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Effect of Urtica dioica Seed Extract on Aquaporin 1 and 7, Caspase-3 and Oxidant/Antioxidant Status in Diethylnitrosamine-induced Kidney Damage in Rats

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ABSTRACT

Diethylnitrosamine (DENA) is known to have a carcinogenic effect on the liver by stimulating oxidative stress and inflammation. There are few studies on the negative effects of DENA on kidney tissue, and the therapeutic effect of *Urtica dioica* Seed Extract (UDSE) against the negative effects of DENA is investigated for the first time in this study. This study investigated the protective effect of UDSE on kidney tissues of rats administered DENA. The rats were divided into four groups (n=8): control, UDSE (1 mL/kg/daily for 16 weeks), DENA (200 mg/kg/first day), DENA+UDSE (200 mg/kg/first-day single dose, and 1 mL/kg/daily for 16 weeks). At the end of the experiment, blood and kidney tissues were taken for biochemical and pathological analysis. DENA administration has been shown to increase oxidative stress in the kidneys and reduce antioxidant levels. Moreover, compared to the DENA group, DENA decreased the level of aquaporin (AQP)-1 in kidney tissue, while UDSE treatment increased both AQP-1 levels in kidney tissue and AQP-1 and 7 levels in the serum samples. Histopathological examination of the kidney revealed significant coagulation necrosis, especially in proximal tubular epithelial cells, hyperemia in capillaries, mononuclear cell infiltration between tubular areas, atrophy in the glomerular cluster, and adhesions with Bowman's capsule. In addition, there was a decrease in both caspase-3 (immunohistochemically) expression and TOS levels in the DENA+UDSE group compared to the DENA group, while an increase was seen in antioxidant levels. These findings indicated that UDSE may be an essential therapeutic agent against DENA-induced kidney injury.

Keywords: Diethylnitrosamine, *Urtica dioica*, kidney, histopathology, oxidative stress, rat

Introduction

Diethylnitrosamine (DENA) is a nitrosamine compound found in agrochemicals, alcoholic beverages, cheddar cheese, dried and fried foods, cigarette smoke, and pharmaceutical products (1,2). Nitrosamines produce their toxic effects primarily in the blood and liver. At the same time, it was reported that the other organs, such as the liver, lung, and kidney, where blood flow is high, are affected by the toxic effects of nitrosamines (3-5). In addition, it has been reported that oral or parenteral administration of nitrosamines in small doses may cause damage to many organs, especially the liver.

Singh et al. reported that DENA application triggered carcinogenesis in rat kidneys and increased serum parameters such as urea, creatine, and uric acid (6). Hassanen et al. determined that the application of DENA revealed epithelial vacuolation covering the renal tubules, vacuolation and obstruction of the glomerular cluster, proteinaceous cast in the lumen of the renal tubules, and focal necrosis of the renal tubules associated with inflammatory cell infiltration in rat kidneys (7). Oxidative stress (OS) is an imbalance between free radical production and body-active antioxidant capacity. It has been stated that excessive levels of OS may cause harmful effects on living things. However, studies have shown that antioxidant products can reduce OS and prevent tissue damage progression and complications (8-10). Cells contain antioxidant enzymes and proteins such as SOD,

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CAT and GSH. Antioxidant enzymes and proteins neutralize exogenous or endogenous free radicals in cells and maintain the oxidant/antioxidant balance.

Urtica dioica (UD) is a simple leafy plant with burning hairs in the Urticales team belonging to the nettle family (Urticaceae). UD was shown to have many pharmacological activities such as immunomodulatory, antioxidant (11,12), antibacterial (13), anti-viral (14), analgesic, anti-inflammatory (15), antihypertensive, anti-hyperlipidemic, anti-diabetic (16), hepatoprotective (17), anti-apoptotic (18), anti-carcinogenic (19), and radioprotective (20). However, it has been reported that taking exogenous antioxidant substances such as UD seed extract (UDSE) may be beneficial in the presence of exogenous oxidative stress stimuli (21). For this reason, in our study, we used UDSE, known to have antioxidant activity, against the adverse effects of DENA on kidney tissue.

