Antitumor activity of *Urtica dioica* seed extract on diethylnitrosamine-induced liver carcinogenesis in rats

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Hepatocellular carcinoma (HCC) is a significant health problem for human life; therefore, new therapeutic approaches are essential. *In vitro* studies have shown that the extract of *Urtica dioica* seed extract (UDSE) may be a crucial protective agent to prevent HCC. Therefore, this study aimed to investigate the antitumor efficacy of UDSE in the process of carcinogenesis induced by diethylnitrosamine (DENA). The antitumor efficacy was evaluated by examining liver tissue histopathology and expression of Hep par-1, alpha-fetoprotein (AFP), caspase-3, and inducible nitric oxide synthase (iNOS) in the liver tissue and activities/levels of aspartate transaminase (AST), alanine transaminase (ALT), lactate dehydrogenase (LDH), carbohydrate antigen (CA) 15-3, CA 19-9, CA 125-II in the serum, and also total oxidative stress (TOS), malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT), total antioxidant status (TAS) in the serum and liver. In addition, real-time PCR was used to evaluate the levels of tumor necrosis factor-alpha (TNF-α), interleukin (IL-1β, IL-6), and proliferating cell nuclear antigen (*PCNA*) in liver tissue. It was observed that DENA application increased liver function tests, cancer markers, apoptosis, and proinflammatory cytokine levels, but UDSE application and DENA suppressed these increases. The findings and histopathological data demonstrated that the UDSE has a very significant antitumor efficacy on the process of DENA-induced hepatocellular carcinogenesis, which appears to be attributable to its antioxidant, anti-apoptotic, and anti-proliferative activity.

Keywords: Biochemical markers, Diethylnitrosamine, Histopathology, Liver carcinogenesis, Rat, *Urtica dioica*

Hepatocellular carcinoma (HCC) represents the majority of primary malignant tumors of the liver. Hepatocellular carcinoma (HCC) ranks 3rd among the causes of death from cancer worldwide and 8th among the most common cancers[1]. Hepatitis B and C viral infections, exposure to aflatoxins, cirrhosis, chronic alcohol consumption, diabetes mellitus, obesity, and nitrosamine group chemicals increase the risk of liver cancer[2]. However, most hepatocellular carcinomas are thought to be due to HBV and HCV infections. Although the mechanism of action of these viruses in tumor formation is not known exactly, it is emphasised that hepatocellular damage, production of active oxygen derivatives that can damage DNA, stimulation of hepatocyte proliferation, and chronic inflammation mediated by the immune system may be effective [3].

Nitrosamines have carcinogenic, teratogenic, toxic, and mutagenic effects and can be found in various food stuffs, drugs, cosmetics, pesticides, and cigarette smoke[4]. DENA, a class of nitrosamines, is widely used as an inducer of HCC in experimental animals in the scientific world[1]. Since nitrosamines are generally metabolised in the liver, they cause cancer in other organs, especially the liver[5]. DENA is hydroxylated by cytochrome P-450 isoenzymes in hepatocytes in the liver and becomes active by the alkylation mechanism. Some radicals (such as the ethyl radical) formed due to this activation are stimulators of the carcinogenesis process[6]. Bioactive DENA interacts with DNA to form pro-mutagenic products such as O_4- and O_6-ethyl deoxythymidine and O_6-ethyl deoxyguanosine. These intermediates can cause mutations in DNA base structures by causing ethylating, inhibiting tumor suppressor genes such as p53, and activating proto-oncogenes. In addition, these reactions result from DNA strand breaks, depurination, damage, and disruptions in coding gene sequences. This usually results in hepatocellular carcinoma[7]. In addition, DENA can cause oxidative stress and cell damage by increasing ROS[8]. An increase in intracellular amounts of ROS causes DNA

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damage, protein carbonylation, mitochondrial damage, and lipid peroxidation. As a result of lipid peroxidation, toxic products such as MDA and 4-hydroxynonenal trigger mutagenicity and carcinogenicity by damaging DNA. This situation can cause many diseases, including cancer[9]. In addition, DENA causes the secretion of mediators such as cytokines and growth factors in cells as an inflammatory response. These cause proliferation in mutated cells[10].

Plants, which form the basis of modern medicine, have been used to protect or treat many diseases for humanity in the past and present. Today, medicinal and aromatic plants used in different conditions have become widespread and scientific studies have gradually increased[11]. Due to its rich phenolic composition, *Urtica dioica* (UD) is one of the most studied natural antioxidant plants, and its economic importance is increasing rapidly. In previous studies, UDwas shown to have many pharmacological activities such as immunomodulatory, antioxidant, antibacterial, anti-viral, analgesic, anti-inflammatory, antihypertensive, anti-hyperlipidemic, anti-diabetic, hepatoprotective, anti-carcinogenic, and radioprotective[12,13]. Although different properties of UD have been revealed in many studies, we did not find a survey of the anti-carcinogenic effect of UD seed extract (UDSE) in experimental in vivo HCC models in our literature review. This study investigated the antitumor efficacy of UDSE in the process of DENA-induced hepatocellular carcinogenesis in rats.

Materials and Methods

Experimental design

Wistar Albino rats were randomly divided into five groups (n=8). The rats were kept in rooms illuminated with a rhythm of 12 h of light and 12 h of dark, with a temperature of $22 \pm 2°C$ and a humidity of 60%. Animals were given standard feed and drinking water *ad libitum* during the experiment. The ethical committee permission for the study was obtained from the Experimental Animals Local Ethics Committee of Van Yuzuncu Yil University (approval protocol number: 2019/01, date 31/01/2019). DENA was also dissolved in 0.9% saline. The dose and duration of DENA were applied by partially modifying the method specified in the literature[14]. The groups were created as follows.

Control group: Standard pellet feed and drinking water were given for 16 weeks.

DENA group: DENA was administered once intraperitoneally at a 200 mg/kg dose on the first day of the experiment, and pelleted feed and drinking water were given for 16 weeks[15].

UDSE group: One mL/kg of UDSE was given daily orogastric and standard pellet feed for 16 weeks.

