perform DNA repair and thus inhibits these enzymes. In addition, these nitrosamines affect the mitochondrial metabolism of the cell, causing the imbalance of reactive oxygen species (ROS) and antioxidants. Because of this situation, they can cause

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oxidative stress and are used to induce liver damage in experimental animals (Fuentes-Hernández et al., 2019).

DEN is mainly used as the initial agent of rodent hepatocarcinogenesis (You et al., 2021). Hepatic bio-activation of DEN produces reactive metabolites and reactive oxygen species (ROS), which leads to the formation of mutagenic DNA adducts (Gupta et al., 2010). Risk factors for liver damage commonly result in the construction of reactive oxygen species in the liver, after which to oxidative stress. This relation between oxidative stress and the development of liver damage has attracted the attention of many researchers investigating the possibility of using antioxidants for treatment (Al-Okbi et al., 2021; Alsahli et al., 2021).

Previous studies show that the use of antioxidants is important in the treatment of liver damage and results in the activation of apoptosis (Ding et al., 2021). Therefore, natural antioxidants may be potential candidates for the treatment of liver injury. The growing interest in plant-derived compounds is mainly attributed to their antioxidant capacity, anticancer and ability to prevent certain diseases. Experimental studies have shown many beneficial effects of melanin and its derivatives, including antiproliferative, anticancer, and anti-oxidative effects. These data suggest that eumelanin may have the potential to reduce the occurrence of various types of cancer and the risk of metastasis. While there are several studies on the effects of melanin on the liver, there are only a few studies on the effects of eumelanin pigment on organs. Eumelanin is a significant pigment, and it was thought that it should be studied in preclinical studies. Therefore, this study aimed to investigate the effects of eumelanin on DEN-induced liver injury using histopathological, immunochemical, stereological, and biochemical analyses. For the first time, the effects of eumelanin doses in a DEN-induced liver injury model were examined in this study.

2. Materials and methods

2.1. Chemicals and reagents

Diethylnitrosamine (DEN, CAS no.: 55-18-5), Dimethylsulfoxide (DMSO, CAS no.: 67-68-5) were purchased from Sigma-Aldrich (St. Louis MO, USA). Caspase-3 (sc-8033), TNF- α (sc-52746), IL-6 (sc-57315) and PCNA (sc-25280) primary antibodies were obtained from Santa Cruz Biotechnology (Texas, USA). The kit and other chemicals required for immunohistochemical examination were obtained from Thermo Fisher Scientific (USA).

2.2. Production and purification of eumelanin pigment

Streptomyces parvus BSB49 strains, which were detected by qualitative methods to produce eumelanin pigment in solid medium (ISP2 medium), were transferred to sterile liquid medium (Nutrient broth) and incubated for a week in an orbital shaker set at 35 °C 200 rpm. At the end of a week incubation period, the pigment-producing samples were transferred to 50 ml Falcon tubes, then centrifuged at 10,000 rpm for 10 min, and this process was repeated twice. After this centrifugation, the cell-free supernatant was adjusted to pH 2.0 with 6 M HCl and incubated at room temperature for 24 h. The obtained suspension was again centrifuged at 10,000 rpm for 10 min and the precipitates were separated from the supernatant. The eumelanin separated from the supernatant was washed twice with distilled water and centrifuged at 10,000 rpm for 10 min. After this treatment, the purified eumelanin pigment was lyophilized and stored at -20 °C until used for the experiment (El-Naggar and El-Ewasy, 2017; El-Naggar, 2021; Haque et al., 2021).

2.3. ^1H NMR spectroscopy

^1H NMR spectrum was obtained from Bruker AVANCE III 400 MHz NMR Spectrometer at 25 °C (9 Tesla). Resonances were got in aliphatic and aromatic regions were recorded and interpreted (Choi et al., 2018;

Satheesh and Preethi, 2019; Sivaperumal et al., 2015).

2.4. Fourier transform-infrared (FT-IR) spectroscopy

Infrared (IR) spectra were recorded on the Perkin-Elmer Spectrum II FT-IR spectrometer and showed as s (strong), m (medium), w (weak), b (thick) band intensities (cm^{-1}) (Capozzi et al., 2006; Bronze-Uhle et al., 2013; El-Naggar, 2021).

2.5. XRD analysis of eumelanin (X-ray diffraction)

Initially, eumelanin samples were ground in a mortar, and analysis was performed with an X-ray diffractometer (Bruker- D8 Discover XRD) with Cu K α radiation ($\lambda = 1.5406 \text{ \AA}$) for 2θ angles ranging from 20° to 90° (Capozzi et al., 2006).

2.6. Dissolution of eumelanin

Ultrasonic-assisted water bath (Kudos) was used to dissolve the eumelanin. For this purpose, eumelanin samples were kept in an ultrasonic water bath at 35 kHz with DMSO for 30 min at 60 °C, thereby dissolving the eumelanin pigment (Bayram et al., 2020).

2.7. UV-VIS absorption spectroscopy

Ultrasonic extraction pulverized and dissolved purified eumelanin in DMSO and then scanned with UV-vis spectrophotometer (Epoch 2, Biotek Instruments) at UV-visible and visible light lengths (200–800 nm). DMSO was used as a blank control in these procedures (Wang et al., 2006, 2019; Bayram et al., 2020).