Aquaporins (AQP) are water channel proteins expressed on the plasma membranes of cells, and they function to facilitate the passive transport of water across the plasma membrane due to osmotic changes (22). They are active in many physiological events, including renal water balance, epithelial fluid secretion, cell migration, and adipocyte metabolism. Inhibition of these water channel proteins by nephrotoxic drugs adversely affects the renal water balance (23). AQP-1 has relatively high water permeability but does not conduct ions or other solutes. AQP-7 is involved in rapidly transporting glycerol and water (24). Owumi et al. investigated DENA administration's effect on rats' hepatorenal function. At the end of the study, they reported that exposure to DENA in rats deteriorated the oxidant/antioxidant status in favor of oxidant, significantly exacerbating an increase in reactive oxygen and nitrogen species and lipid peroxidation, and histopathological lesions in the liver and kidneys (25).

Apoptosis is programmed cell death observed in many physiological or pathological events from embryonic to death. Expression of caspase-3 is the hallmark of apoptosis and can be used in cellular assays to quantify activators and inhibitors of the "death cascade" (26). Therefore, we evaluated caspase 3, an important marker in the apoptosis pathway.

Although many studies have revealed different properties of UD, we were still looking for research in our literature review on the protective effect of UDSE on DENA-induced kidney damage. Therefore, this study investigated the effect of AQP-1 and 7, caspase-3, and the oxidant/antioxidant status of UDSE on DENA-induced kidney damage in rats.

Materials and Methods

Chemicals and ELISA Kits: DENA was purchased from Sigma-Aldrich (Sigma-Aldrich, Merck KGaA, Germany, Catalog No: 62-75-9). Na_2HPO_4 was purchased from Sigma Aldrich Company to prepare the phosphate buffer. In addition, ELISA kits were purchased commercially from YL Biont (AQP-1: Catalog No: YLA1024RA, AQP-7: Catalog No: YLA1593RA, GSH: Catalog No: YLA0121RA, TOS: Catalog No: YLA1392RA, SOD: Catalog No: YLA0115RA, TAS: Catalog No: YLA1389RA, Catalog No: YLA0123RA), and GSH-Px: Catalog No: YLA0119RA) to measure all the biochemical parameters in the kidney and serum samples.

Plant Material and Preparation of UDSE: The UDS was purchased from a local commercial firm (Velibaba Spice Shop, Van, Turkey). Voucher samples are preserved in the herbarium of Van Yuzuncu Yil University, Faculty of Science, Department of Biology (Herbarium code: VANF-16778). The seed extract was prepared according to the method used by Yıldızhan et al. (20).

Animals and Experimental Design: The ethical committee permission for the study was obtained from the Experimental Animals Local Ethics Committee of Van Yuzuncu Yil University (approval protocol number: 2022/13-02, date 29/12/2022). In this study, 32 Albino Wistar rats, 2-3 months old, were used. The rats were housed in plastic cages with a 12-hour light and dark photoperiod at 24°C. Animals were given standard feed and drinking water ad libitum during the experiment. DENA was also dissolved in 0.9% saline. The dose and duration of DENA (27) and UDSE (28) were applied by partially modifying the method specified in the literature.

The rats were randomly divided into four groups (n=8).

Control: No administration was made (16 weeks).

DENA: DENA was administered once intraperitoneally at a 200 mg/kg dose on the first day of the trial (16 weeks).

UDSE: 1 mL/kg of UDSE was given daily orogastric for 16 weeks.

DENA+UDSE: DENA at 200 mg/kg was administered intraperitoneally on the first day of the trial, and 1 mL/kg of UDSE was given daily orogastric for 16 weeks.