DENA+UDSE-1 group: DENA at a dose of 200 mg/kg was administered intraperitoneally on the first day of the experiment, and 1 mL/kg of UDS ethanol extract was given daily orogastric for 16 weeks.

DENA+UDSE-2 group: DENA at a dose of 200 mg/kg was administered intraperitoneally on the first day of the experiment, and 1 mL/kg of UDS ether oil extract was administered daily orogastric to each rat for 16 weeks.

Termination of the experiment and collection of samples

At the end of the application, the rats were anaesthetized with 50 mg/kg Ketamine hydrochloride+10 mg/kg dose of Xylazine hydrochloride, and the blood was collected intracardiac from all the rats. The obtained serum and plasma samples were stored in a deep freezer (-80°C) until the relevant analyses were performed. In addition, systemic necropsies of the rats were performed, and the liver tissue was macroscopically examined and photographed. Tissue samples were taken for histopathological examination.

Plant Material and Preparation of UDSE extracts

The UDS was purchased from a local commercial firm (Van, Turkey). Voucher samples are preserved in the herbarium of Van Yuzuncu Yil University, Faculty of Science, Department of Biology (Herbarium code: VANF-16778).

Alcohol Extract

UDS were ground in an electric mill. Then, 50 g of the stinging nettle seed sample was placed in a glass beaker and mixed with 500 mL of ethyl alcohol.The beaker was covered with aluminium foil and homogenised in a shaker for 2 h. After the homogenate was passed through a sieve, the pulp was discarded, and the remaining mixture was transferred to 10 mL falcon tubes and centrifuged at $3500 \times g$ for 5 min.400 mL of the supernatant sample was removed from the solvent by evaporation in an evaporator at +37°C for approximately 1 h and 45 min.At the end of the process, the concentrated extract was placed in falcon tubes and kept at -80°C for 48 h. Then, the frozen samples were dried in a lyophilizer at 0.030 mBar pressure and -54°C for three days. The

lyophilized saline fraction was stored at -20°C until starting the analysis.

Diethyl Ether Extract

The UD seeds were ground in an electric mill. The ground seed was placed on filter paper and in the Soxhlet extractor. Diethyl ether was used as the solvent. After extraction, the mixture was filtered. Oil extraction and diethyl ether were separated using a rotary evaporator and obtained the oil extract[16].

Chemicals

DENA was purchased from Sigma-Aldrich (Sigma-Aldrich, Merck KGaA, Germany). Na_2HPO_4 used to prepare the phosphate buffer was purchased from Sigma Aldrich Company. In addition, ELISA kits were purchased commercially from YL Biont (Shanghai, China) to measure all the biochemical parameters in liver and serum samples.

Separation of serum samples from blood

Intracardiac blood samples were placed in yellow-capped biochemistry tubes and centrifuged at $3500 \times g$ for 10 min. The remaining serum samples were transferred to another Eppendorf tube and kept at -80°C until they were studied.

Obtaining homogenate from liver samples

1.8 mL of phosphate buffer with pH: 7.4 and 50 mM was added to 0.2 g of liver tissue. After homogenisation with Ultraturrax (Ultra Turrax-T25) for about 15 sec, the mixture was centrifuged at $3000 \times g$ for 20 min. The homogenate was transferred to another tube and kept at -80°C until studied.

Measurement of routine biochemistry parameters

AST, ALT, LDH, CA-15-3, CA-19-9, CA-125 II, AFP, and CEA activities or levels in serum samples were measured by using the appropriate calibrator, control, and kit for each parameter with the chemiluminescent microparticle immunological method at the device of the routine biochemical analyser (Abbott Architect c16000, U.S.A). The AST, ALT, and LDH activities were expressed as U/L, and the results of other parameters were expressed as U/mL or U/dL.

Measurement method of apoptosis, oxidative stress, and antioxidant parameters

Caspase-3, BcL-2, TAS, TOS, GSH, SOD, CAT, and GSH-Pxvalues were measured with enzyme-linked immunosorbent assay (ELISA) kits. ELISA kits were purchased commercially from YL Biont (Shanghai, China) to measure all the biochemical parameters in liver and serum samples.

Measurement of MDA by high-pressure liquid chromatography (HPLC) method

The serum (50 μL) or tissue homogenate was placed in a tube. 750 μL of 0.44 M H_3PO_4, 250 μL of TBA, and 450 µL of distilled water were added to the samples. The tubes were tightly capped and placed in a boiling water bath for 60 min. The tubes were then cooled on ice or in tap water. Alkaline methanol (50 mL + 4.5 mL and 1 M of NaOH) was added to the mixture in a 1:1 ratio (1.5 mL). The mixture was centrifuged at $2500 \times g$ for 3 min. 200 μL of the supernatant remaining in the upper part was placed in a vial and loaded into the HPLC device.

RP18 with 150x4.6 mm and 5μm particle width was used as a measurement column in the HPLC device. For the mobile phase, 400 mL of 50 mM phosphate buffer (pH: 6.8) and 600 mL of methanol were mixed. The device's flow rate was set to 0.8 mL/min, and the injection volume was set to 20 μL. Readings were done against standard samples prepared at different concentrations with 1,1,3,3-tetra ethoxy propane in a fluorescent detector at 527 nm excitation and 551 nm emission wavelengths.

Histopathological Examination

After the tissue pieces were fixed in buffered formaldehyde solution, a routine follow-up was performed and embedded in paraffin blocks. Then, sections of 4 μm were taken with a microtome.