2.8. Experimental design

Thirty-six adult *Wistar albino* male rats approximately 200–300 g. weight was utilized in this study. Standard pellet diet and tap water were ad libitum given to the animals. The animals were obtained from Van Yuzuncu Yil University Research Center for Experimental Medicine (Van, Turkey). The animals were kept in a room with 12-hour light/dark cycles, with free access to food. All experimental procedures have been approved by Van Yuzuncu Yil University Bioethics Committee (2021/02-09). Animals were divided into 6 groups Control, DMSO, DEN, DEN + EU10, DEN + EU15 and DEN + EU20 groups. The Control group was intraperitoneally (i.p.) injected with 1 ml/kg saline for a week. DMSO group was intraperitoneally (i.p.) injected with 1 ml/kg DMSO for one week. DEN (200 mg/kg) was administrated to DEN, DEN + EU10, DEN + EU15 and DEN + EU20 groups for a single dose (i.p) (Adebayo et al., 2020). Subsequently, 10 mg/kg/day EU, 15 mg/kg/day EU and 20 mg/kg/day EU were dissolved in DMSO and given to the DEN + EU10, DEN + EU15, and DEN + EU20 groups, respectively. This procedure was administered to all treatment groups by i.p.injection for a week before DEN administration (Sava et al., 2003; Hung et al., 2003, 2004). Following seven days experimental period, blood samples were taken from the heart under the anesthesia subsequently the rats were euthanized, and liver samples were collected.

2.9. Measurement of serum AST and ALT levels

At the end of the study, blood samples were taken by cannulation of the animal hearts with a sterile injector. Blood samples were taken into sample tubes. They were centrifuged for 10 min at 3500 rpm. In serum samples, AST and ALT were evaluated in an autoanalyzer (Roche Hitachi, C502) using commercial kits (Roche Diagnostics).

2.10. Measurement of oxidative stress-associated enzymes in liver

To evaluate the antioxidant, CAT activity was measured, and to

determine lipid peroxidation, MDA content was measured. Liver tissues were homogenized in a homogenizer. The whole homogenate was centrifuged at 3500 rpm for 15 min to analyze MDA levels and CAT activities. MDA levels and CAT activities were evaluated spectrophotometrically using commercial kits in liver tissues.

2.11. Histopathological analysis

The liver tissues were fixed in buffered 10 % formaldehyde for histological analysis. After the tissue processing stages, the tissues were embedded in paraffin blocks, and the blocks were cut into 4 µm thickness. The tissue sections were stained with H&E, periodic acid Schiff (PAS), and Masson's trichrome (M-T) stains for histological evaluation. For histopathological evaluation, an average of 15–20 areas was evaluated by random sampling for each animal in the groups. The findings were semiquantitatively evaluated according to the number of lesions observed in examined areas; thus, it was performed as normal 0–3: (0) none, (1) weak damage, (2) moderate damage, (3) severe damage.

2.12. Immunohistochemical analysis of Caspase-3, TNF-α, IL-6, and PCNA

In DEN-induced hepatotoxicity, expressions of caspase-3, TNF-α, IL-6, and PCNA primary antibodies were measured by the immunohistochemical method according to a reported previous study (Demir and Altındağ, 2022).

2.13. Stereological analysis

For stereological analysis, liver tissue was evaluated by random sampling and separated into fractions (f). For this, the liver tissue was divided into equal pieces (f1). One of the equal pieces was randomly selected and embedded in paraffin after histological tissue processing stages. 4 µm thickness was cut from paraffin blocks by a rotary microtome (Leica RM 2125, China). The first section was taken randomly, and the next sections were taken every 100 steps (f2). An average of 13–17 sections was taken. The sections were stained with H&E and examined under a light microscope (Nikon Eclipse Ni, Japan) using a camera (Nikon DSi Fi2 Japan) and microscope imaging systems (NIS-ELEMENTS F4.00.00, Japan). Cavalieri principle was used to estimate the total volume of the liver and calculate the vessel volume density. The following formula was used to calculate the volume of the liver and vessel volume density:

$$V = \sum P \times a/p \times t$$

V: The volume of the liver, $\sum P$: Total number of points on liver sections, a/p: Area of a point, t: Section thickness.

2.14. Statistical analysis

Descriptive statistics are presented as mean ± standard error for continuous variables. For continuous variables, descriptive statistics are present as mean and standard deviation, and for categorical variables, expressed as numbers and percentages. As all the continuous dependent variables are violated by the homogeneity and normality assumption, the Kruskal-Wallis test was performed to show the difference among the five experimental groups. In case of any statistical difference, the Mann-Whitney U test with Bonferroni correction was used as posthoc test. Statistical significance level was taken as 0.05 % in calculations and IBM SPSS Statistics for Windows package program was used for calculations.

3. Results

3.1. Effects of EU on AST and ALT levels in the DEN-induced rats

ALT and AST levels of the DEN group were significantly increased compared to the control group ($p < 0.05$). Both ALT and AST levels of the DEN + EM10 and the DEN + EM15 groups were significantly decreased compared to the DEN group ($p < 0.05$). ALT level of the DEN + EM20 group was significantly decreased compared to the DEN group ($p < 0.05$), however, the AST level was not significantly different from the DEN group (Table 1).