At the end of the study, the abdominal areas of the rats were opened under anesthesia (ketamine hydrochloride: 50 mg/kg and xylazine: 10 mg/kg), intracardiac blood samples and right kidney tissues were taken for biochemical analysis, and left kidney tissues were placed in a 10% formaldehyde solution for histopathological and immunohistochemical examinations.

Separation of serum samples from blood: Blood samples taken into yellow-capped biochemistry tubes were centrifuged at 3500 xg for 10 min., and serum samples were stored at -80℃ until they were analyzed (29).

Homogenization of kidney tissues and supernatant production: The right kidney tissues were homogenised with an ultraturrax device (Ultra Turrax-T25) (30).

Measurement of Protein Quantity: The amount of kidney tissue protein was measured using the Bradford method (30).

Histopathological and Immunohistochemical Examination: At the end of the experiment, rats were necropsied, and kidney tissue samples were taken. The tissue samples were fixed in a 10% buffered formaldehyde solution and embedded in paraffin blocks after routine follow-up, and 4 µm sections were taken with a microtome. Normal slides were used for Hematoxylin and Eosin (H&E) staining, and poly-L-lysin-coated slides were used for immunohistochemical staining.

Immunohistochemical examination was performed according to the streptavidin-peroxidase method (ABC) using a streptavidin/biotin immunoperoxidase kit (Histostain-Plus Bulk Kit; Zymed, South San Francisco, CA, USA) to determine caspase-3 expression. Prepared sections were taken on adhesive slides and passed through the xylene and alcohol series. After washing the sections with PBS (phosphate buffer solution), endogenous peroxidase was inactivated in H_2O_2 (3%) for 20 min. After being placed in antigen retrieval solution (citrate buffer) and covered, the samples were subjected to heat treatment twice for 20 min. After removal from the oven, the samples were allowed to cool to room temperature. The samples were washed again with PBS and blocked with protein blocking for 20 min. The samples were incubated overnight at +4°C with caspase-3 (Abcam, ab 4051; 1/100 dilution) polyclonal antibodies. Sections were washed with PBS and incubated with biotinized secondary antibody at room temperature for 20 min. The sections were washed again with PBS and incubated in streptavidin peroxidase for 20 min. Then, they were washed in the same way with PBS. After washing, diaminobenzidine was added and kept for 1-2 min. Afterward, all the tissues were fixed in Mayer's hematoxylin for 1-2 min and washed in tap water. The sections were again passed through alcohol and xylene series and sealed using Entallen. Negative controls were used to confirm staining; these slides were reacted with PBS instead of primary antibodies. Sections were examined under a light microscope (Nikon 80i-DS-RI2) and photographed. As a result of this staining, each sample examined in 10 different fields was examined under a microscope at 20 magnification, the number of positively stained cells was counted, and the mean was calculated (21).

Statistical Analysis: The data determined were all expressed as the mean ± the standard error of the mean (SEM). The normality of the data was examined with the Shapiro-Wilk test, while the homogeneity of the variances of the independent groups was checked with the Levene test. All the biochemical biomarkers showed normal distribution at the end of the study. Since the data were normally distributed, following the One-Way ANOVA analysis, the post hoc (Tukey HSD) test was performed to determine which groups caused the difference separately. The statistical significance level was accepted as 5%. IBM SPSS Statistics for Windows 21.0 was used for Statistical computations (IBM Corp., Armonk, NY, USA).