Immunohistochemical Examination

AFP, HSA (Hep par-1), caspase-3, and iNOS expressions were determined using the streptavidin/biotin immunoperoxidase kit (Histostain-Plus Bulk Kit; Zymed, South San Francisco, CA, USA) according to the streptavidin-peroxidase method (ABC). Prepared sections were passed through the xylene and alcohol series. After washing the coverslips with PBS (phosphate buffer solution), endogenous peroxidase was inactivated, keeping the samples in 3% H_2O_2 for 20 min. After the samples were placed in antigen retrieval solution (citrate buffer) and covered, they were heated twice for 20 min. After the samples were removed from the oven, they were kept at room temperature. After the tissues were rewashed with PBS, they were blocked with protein blocking (non-immune serum) for 20 min. AFP (Santa Cruz, sc-8399; 1/100 dilution), HSA (Santa Cruz, sc-58693; 1/100 dilution), caspase-3

(Abcam, ab-4051; 1/100 dilution), and iNOS polyclonal antibodies (Abcam, ab-15323; 1/100 dilution) were dropped on tissue samples and incubated overnight at +4°C. Coverslips were washed with PBS and incubated with a biotinized secondary antibody at room temperature for 20 min. The slides washed with PBS were incubated in streptavidin-peroxidase for 20 min and then washed with PBS in the same way. Diaminobenzidine (DAB) was dripped on the samples and left for 1-2 min. All tissues were kept in Mayer's hematoxylin for 1-2 min and washed in tap water. Sections passed through alcohol and xylene series were closed using enthralled. Negative controls were used to confirm the staining process. These slides were reacted with PBS instead of primary antibodies. Positive staining cells were counted, and the averages were calculated by examining 10 different areas at 20 magnifications on the microscope.

Real-time PCR analysis

Liver tissue samples were sampled during the necropsy and stored at -80°C until RNA extractions. RNA isolations from the tissues were carried out using the GenJET RNA purification Kit (Thermo Scientific, USA). Following the manufacturer's protocol, total RNAs were extracted from the liver tissues. RNA quality (260/280, 260/230) and RNA concentration (ng/μL) were determined spectrophotometrically by using NanoDrop 2000c (Thermo Scientific, USA) and stored at -80°C until other processes. Before cDNA synthesis, RNA integrities were investigated by agarose gel electrophoresis. RNA with desired integrity and quality was determined, and cDNA synthesis was carried out. High-capacity cDNA Reverse Transcription Kit (Thermo Scientific, USA) was used for cDNA synthesis. Real-time quantitative PCR (RT-qPCR) was performed with 2x AmplifyMe Syber Green Master Mix (Blirt, Poland) and carried out in duplicate reactions. Primers from literature or newly designed primers were used to determine gene expression levels of IL-6, IL-1β, TNF-α, proliferating cell nuclear antigen (*PCNA*), and housekeeping gene beta-actin (*ACTB*) (Table 1). For designing new primers, the online Primer-Blast tool was used. Hairpin, self, and hetero-dimer properties were checked as described previously[17]. The ensemble genome database also contained SNP and transcript variants (www.ensembl.org). Single Nucleotide Polymorphisms (SNPs) and transcript variants of the gene were also checked on the ensemble genome database (www.ensembl.org). PCR cycling conditions were as follows: initial denaturation at 95°C for 5 min, denaturation at 95°C for 5 s, annealing at 60°C for 20 s, and extension at 72°C for 5 s. Amplicon specificities were checked by melting curve and gel electrophoresis. Relative gene expression levels were determined using the $2^{-\Delta\Delta CT}$ method in which *ACTB* was used as a calibrator gene[18].

Statistical Analysis

SPSS package program was used for biochemical analyzes (version 21). Descriptive statistics of the groups were given as mean and standard deviation. The Shapiro-Wilk test was used to determine whether the data were normally distributed. Since the data were normally distributed, whether there were significant differences between the groups within the same parameter was evaluated with the Kruskal-Wallis test. One-way ANOVA was carried out to assess gene expression. Results with a p-value of 0.05 or less were considered significant.

Results

Biochemical Findings

The levels of AST, ALT, and LDH from serum liver function tests in the DENA group were higher than in the other experimental groups ($P =0.001$). Still, these parameter values were considerably lower

Table 1 — Primers used in RT-qPCR for the assessment of several pro-inflammatory and proliferative gene expressions

Gene	Primers (5′>3′)	PCR product	Reference
ACTB	F: GCCTTCCTTCCTGGGTATGG	91 bp	In this study
	R: GAGGTCTTTACGGATGTCAACG		
PCNA	F: TGCAGATGTACCCCTTGTTGT	105 bp	In this study
	R: AGCAACGCCTAAGATCCTTCT		
TNF-α	F: ACCACGCTCTTCTGTCTACTG	169 bp	[19]
	R: CTTGGTGGTTTGCTACGAC		
IL-1β	F: GCCTCAAGGGGAAGAATCTAT	117 bp	[20]
	R: CAAACCGCTTTTCCATCTTCT		
IL-6	F: GCCCTTCAGGAACAGCTATGA	80 bp	[21]
	R:TGTCAACAACATCAGTCCCAAGA		

F: Forward, R: Reverse, ACTB: Actin beta, *PCNA*: Proliferating cell nuclear antigen, TNF-α: Tumornecrosis factor alpha, IL: Interleukin

in the DENA+UDSE groups compared to the DENA group (P =0.001). In addition, AST, ALT, and LDH values in the UDSE group were remarkably lower than in the control and other experimental groups (P =0.001). The different results are detailed in (Fig. 1A- C).

Tumor activities such as CA 15-3, CA 19-9, and CA 125-II of serum samples in the DENA group were significantly higher than in the other experimental groups (P =0.001). AFP and CEA values in the DENA group were higher than in the control and UDSE groups (CEA, P =0.001, AFP; P =0.029). However, CA 15-3, CA 19-9, and CA 125-II activities in the DENA+UDSE groups were observed to be considerably lower than in the DENA group (P =0.001), while AFP and CEA values were partially decreased (P >0.05). Apoptotic caspase-3 values in the DENA group were higher than in the other experimental groups (P =0.002), while these values in the DENA+UDSE group, especially the DENA+UDSE-2 group, were considerably lower than the other experimental groups (P =0.002). While anti-apoptotic BcL-2 levels in the DENA group were relatively low compared to the control and UDSE groups (P =0.010), they were slightly higher in the DENA+UDSE groups compared to the DENA group (P ≥0.05). Detailed results are represented in (Fig. 2).

TAS values in serum samples of the DENA group were lower than in the control, UDSE, and DENA+UDSE groups. Still, these data were significantly higher in the UDSE and DENA+UDSE-2 groups compared to the DENA group (Fig. 3A, P =0.049). In addition, serum TOS values of the DENA group were partially high than in the other experimental groups (P =0.399), but there was no significant difference between them (Fig. 3B).