3.2. Effects of EU on oxidative stress-associated enzymes in the liver

To evaluate the DEN-induced hepatotoxicity, MDA (the main oxidative stress marker), and CAT (the antioxidant enzyme) were estimated in liver tissue samples. Compared to the control group, MDA was significantly increased while CAT was significantly decreased in the DEN group. Compared to the DEN group, MDA significantly decreased, while the CAT level increased in the DEN + EU10 and EU + 15 groups. However, the MDA was significantly increased in the DEN+ 20 group, while the CAT level was not significantly different in the DEN + EU20 group compared to the DEN group (Table 1).

3.3. Histopathological findings

Normal liver histology was seen in the Control and DMSO groups, while severe cell infiltration, severe hydropic degeneration, and moderate necrotic cells were observed in the DEN group. DEN + EU10 and DEN + EU15 treatment groups had moderate cell infiltration, weak hydropic degeneration, and weak necrotic cells. In the DEN + EU20 group, weak cell infiltration, moderate hydropic degeneration, and weak necrotic cells were found (Fig. 1). Evaluation of the extracellular matrix by Masson's Trichrome staining revealed no significant enlargement of the extracellular matrix between all groups (Fig. 2). For glycogen accumulations in hepatocytes, PAS staining examinations were performed, and severe PAS-positive cells were found in the control and DMSO groups, but DEN group has a wear PAS-positive cell. Moderate PAS-positive cells were found in the DEN + EM10 and DEN + EM15 treatment groups, whereas wear PAS-positive cells were in the DEN + EM20 treatment group (Fig. 3).

Table 1
Effect of DEN and EU on biochemical parameters.

Groups	AST (IU/L)	ALT (IU/L)	CAT (IU/ml tissue)	MDA (µmol/g protein)
Control	74.83 ± 8.68**	27.66 ± 3.82**	837.75 ± 13.58**	621.69 ± 15.45**
DMSO	75.16 ± 4.49**	24.83 ± 3.84**	819.34 ± 46.50**	635.04 ± 16.27**
DEN	99.16 ± 4.95 ^a	48.66 ± 5.57 ^a	675.64 ± 9.16 ^a	762.51 ± 18.95 ^a
DEN + EU10	69.33 ± 6.18**	22.66 ± 3.77**	754.03 ± 11.47**	702.41 ± 17.46 ^{a,b}
DEN + EU15	81.33 ± 9.62 ^{a,b}	21.66 ± 1.63**	708.84 ± 19.84 ^{a,b}	731.60 ± 18.18 ^{a,b}
DEN + EU20	93.83 ± 12.89 ^a	21.16 ± 2.85**	654.88 ± 9.17 ^a	832.93 ± 20.70 ^c

Data were represented as mean ± S.D. Significance is at $p < 0.05$.

^a Significant difference from DEN group ($p < 0.05$).

^{**} Significant difference from DEN group ($p < 0.01$).

^a Significant difference from control group ($p < 0.01$).

^b Significant difference from control group ($p < 0.05$).

^c Significant difference from control group ($p < 0.005$).