Results

AQP-1 and 7 levels in the serum and kidney tissue: AQP-1 levels in serum samples were similar in all the groups, and there was no significant difference between them ($p > 0.05$, Figure 1A). The UDSE group had the highest AQP-7 level in serum samples compared to the other groups ($p < 0.05$). In addition, it was determined that the AQP-7 level of the DENA+UDSE group was significantly higher than the DENA group ($p < 0.05$, Figure 1B). When kidney AQP-1 levels were examined, the DENA group had the lowest level of AQP-1 compared to the other groups ($p < 0.05$), while there was no significant difference between the other groups (Figure 1C). The UDSE group had the highest value AQP-7 level in kidney tissue compared to the other groups ($p < 0.05$). It was determined that the AQP-7 level of the

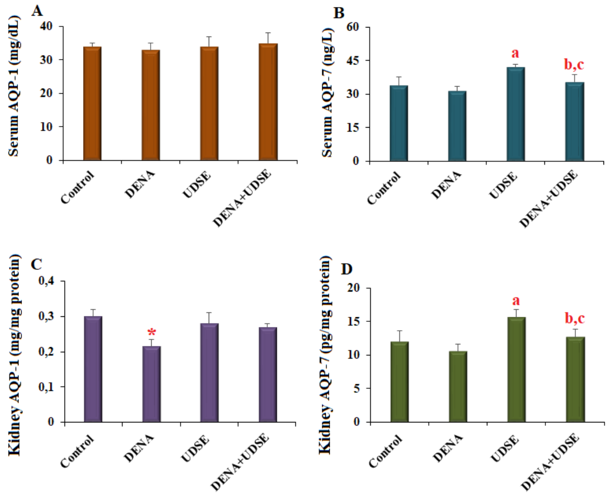

Fig. 1. Effect of UDSE on AQP-1 and 7 levels in the serum and kidney tissue samples of DENA-administered rats. (Values were given as mean ± SD). ([a]p< 0.05 compared with the control and DENA groups; [b]p< 0.05 compared with the UDSE group; [c]p< 0.05 compared with the DENA group; *p< 0.05 compared with the others groups)

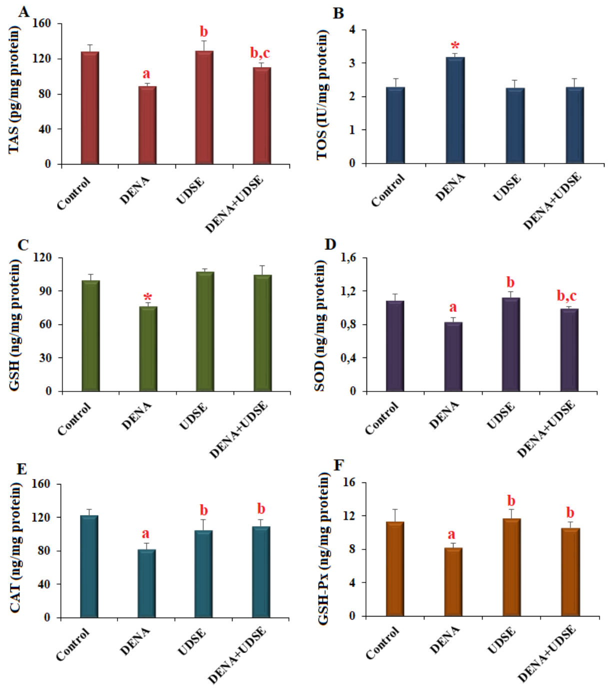

Fig. 2. Effect of UDSE on oxidant and antioxidant parameters in kidney tissue samples of DENA-administered rats. (Values were given as mean ± SD). ([a]p< 0.05 compared with the control group; [b]p< 0.05 compared with the DENA group; [c]p< 0.05 compared with the UDSE group; *p< 0.05 compared with the others groups)

The DENA+UDSE group was significantly higher than the DENA group (p< 0.05, Figure 1D).