When the antioxidant enzyme and protein levels were examined, the serum GSH values of the DENA group were partially lower than the other experimental groups. At the same time, they were somewhat higher in all the groups with UDSE compared to the control and DENA groups (Fig. 3C, P >0.05). While the SOD levels of the DENA group were slightly lower than the other experimental groups, these values were partially high in the DENA+UDSE-2 group according to the DENA group (Fig. 3D, P =0.078).There was a slightly different situation in CAT and GSH-Px values. While the CAT and GSH-Px values in the DENA group were relatively low compared to the other experimental groups, they were pretty high in the UDSE and DENA+UDSE-1 groups and were similar to the control values (CAT, P =0.002; GSH-Px, P =0.001; Fig. 3E and F).

When the oxidative stress indicator lipid peroxidation levels were examined, the MDA levels of the DENA group were the highest compared to the other experimental groups (P =0.001), while serum MDA levels in the UDSE group were the lowest according to the other experimental groups. In addition, serum MDA values of the DENA+UDSE-2 group were similar to the control group. The other results are detailed in (Fig. 3G).

Antioxidant and oxidative stress values in liver tissue were parallel to serum samples' parameters. While the liver TAS values of the DENA group were lower than those of the other experimental groups (Fig. 4A), TOS activities were higher than those of the control, UDSE, and DENA+UDSE-2 groups (P =0.004).In addition, liver TOS values of the DENA+ UDSE-1 group were slightly lower than those of the DENA group (P >0.05, Fig. 4B). When the antioxidant enzyme and protein values were examined, liver GSH, SOD, and CAT values of the DENA group were relatively low according to the other experimental groups (P <0.05). But these values
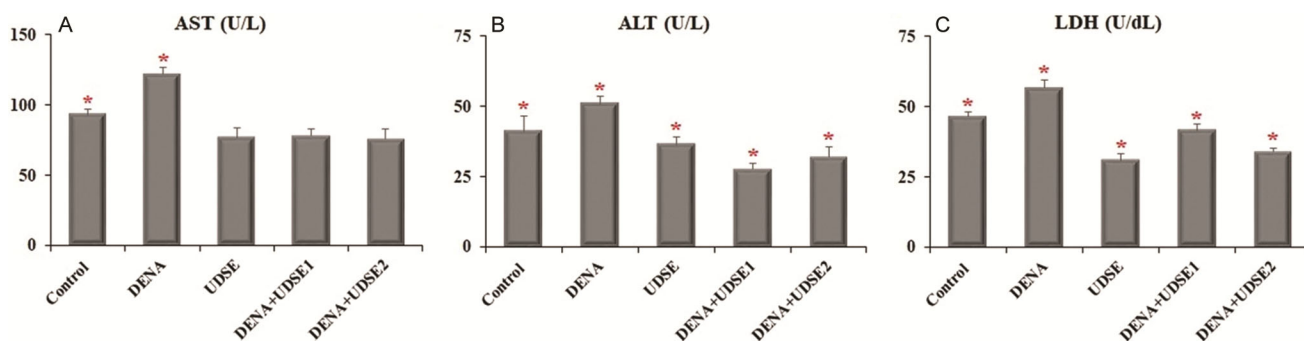

Fig. 1 — A: Comparison of liver function test results between experimental groups. ALT: Alanine transaminase, AST: Aspartate transaminase, LDH: lactate dehydrogenase, *p: It is significant compared to other experimental groups (P =0.001)
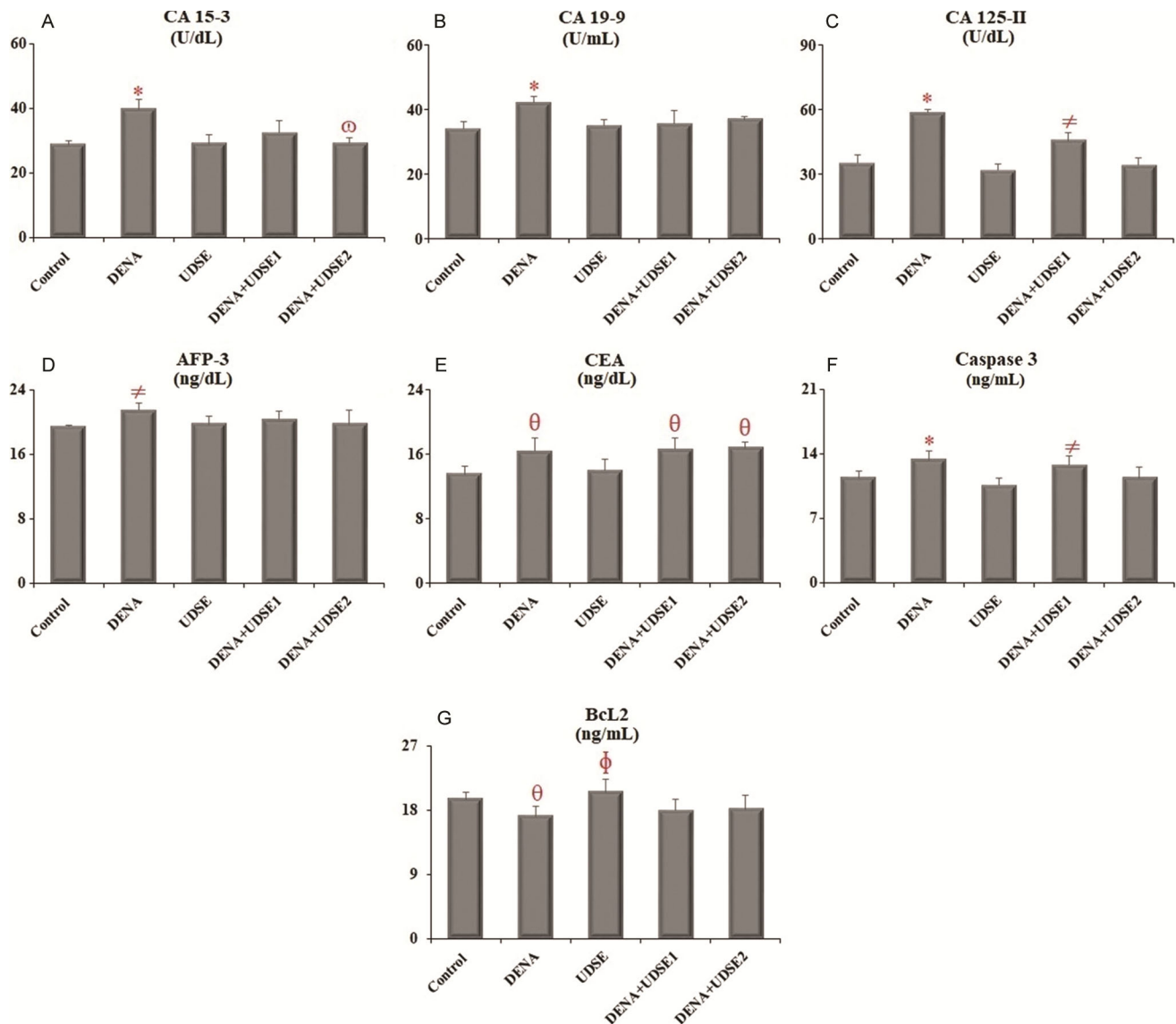
Fig. 2 — The mean and standard deviation values of serum cancer and apoptosis markers. CA 15-3: carbohydrate antigen 15-3 (U/dL), CA 19-9: carbohydrate antigen 19-9 (U/20 mL), CA 125-II: carbohydrate antigen 125-II (U/dL), AFP-3: alpha fetoprotein-3 (ng/dL), CEA: carcinoma embryonic antigen (ng/dL), Caspase-3 (ng/20 mL), BcL2 (ng/20 mL), *p: It is significant according to the other experimental groups (P <0.05). ‡p: It is significant according to the control, UDSE, and DENA+UDSE2 groups (P <0.05). θp: It is significant according to the control and UDSE groups (P <0.05). ϕp: It is significant according to the DENA+UDSE-1 and DENA+UDSE-2 groups (P <0.05). ωp: It is significant according to the DENA+UDSE-1 group (P <0.05)