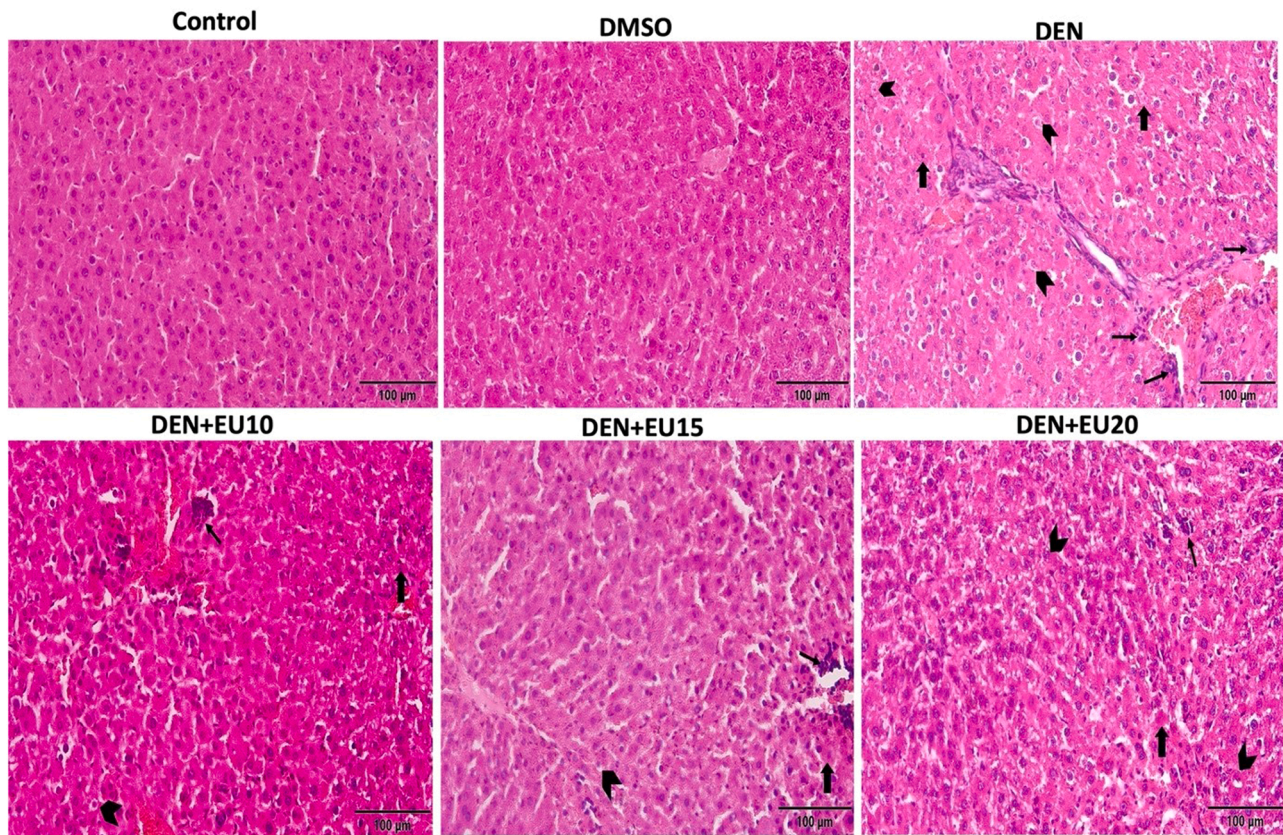


Fig. 1. Microscopic images of Hematoxylin & Eosin staining of the liver. Histological examination of the liver of rats: Control and DMSO groups; liver in normal histological architecture. DEN group; Severe inflammatory cell infiltration (thin arrow), severe hydropic degeneration (arrowhead) and moderate necrotic cells (thick arrow) were seen. DEN + EU10 and DEN + EU15 groups; moderate inflammatory cell infiltration (thin arrow), weak hydropic degeneration (arrowhead) and weak necrotic cells (thick arrow) were seen. DEN + EU20 group; weak inflammation cell infiltration (thin arrow), moderate hydropic degeneration (arrowhead) and weak necrotic cells (thick arrow) were seen. H&E. $\times 400$.

3.4. Effects of EU on IL-6, TNF- α , Caspase-3 and PCNA expressions in the DEN-induced rat liver

While wear caspase-3 expression was found in the DEN group, caspase-3 expression was negative in the other groups. PCNA expression was negative in the control and DMSO groups. PCNA expression of the DEN group was severe, while the PCNA expression of the DEN+PM10, DEN+PM15 and DEN+PM20 groups was wear. IL-6 expression was not observed in the control and DMSO groups. IL-6 and TNF- α expressions were severe in the DEN group, moderate in the DEN + EU20 group, and wear in the DEN + EU10 and DEN + EU15 groups (Fig. 4).

3.5. Stereological findings

In the stereological analyzes, there was no significant difference between the groups in terms of liver vessel volume densities of all groups ($p > 0.05$) (Table 2).

4. Discussion

The liver is a central vital organ that helps to regulate vital events and to detect and destroy pathogens that enter the organism through the intestines (Trefts et al., 2019). Because of the liver's role in important metabolic events and its connection with the gastrointestinal system, it is exposed to the toxicity of drugs and xenobiotics (Won et al., 2021; Vacani-Martins et al., 2021). Since the liver provides an average of 80 % of its blood flow from the vessels, compared to other organs, it is an extremely sensitive organ against toxic agents. Because some drugs and xenobiotics, which are absorbed in the stomach and intestines, pass

through the liver before entering the large circulation. Hepatocytes are metabolically active cells that are essential for numerous biological functions (Clemens et al., 2019). Nitrosamines are a large group of extremely potent carcinogen chemical compounds formed by the reaction of nitrite with primary, secondary, or tertiary amine precursors through external environmental conditions such as high temperature or acidic environment. DEN is a toxic substance that can cause liver damage. DNA damage from DEN can occur even after 24 h (Farzaei et al., 2018). Oral or parenteral administration of DEN causes serious damage to the liver even at very low doses (Tolba et al., 2015).

Oxidative stress is associated with the deterioration of the oxidant-antioxidant balance. The liver is structurally and functionally negatively affected by ROS. In addition, oxidative stress in the liver causes apoptosis in hepatocytes. Oxidative stress causes cellular deterioration in proteins, lipids and DNA, as well as degeneration, apoptosis and necrosis (Farzaei et al., 2018). It has been reported that the evaluation of ROS markers in hepatocytes and the determination of the degree of damage in the liver will contribute positively to treatments. In addition, it has been reported that it may help in explaining the pathogenesis of liver disorders (Cichoz-Lach and Michalak, 2014). MDA is released as a result of the effects of free radicals and oxidative damage to the lipid structures of cell membranes. To prevent the damage caused by ROS, there is a mechanism that will provide the defense, which is described as an antioxidant, and CAT is a member of this system (Özcan et al., 2015). Nitrosamines, which cause DNA damage, affect the mitochondrial metabolism of the cell, causing a disruption in the balance of reactive oxygen species (ROS) and antioxidants, resulting in oxidative stress (Santos et al., 2014; Kaya et al., 2020). In most studies, CAT activity and oxidative stress indicator MDA levels were evaluated to determine