Results of Oxidant/Antioxidant parameters: The results obtained showed that the TAS level of

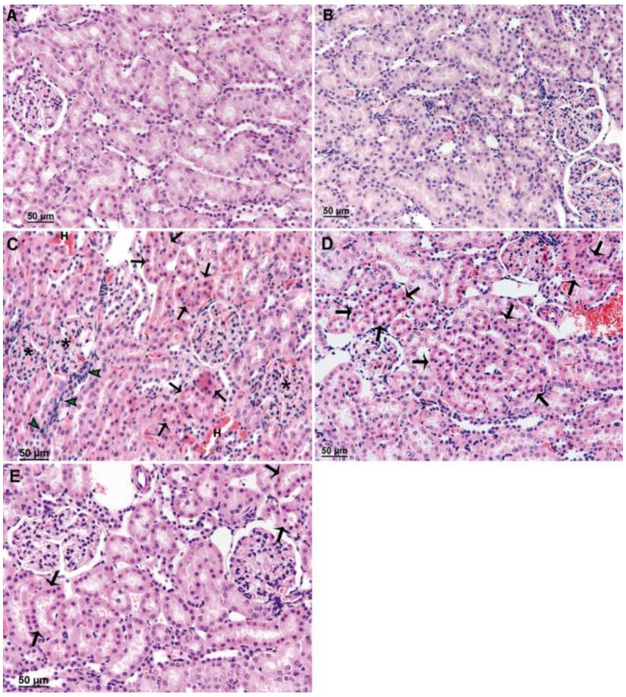

Fig. 3. Histopathological Photomicrographs (H&E staining) regard the effect of UDSE in kidney tissue of DENA-administered rats. (**A-B**) Normal histological appearance of the kidney parenchyma is observed in the control and UDSE groups. (**C-D**) In the DENA group, atrophy in the glomerulus and adhesions with Bowman's capsule (*), intertubular low-density inflammatory cell infiltration (arrowheads), coagulation necrosis in tubular epithelial cells (arrows), hyperemia in capillaries (H) are observed. (**E**) Coagulation necrosis (arrows) is observed in epithelial cells in some tubules in the DENA+UDSE group (Bar; 50 µm)

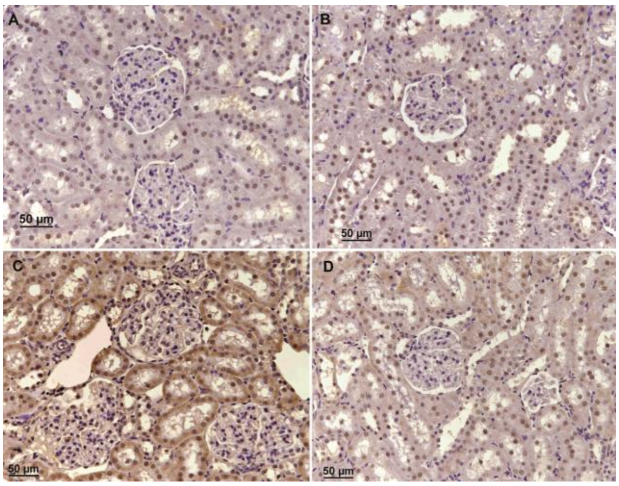

Fig. 4. Downregulation effect of UDSE on caspase-3 expression in DENA-induced nephrotoxicity. The caspase-3 expression was detected by immunohistochemical staining. Control group (**A**): UDSE group (**B**); DENA group (**C,** severe expression); DENA+UDSE group (**D**). (Bar; 50 µm)

Table 1: Effects of UDSE and DENA on The Kidney Structures (Affected Rats/Total Number of Rats)

Changes/lesions in kidney		Control	UDSE	DENA	DENA+UDSE	*p-Values
Necrosis		-/8b	-/8b	8/8a	6/8c	*
	Slight	-	-	5	5	
	Moderate	-	-	3	1	
	Severe	-	-	-	-	
Inflammation		-/8b	-/8b	8/8a	4/8c	
	Slight	-	-	6	4	*
	Moderate	-	-	2	-	
	Severe	-	-	-	-	
Hyperemia/congestion		-/8b	-/8b	8/8a	5/8c	
	Slight	-	-	3	5	*
	Moderate	-	-	4	-	
	Severe	-	-	1	-	
Glomerulopathy		-/8b	-/8b	8/8a	3/8c	
	Slight	-	-	6	3	*
	Moderate	-	-	2	-	
	Severe	-	-	-	-	

[a,b,c;] Values in a row with no common superscript letter differ significantly from each other. * $p < 0.01$.