were higher than in the DEN group in all the groups with UDSE and especially in the DENA+UDSE-1 group (P <0.05, Fig. 4C-E). In addition, GSH-Px levels were lower in the DENA group compared to the other experimental groups. However, in the DENA+UDSE groups, these values were similar to the control group and were significantly restored (Fig. 4F).

Liver lipid peroxidation levels were also similar to serum MDA levels. While the liver MDA levels of the DENA group were higher than the other

experimental groups, these values in all the groups with UDSE were lower than in the DENA group (P =0.001, Fig. 4G).

When liver apoptosis markers were examined biochemically, liver caspase-3 values of the DENA group were higher than in the other experimental groups. However, caspase-3 values in all the groups with UDSE were lower than in the DEN group (P =0.001), and these values were similar to the control values (Fig. 4H). In addition, BcL-2 levels in the DENA group were lower than in the other
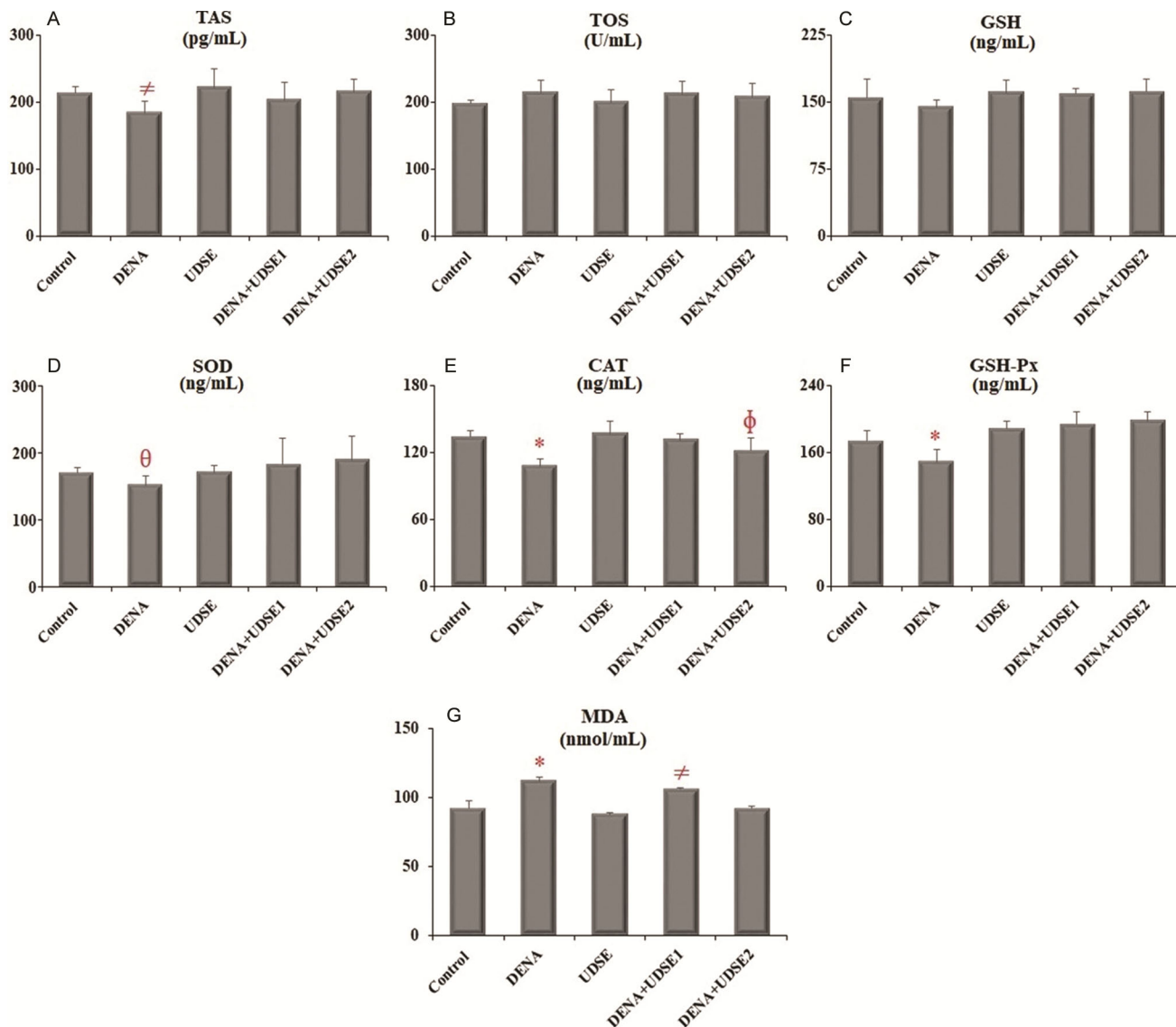
Fig. 3 — Comparison between experimental groups of mean and standard deviation values of some oxidant/antioxidant parameters and lipid peroxidation. TAS: total antioxidant capacity (pg/10 mL), TOS: total oxidative stress (U/50 mL), GSH: reduced glutathione (ng/mL), SOD: superoxide dismutase (ng/dL), CAT: catalase (ng/5 mL), GSH-Px: glutathione peroxidase (ng/10 mL),MDA: malondialdehyde (nmol/dL).*p: It is significant compared to other experimental groups (P <0.05). $^{\neq}$p: It is significant according to the control, *Urtica dioica* seed extract (UDSE), and Diethyl nitrosamine (DENA)+ (UDSE-2) groups (P <0.05). $^{\theta}$p: It is significant according to the DENA+UDSE-2 group (P <0.05).$^{\phi}$p: It is significant according to the control, DENA, and UDSE groups (P <0.05)