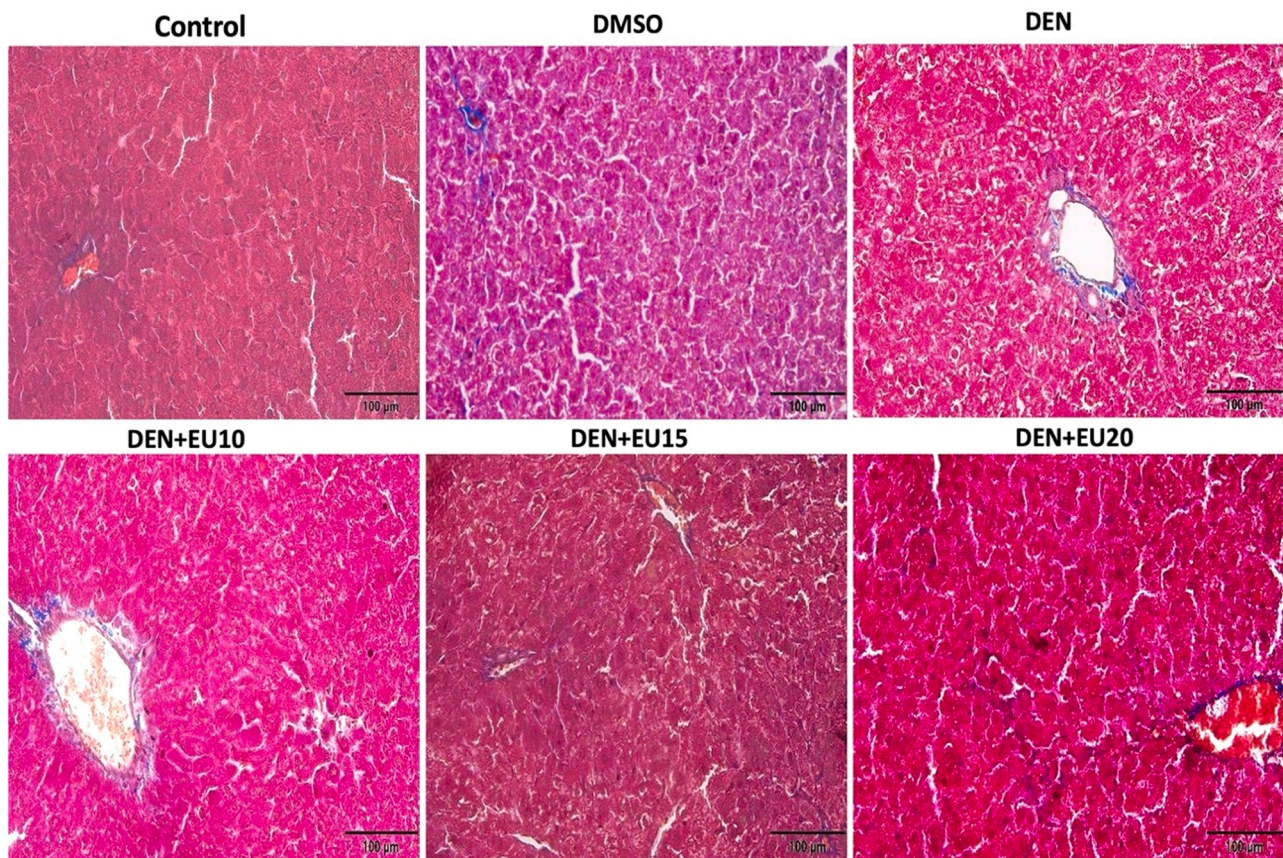


Fig. 2. Microscopic images of Masson-Trichrome staining of liver. No significant difference between groups in liver of all groups. Masson trichrome. $\times 400$.

antioxidant capacity (Calabrese and Rose-John, 2014; Özcan et al., 2015; You et al., 2021). Previous studies have reported that DEN causes an increase in the level of MDA, a marker of oxidative stress, and a decrease in the level of CAT (Mansour et al., 2019; Adebayo et al., 2020; Alsahli et al., 2021; You et al., 2021). In this study, CAT and MDA activities of liver tissue were investigated. DEN caused a decrease in CAT level, while an increase in MDA level. This indicates that DEN induced oxidative stress. This effect of DEN is consistent with the literature (Adebayo et al., 2020; You et al., 2021). Eumelanin is a pigment that affects reducing ROS thanks to its antioxidant property (Pinna et al., 2014; Swope and Abdel-Malek, 2018). The antioxidant properties of eumelanin have been reported in previous studies (Brenner and Hearing, 2008; El-Naggar and El-Ewasy, 2017). Hou et al. (2019) reported that eumelanin decreased the increased MDA level in ethanol-induced liver damage and increased the CAT level. In the present study, it was observed that 10 and 15 mg/kg doses of eumelanin decreased the increased DEN-induced MDA level and increased the decreased CAT level. These findings of our study prove that these doses of eumelanin can have an antioxidative effect on the liver. However, the fact that 20 mg/kg dose of eumelanin increases the MDA level compared to the DEN group, indicating that co-administration of this dose with DEN may increase the formation of free radicals in the liver and may have a cytotoxic effect (Kurian et al., 2015; Shalaby et al., 2019).

DEN causes cell damage by inducing oxidative stress in tissues. Hepatocellular necrosis is accompanied by increased cell proliferation in DEN-induced hepatic damage (Mansour et al., 2019). In previous studies, pathological changes such as infiltration, degeneration, and necrosis have been reported in the histopathological evaluation of the liver tissue of animals treated with DEN (Kang et al., 2013; Khan et al., 2017). In accordance with the literature, inflammatory cell infiltration, degeneration and necrosis were observed in the liver tissue of rats treated with DEN in our study. Abdelhalim et al. (2018) reported that

the vacuolar degeneration and necrosis observed in liver damage induced by gold nanoparticles were reduced by melanin treatment. In our study, we found that EU significantly reduced DEN-induced infiltration, hydropic degeneration, and necrosis in the liver. Our findings are compatible with the literature.