The DENA group was the lowest compared to the other groups. It was determined that the TAS level of the UDSE and DENA+UDSE groups was higher than the DENA group ($p< 0.05$, Figure 2A). When the TOS levels between the groups were examined, the TOS level of the DENA group was the highest according to the other groups ($p< 0.05$, Figure 2B). At the same time, there was no significant difference between the other groups. When the GSH levels were examined between the groups, the GSH level of the DENA group was the lowest, while no significant difference was found between the other groups ($p< 0.05$, Figure 2C). A decrease was observed in the SOD, CAT, and GSH-Px levels in the kidney tissue after DENA administration (Figure 2D-E). However, SOD, CAT, and GSH-Px levels of the DENA+UDSE group were higher than the DENA group ($p< 0.05$).

Histopathological Findings: The findings observed in the kidneys as a result of the histopathological examinations were summarized in Table 1. Microscopically, a normal microscopic appearance was detected in the kidneys of the control and UDSE groups. The most important morphological changes in the DENA group are significant coagulation necrosis, especially in proximal tubular epithelial cells, hyperemia in capillaries, infiltration of mononuclear cells in intertubular areas, atrophy of glomerular tuft and adhesions with Bowman's capsule were detected. It was noted that the findings in the DENA+UDSE group were significantly milder compared to the DENA group (Figure 3).

Caspase-3 Expressions: The immunohistochemical method evaluated the expression caspase-3 in kidney tissue (Figure 4). DENA administration significantly increased the expression of caspase-3 in the kidney. However, compared to the DENA group, caspase-3 was significantly lower than in the UDSE and DENA+UDSE groups.

Discussion

DENA is considered a highly toxic environmental carcinogen, leading to the generation of ROS and causing cellular damage and oxidative stress (6). The current study demonstrated that the administration of DENA proved kidney damage by reducing AQP1 and 7 levels, reducing antioxidant status, and impairing kidney histopathology (29). Also, the oxidant and antioxidant parameters in the kidneys were evaluated to determine the occurrence of kidney damage caused by DENA administration. TOS levels showed that administration of DENA increased oxidative stress in kidneys. In addition, DENA decreased the level of antioxidants such as GSH, SOD, GSH-Px, catalase and TAS. In the histopathological examination of the kidney tissue, significant coagulation necrosis, especially in proximal tubular epithelial cells, hyperemia in capillaries, infiltration of mononuclear cells in intertubular areas, atrophy in the glomerular cluster and adhesions with Bowman's capsule were detected. In addition, increased caspase-3 expression was observed in the kidneys after DENA administration. We observed that UDSE treatment decreased kidney damage by reducing DENA-induced oxidative stress through its antioxidant properties (11).

It has been reported that toxicity and dysfunction in the kidneys significantly affect AQP levels, and AQP levels in kidney tissues of rats were significantly reduced with different toxic agents (31). Kucukler et al. reported a decrease in AQP-1 levels caused by oxidative stress and inflammation in rat kidneys (32). This study determined that DENA had toxic effects on the kidney, leading to renal damage and significantly reducing the level of AQP-1 in the kidney. Also, this study revealed that treating UDSE decreased the toxic effects of DENA, alleviated renal dysfunction, and increased AQP-1 levels in kidney tissues (Figure 1). In a study conducted by Shahzad et al. on the effect of quercetin on AQP-7 protein levels in the presence of sex hormones, they showed that AQP-7 mRNA expression was increased approximately 2.5 times in the uterus depending on quercetin doses (10, 50 and 100 mg/kg). In the current study, similar to the above study, there was a significant increase in AQP-7 levels in serum and kidney tissues in the DENA-UDSE group compared to the DENA group (33).