experimental groups. But, liver BcL-2 levels in all the groups with UDSE were similar to the control values (Fig. 4I).

Macroscopic Findings

The livers of the control (Fig. 5A) and UDSE group (Fig. 5B) rats were standard view. However, the livers of the rats in the DENA group were partially larger and blunted at the edges. In addition, they were darker in colour and had a delicate granular appearance (Fig. 5C). The livers of rats in the DENA+UDSE groups had an almost similar appearance to the control group. However, a fine delicate appearance in the DENA+UDSE-2 group was observed in two livers similar to the DENA group but at a milder level (Fig. 5D).

Histopathological Findings

Livers in control and UDSE groups had normal histological appearance (Fig. 5E). Tumoral cell proliferations were not observed in the livers of the DENA group. However, dysplasia hepatocytes, characterized by large and small cell changes and considered histological precursors of hepatocellular
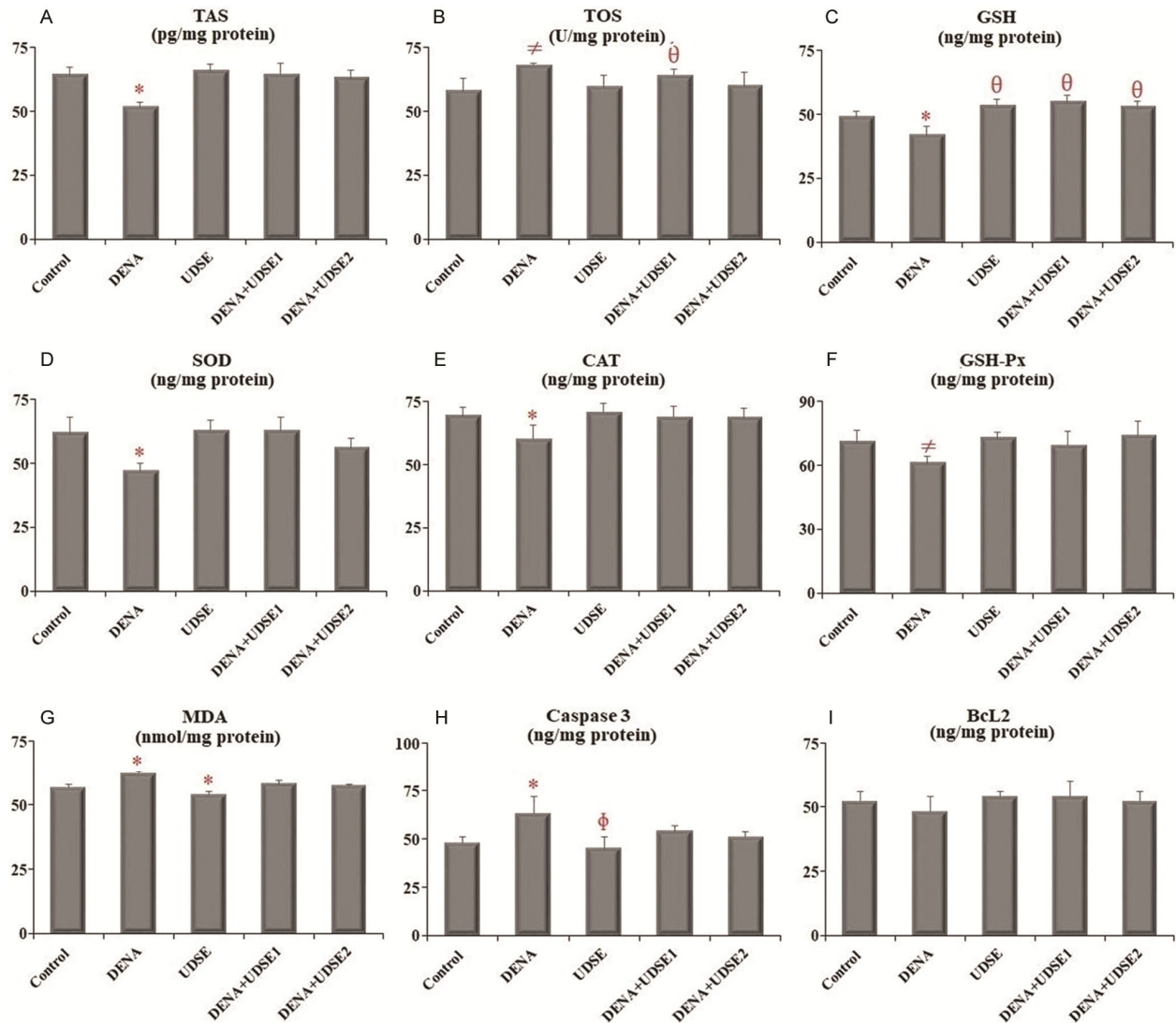
Fig. 4 — Collective comparison of the mean and standard deviation values of apoptosis and some oxidant/antioxidant parameters in liver tissue. TAS: total antioxidant capacity (pg/10 mg protein), TOS total oxidative stress (U/50 mg protein), GSH: reduced glutathione (ng/mg protein), SOD: superoxide dismutase (ng/100 mg protein), CAT: catalase (ng/10 mg protein), GSH-Px: glutathione peroxidase (ng/10 mg protein), MDA: malondialdehyde (nmol/20 mg protein), Caspase-3 (ng/300 mg protein), BcL2 (ng/200 mg protein). *p: It is significant compared to other experimental groups ($P < 0.05$). $^{\neq}$p: It is significant according to the control, UDSE, and DENA+UDSE-2 groups ($P < 0.05$). $^{\theta}$p: It is significant according to the control group ($P < 0.05$). $^{\phi}$p: It is significant according to the UDSE group ($P < 0.05$)