DEN is considered one of the toxic agents that cause necrosis and subsequent fibrosis in the liver (Rodriguez et al., 2020). The previous studies reported that fibrosis developed after DEN was administered at a dose of 50 mg/kg three times a week for 8 weeks or at a dose of 30 mg/kg twice a week for 11 weeks (Alsahli et al., 2021; Ding et al., 2021). However, in our study, there was no difference between DEN and other groups in the sections stained by Masson Trichrome staining. The absence of fibrosis in our study is thought to be dependent on the dose or duration of administration of DEN. Periodic acid releases aldehyde groups by separating the bonds between carbon atoms. As a result of the interaction of the aldehyde groups with the Schiff reagent, they acquire a purplish red color (Lefkowitz, 2006). With the PAS reaction, the glycogen and basement membrane in the cells are displayed. Besides determining glycogen synthesis by PAS staining, hepatocyte function is also evaluated (Hui et al., 2017). In the present study, it was observed that DEN caused hepatotoxicity by significantly decreasing the PAS staining intensity in the liver. However, 10 and 15 mg/kg EU doses reduced DEN-induced hepatotoxicity by significantly increasing the PAS staining intensity in the liver.

IL-6 is known to be associated with some types of cancer and various diseases associated with inflammation (Calabrese and Rose-John, 2014). Permanent activation of the signaling pathways of IL-6, which has a role in the metabolic functions of the liver, is harmful to the liver and may lead to tumor development (Calabrese and Rose-John, 2014; Özcan et al., 2015). TNF- α is synthesized by activated macrophages, endothelial cells, fibroblasts and lymphocytes; it causes tumor necrosis, synthesis of liver acute phase reactants and inflammation (Dursunoglu and

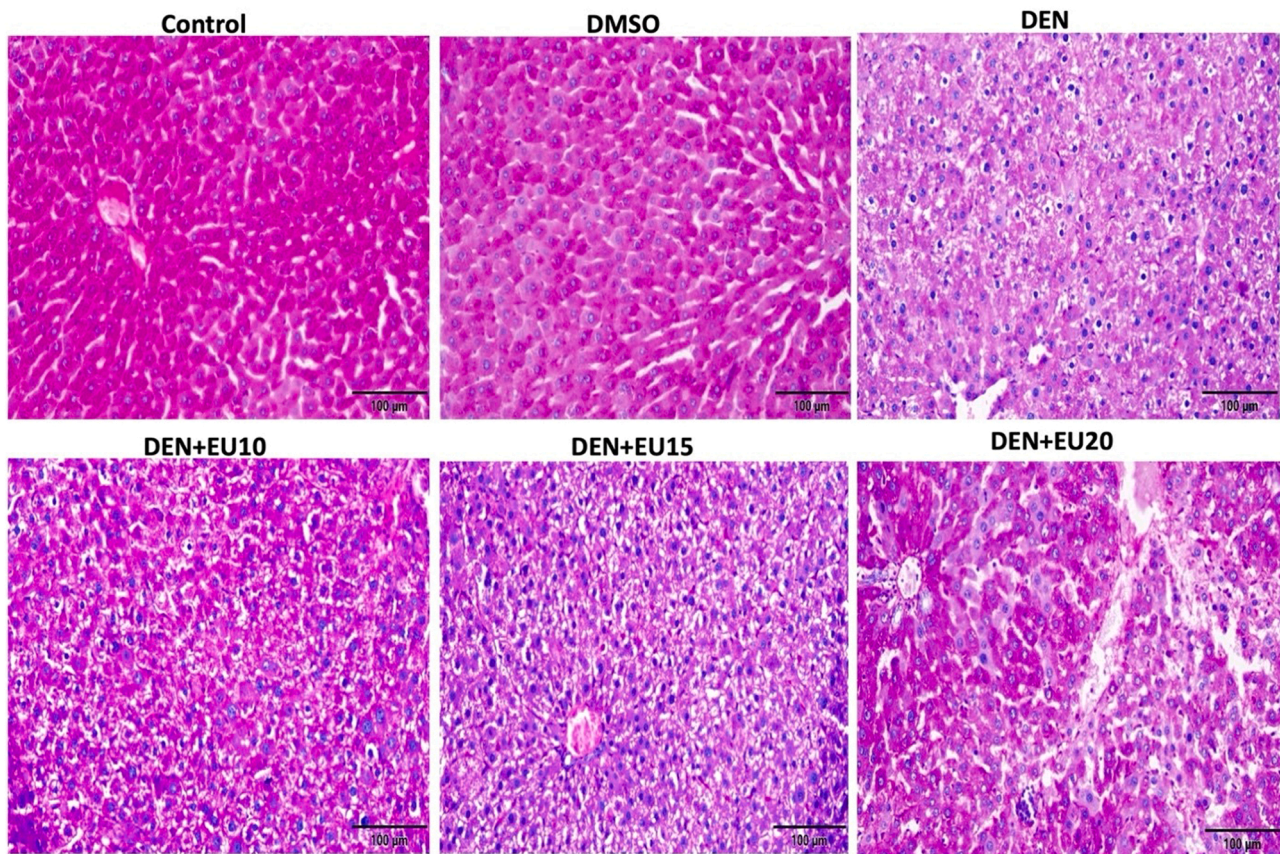


Fig. 3. Microscopic images of Periodic acid Schiff staining of the liver. Severe PAS (+) cells are observed in the control and DMSO groups. DEN and DEN + EU20 groups have wear PAS (+) cells. DEN + EU10 and DEN + EU15 groups have moderate PAS (+) cells. PAS. $\times$ 400.