DENA is commonly found in smoked and fried foods. DENA has been proven hepatotoxic in rodent models, although its mechanism of kidney damage is not fully understood. Adebayo et al. reported that the administration of DENA induced hepatic oxidative stress in mice through mechanisms including activation of proinflammatory enzymes and proteins, suppression of apoptosis, and alteration of oxidant/antioxidant balance (34). Owumi et al., In their study on kidney toxicity caused by combined exposure to fluoride and DENA in rats, reported that besides the decrease in antioxidant enzyme activities, the increase in ROS and nitrogen species and lipid peroxidation were significantly exacerbated in kidney toxicity (35). Hassanen et al., in the study investigating the protective effect of rosemary against kidney damage caused by DENA exposure in rats, observed an increase in lipid peroxidation levels in rats in the DENA group. In contrast, they noted a significant decrease in CAT and GSH-PX levels (7). Our study results, which support the literature data, showed that the application of DENA significantly reduced the TAS level in the kidney tissue. However, the use of UDSE and the DENA increased the decreased TAS level by DENA in the kidney tissue. Also, the TOS level of the DENA group was the highest, while a significant decrease was observed in the TOS level in the groups that received UDSE treatment. Furthermore, we determined that the use of UDSE against kidney damage caused by DENA significantly increased the levels of SOD, CAT, and GSH-Px compared to the DENA group (Figure 2). These results showed that using UDSE decreased the oxidative stress caused by DENA.

In tissue toxicity studies, evaluating whether the tissue is in a normal structure (29,36) is important. A study reported that the administration of DENA to rats revealed abnormal results in the structure of the kidney tissue and caused glomerular congestion, tubular congestion, and inflammatory cell invasion (6). In a different study, rats treated with DENA showed severe diffuse congestion, infiltration by inflammatory cells, and mild disseminated segmented glomeruli necrosis. In contrast, rats treated with fluoride alone showed tubular desquamation and disseminated glomerular congestion with cellular infiltration by inflammatory cells in the kidney (35). Our study detected a normal microscopic appearance in the kidneys of the control and UDSE group. The most important morphological changes in the DENA group were detected, especially prominent coagulation necrosis in proximal tubular epithelial cells, hyperemia in capillaries, infiltration of mononuclear cells in intertubular areas, atrophy in the glomerular cluster and adhesions with Bowman's capsule. In addition, it was observed that the findings in the DENA+UDSE group were significantly milder than those in the DENA group (Table 1 and Figure 3).

Caspase-3 belongs to the cysteine protease family and has been recognized as a critical effector enzyme in inducing cell apoptosis (37). Furthermore, caspase-3 is an inactive pro-caspase in living cells and activates during apoptosis, ultimately achieving cell death. Therefore, it is an essential marker for toxicological studies (38). Owumi et al. also noted that exposure to DENA exacerbates hepatorenal injury through increased oxido-inflammatory responses and caspase-3 activation in rats (35). Subramanian et al. reported increased protein expressions of Bcl2, Bax, and cleaved-caspase 3 in DENA-induced hepatocarcinogenesis in rats (39). In our study, DENA administration significantly increased caspase-3 expression in the kidney. In addition, it was observed that caspase-3 levels decreased with UDSE treatment compared to the DENA group (Figure 4).

This study showed that the administration of DENA decreased AQP-1 and antioxidant enzyme levels, increased oxidant status, and caused oxidative damage in kidney tissue samples. However, it was determined that co-administration of DENA and UDSE reduced kidney and oxidative damage. These results showed that UDSE treatment may suppress DENA-induced kidney and oxidative damage. However, there is a need for studies investigating the signaling pathways at the molecular level on how exactly DENA increases kidney damage and how DENA increases kidney damage and UDSE inhibits these parameters and kidney damage.

Conflict of Interest: All authors declare no conflict of interest.

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