carcinomas, were commonly seen. It was observed that dysplastic changes in hepatocytes were more prominent, especially around the vena centralis, mainly covering the midzoanal regions and sometimes even the portal areas. It was noted that these dysplasias around the vena centralis were mostly in the form of small cell changes. Minor cell changes included the formation of more prominent and darker basophilic stained nucleolus in the nuclei of hepatocytes, marginal hyperchromasia and major vesicular appearance, dark basophilic or pale staining in the cytoplasm, and fine powdering of cytoplasmic

structures (cytoplasmolysis) (Fig. 5F). It was determined that in hepatocytes with significant cell changes, the ratio of the nucleus to the cytoplasm was impaired in favour of the nucleus. The nucleus was larger at varying rates and had different morphological structures (karyocytomegaly), darker stains, and many nucleoli of various sizes (Fig. 5G). Hydropic degeneration was observed in one or a few hepatocytes in the liver parenchyma and many hepatocyte assemblies. As a result of the degenerations, the alignment of the remark cords was distorted, and the sinusoids were narrowed.
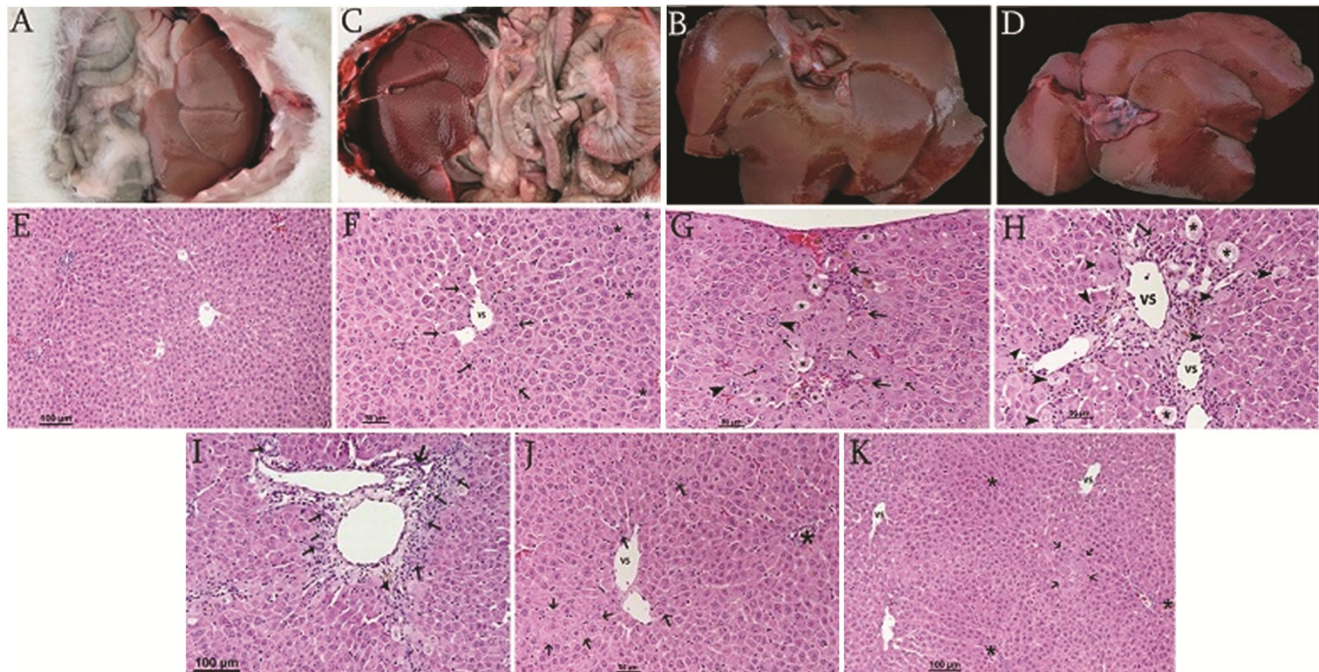
Fig. 5 — Macroscopic views of the livers of the study groups and histopathological appearance of cross-sections of the rat liver. In the control group (A) and UDSE group (B), the normal formation of the liver is observed. DENA group (C); partial enlargement of the liver, mass at the edges, darker colour and a diffuse fine granular appearance on the surface are observed. DENA+UDSE groups (D); although the liver appears primarily normal, a slightly fine granular surface (*) is observed in some parts, similar to that in the DENA group. Control group (E); the normal histological appearance of the liver. H.E. Bar; 100 μm. DENA group (F); small dysplastic cell (arrows) changes are observed in hepatocytes around the vena centralis, and normal hepatocytes (*) are kept in the periportal regions. Dysplastic small cell (thin arrows) and megakaryocytic large cell (arrowheads) changes, degenerate-necrotic (*) and hyalinized degenerate-necrotic (arrows) hepatocytes are observed in hepatocytes (G); Fibrosis, cholestasis, mononuclear cell infiltration, cystic degenerate hepatocytes (*), hyaline body (arrow) and degenerated necrotic hepatocytes at different stages (arrow heads) around the vena centralis (VS) in the liver (H). H.E. Bar; 50 μm. Newly proliferating bile ducts in the portal region of the liver, increased loose connective tissue around the portal vein (fibrosis), cholestasis (arrowhead), inflammatory cell infiltration, including a small number of eosinophil leukocytes (I). H.E. Bar; 100 μm. DENA+UDSE-1 group (J); dysplastic small cell (arrows) changes are observed in hepatocytes around the vena centralis, and a focus of degeneration (area between thick arrows) including normal hepatocytes (*) and a small number of hepatocytes in the periportal region. H.E. Bar; 50 μm. DENA+UDSE-2 group (K); dysplastic small cell changes in hepatocytes around the vena centralis, normal hepatocytes (*) in the periportal region, and degeneration focus (area between arrows) including a small number of hepatocytes are observed. H.E. Bar; 100 μm