Dursunoğlu, 2018).

Previous studies have reported that TNF- α plays a role in inflammation, apoptosis, and angiogenesis (Postal and Appenzeller, 2011; Aggarwal et al., 2012). Previous studies have revealed that IL-6 and TNF- α expression, which are important markers of inflammation, increase with DEN administration (El-Naggar and El-Ewasy, 2017; Mansour et al., 2019; Alsahli et al., 2021; Ding et al., 2021; You et al., 2021). Consistent with previous studies, in the current study, it was determined that DEN induces inflammation by increasing the expression of IL-6 and TNF- α in the liver. Abdelhalim et al. (2018) demonstrated that melanin inhibits liver inflammation in experimentally induced liver toxicity. Similarly, Kunwar et al. (2012) reported that melanin decreased the expression of proinflammatory mediators IL-6 and TNF- α . The immunohistochemical findings of our study revealed that especially the 10 and 15 mg/kg doses of EU showed anti-inflammatory effects by reducing the increased IL-6 and TNF- α expressions caused by DEN.

The presence of caspase-3 expression in tissues is one of the most important indicators of apoptosis (Choudhary et al., 2015). Apoptosis is necessary for the normal physiological process as well as for the elimination of cells that are considered a threat to the organism. Apoptosis occurs in immune system diseases, tumor formation and the presence of oxidative stress (Coşkun and Özgür, 2011). In DEN-induced hepatic injury, besides cell proliferation, hepatic cell death also occurs (Mansour et al., 2019). Previous studies have demonstrated that DEN induces cell apoptosis (Gupta et al., 2010). In the present study, low caspase-3 expression was observed in the DEN group. It was reported that melanin caused a decrease in the Bax/Bcl-XL ratio (Kunwar et al., 2012). The negative expression of caspase-3 in the groups given eumelanin together with DEN, compared to the DEN group, suggests that eumelanin suppresses apoptosis.

PCNA is one of the alternative methods used to demonstrate cells

(Foley et al., 2013). The balance between apoptosis and proliferation must be normal in maintaining homeostasis in tissues. It has been reported that decreasing apoptosis versus increasing proliferation is involved in the formation of carcinogenesis (Akşit and Bildik, 2008). Previous studies have reported that DEN causes an increase in PCNA expression (Chuang et al., 2000; Chakraborty et al., 2006; Subramaniam et al., 2021). In parallel with the literature, intense PCNA expression was observed in the DEN group in this study. However, PCNA expressions were lower in the DEN + EU10, DEN + EU15, and DEN + EU20 groups compared to the DEN group. The increase in PCNA levels in DEN-administered rats may prove that DEN causes hepatic cell proliferation. It is thought that the low PCNA expression in eumelanin groups suppresses uncontrolled cell proliferation in hepatocytes due to its hepatoprotective effects (Akşit and Bildik, 2008).

Morphological changes in the liver can affect the metabolic activity of the organism. In case of a change in the permeability status of the cell membrane or lysis of the cell, liver enzymes pass into the blood and values such as serum ALT and AST increase (Çömelekoğlu et al., 2000). It has been reported that DEN has detrimental effects on liver enzyme functions and has an effect on the transition of enzymes to the cytosol by disrupting the cell membrane (Adelani et al., 2020). With DEN and oxidative stress in the liver, liver enzyme levels increase and hepatocellular damage is determined (Pradeep et al., 2007). In most studies induced by DEN, DEN has been reported to cause an increase in ALT and AST enzyme levels (Gupta et al., 2010; Khan et al., 2017; Adebayo et al., 2020; Adelani et al., 2020; Ding et al., 2021; You et al., 2021). Increased AST and ALT levels indicate hepatotoxicity (Rezaie et al., 2013). In this study, it was observed that AST and ALT levels in rats in the DEN group were significantly higher than in the control group. The fact that AST and ALT levels in the DEN group are higher than in all other groups indicates that DEN causes damage to the liver (Rezaie et al., 2013). In a

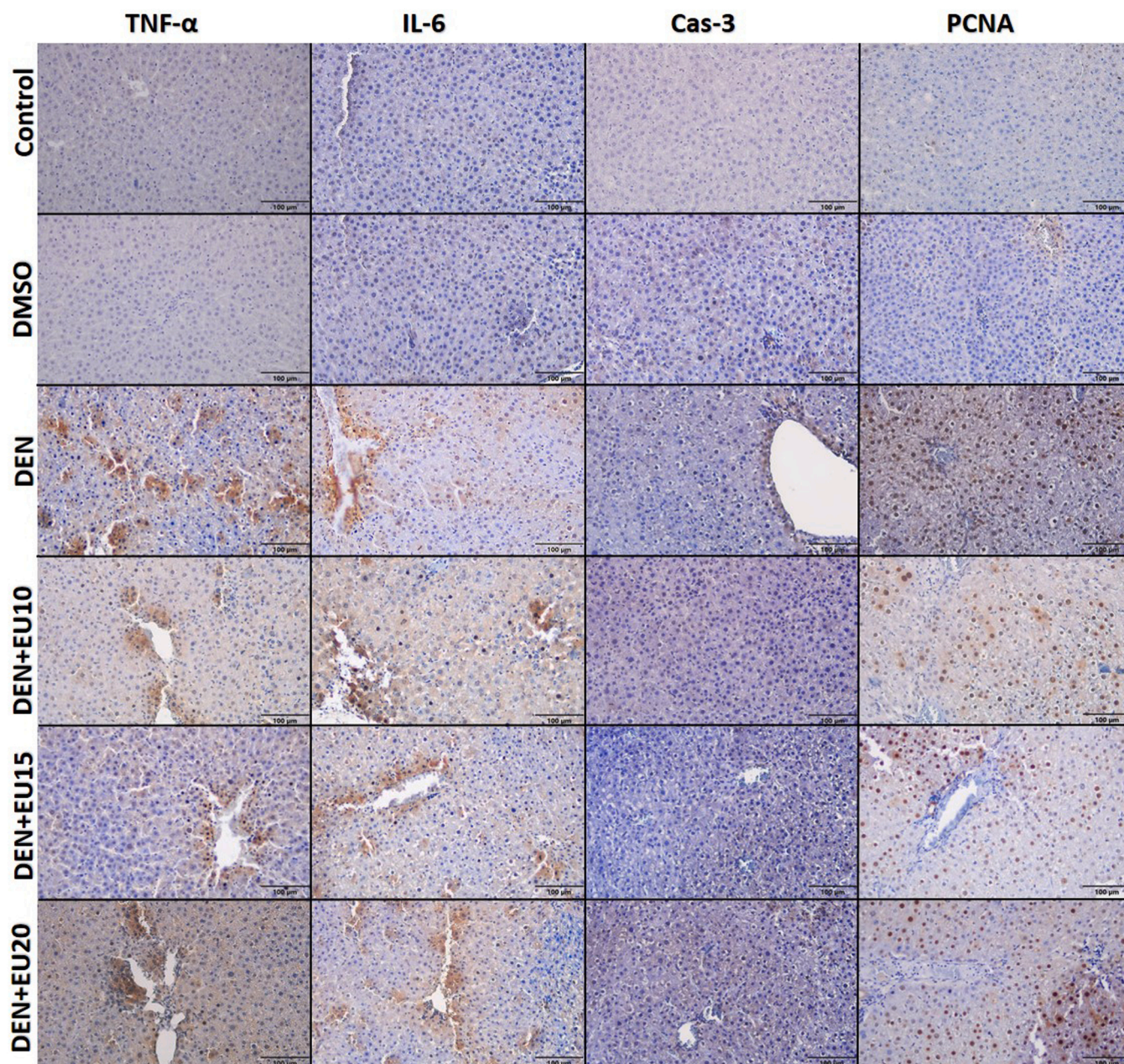


Fig. 4. Photomicrographs of TNF- α , IL-6, Caspase-3 and PCNA immunostaining in the liver. TNF- α and IL-6 expression of the DEN group was more intense than in the control group. TNF- α and IL-6 expressions of the DEN + EU10, DEN + EU15 and DEN + EU20 groups were lower than in the DEN group, but TNF- α and IL-6 expressions of the DEN + EU20 group were higher than in the DEN + EU10 and DEN + EU15 groups. While low caspase-3 expression was found in the DEN group, caspase-3 expression was negative in the other groups. PCNA expression of the DEN group was higher compared to the control group, while the PCNA expression of the DEN + EU10, DEN + EU15 and DEN + EU20 groups was lower than the DEN group. $\times 400$.

Table 2

Total vessel volume densities of the groups.

Groups	Total vein numerical density of liver
Control	1,61 $\pm$ 0,16
DMSO	1,47 $\pm$ 0,06
DEN	1,84 $\pm$ 0,72
DEN + EU10	1,96 $\pm$ 0,38
DEN + EU15	1,85 $\pm$ 0,49
DEN + EU20	2,02 $\pm$ 0,81

No significant difference between the groups ($p > 0.05$).

study with ethanol, it was reported that melanin provided a decrease in ethanol-induced increased ALT and AST levels. It has been reported that melanin has a protective effect against ethanol-induced liver damage (Hou et al., 2019). The ALT level of all groups of this study, in which DEN was given together with eumelanin, was found to be significantly lower than the DEN group. While the AST level in the DEN + EU10 group was significantly lower than in the DEN group and similar to the control group, it was observed that although the AST level of the DEN + EU15 group was significantly lower than the DEN group, there was a significant increase compared to the control group. Although the AST level of the DEN + EU20 group was less than the AST level of the DEN group, it was not statistically significant. However, all eumelanin doses significantly decreased the increase in DEN-induced ALT levels

and had similar values with the control group. The AST findings of our study revealed that the EU10 dose was the most effective dose in reducing the increase in DEN-induced AST, its effect decreased as the eumelanin dose increased, and the EU20 dose had no effect.

5. Conclusion

As a result, in this study, which was performed by administering a single dose of 200 mg/kg dose to rats, the structural and functional impairments in the liver caused by DEN were significantly restored by eumelanin treatment. DEN-induced apoptosis, inflammation and hepatocyte proliferation were inhibited by eumelanin. In addition, the increased oxidant and decreased antioxidant enzyme system caused by DEN were improved by eumelanin treatment. Moreover, eumelanin 10 mg/kg dose was determined to be the most effective dose.

CRedit authorship contribution statement

Seda Boğokşayan and **Fikret Altındağ**: conducted histopathological, immunohistochemical, biochemical and stereological experiments. **Fikret Altındağ**: interpreted the findings and wrote the manuscript. **Sinan Bayram**: synthesized Eumelanin. All authors read and approved the final manuscript.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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