It was noted that a few hepatocytes around the portal and periacinar fibrosis areas underwent severe balloon-like degeneration and eventually became large cystic spaces. Some degenerate-necrotic changes were in hyalinisation or typically hyaline bodies (apoptotic cells) (Fig. 5G and H). In some localisations, thin fibrous bands were seen extending from the portal and periacinar fibrosis areas to the liver capsule. A slight or prominent collapse was observed in the liver capsule, where the fibrous bands reached (Fig. 5G). Hyperplasia in the bile ducts and intrahepatic cholestasis were detected in the portal areas with fibrosis (Fig. 5I and E). In addition, lymphocytic-eosinophilic cell infiltrates were observed mainly in the portal and periacinar fibrosis regions and less frequently in the parenchyma. In addition, some sinusoids detected mild macro and microvesicular adiposity in hepatocytes, hyperplasia in Kupffer cells, and dilatation in some sinusoids.

Almost similar morphological changes were seen in the groups that received DENA and UDSE. Morphological changes in the livers of all the rats in these groups were significantly reduced compared to the DENA group. Unlike the DENA group, large hydropic degeneration areas and necrotic changes in the DENA+UDSE groups were not detected in these groups. In addition, lesions in these groups were a few lobules in the form of degenerative cell foci consisting of a few cells (Fig. 5J). In addition, dysplastic small cell changes in the DENA+UDSE groups were limited, especially to the periacinar regions. However, in contrast to the DENA group,

Lesions	Control	DENA	DENA+UDSE-1	DENA+UDSE-2	P value
Table 2 — Comparison of lesions observed in the liver between experimental groups					
Enlargement -fine-grained surface	-/8[b]	8/8[a]	2/8[b]	1/8[b]	*
Slight	-	1	2	1	
Moderate	-	7	-	-	
Severe	-	-	-	-	
		-	-	-	
Hydropic degeneration	-/8[b]	8/8[a]	8/8[c]	8/8[c]	* *
Slight	-	1	7	6	
Moderate	-	5	1	2	
Severe	-	2	-	-	
Dysplastic hepatocytes	-/8[b]	8/8[a]	8/8[c]	8/8[c]	**
Slight	-	3	8	6	
Moderate	-	3	-	-	
Severe	-	2	-	-	
Portal fibrosis	-/8[b]	8/8[a]	-/8[b]	-8[b]	*
Slight	-	1	-	-	
Moderate	-	7	-	-	
Severe	-	-	-	-	
Bile Duct proliferation	-/8[b]	8/8[a]	2/8[b]	1/8[b]	*
Slight	-	5	2	1	
Moderate	-	3	-	-	
Severe	-	-	-	-	

[a, b]: The difference between values with different letters on the same line is significant (*P* <0.001). * *P* <0.05. ** *P* <0.01). DENA: Diethyl nitrosamine, UDSE: *Urtica dioica* seed extract

degeneration of hepatocytes as cystic spaces and hyalinized degenerative-necrotic appearance (apoptotic cells) were not observed in these groups. Bile duct proliferation and portal fibrosis were mild only in the DENA+UDSE-2 group, in two and one rats, respectively (Fig. 5K, Table 2).

Immunohistochemical Findings

Hepatocyte specific antigen (HSA)/Hepatocyte paraffin-1 (Hep Par-1): It was determined that the mean value of positive cell number in the DENA group (60.1 ± 9.1, Fig. 6A and D) was higher than in the DENA+UDSE-1 (45.6 ± 20.3, Fig. 6A and E) and DENA+UDSE-2 (53 ± 8.2, Fig. 6A and F) groups.

Alfa fetoprotein (AFP): Mean value of AFP positive cell number of the DENA group (100.5±5.5, Fig.6A and D) was higher than the mean values of the DENA+UDSE-1 (40.3±5.1, Fig. 6A and E) and DENA+ UDSE-2 (29.1±4, Fig. 6A and F).

Caspase 3: No immunopositive staining was observed in control (Fig. 6A) and UDSE (Fig. 6B) groups. The mean value of caspase-3 positive cells in the DENA group (37.8 ± 5.4, Fig. 6A and D) was higher than the mean values of the DENA+UDSE-1 (21.6±5, Fig. 6A and E) and DENA+ UDSE-2 (18.5±4.5, Fig. 6A and F).

Inducible nitric oxide synthase (iNOS): in all the groups, iNOS immunoreaction was observed intracytoplasmically in Kupffer cells and fixed liver macrophages. The reactions were more pronounced, especially in the centrilobular regions. The strongest immunepositivity was found in the UDSE group (Fig. 6C), the weakest reaction was in the DENA group (Fig. 6D), and moderate levels were found in the DENA+UDSE group. However, the expression in UDSE-1 was stronger than in UDSE-2 (Fig. 6E).

RT-qPCR Findings

PCNA mRNA expression levels were investigated to evaluate proliferation in liver tissue in the experimental groups. As shown in Figure 7A, upregulation of *PCNA* was observed in the DENA-treated group, which may indicate carcinogenesis in the liver of the DENA-treated group. However, *PCNA* expression was insignificant in the DENA+UDSE-1 and DENA+UDSE-2, indicating that UD extracts protected the liver from DENA-induced carcinogenesis.

TNF-α, IL-1β, and IL-6 are pro-inflammatory cytokines, and their expression triggers tissue inflammation. Therefore, TNF-α, IL-1β, and IL-6 expression levels were investigated to evaluate the role of the anti-inflammatory action of UDSE in DENA-
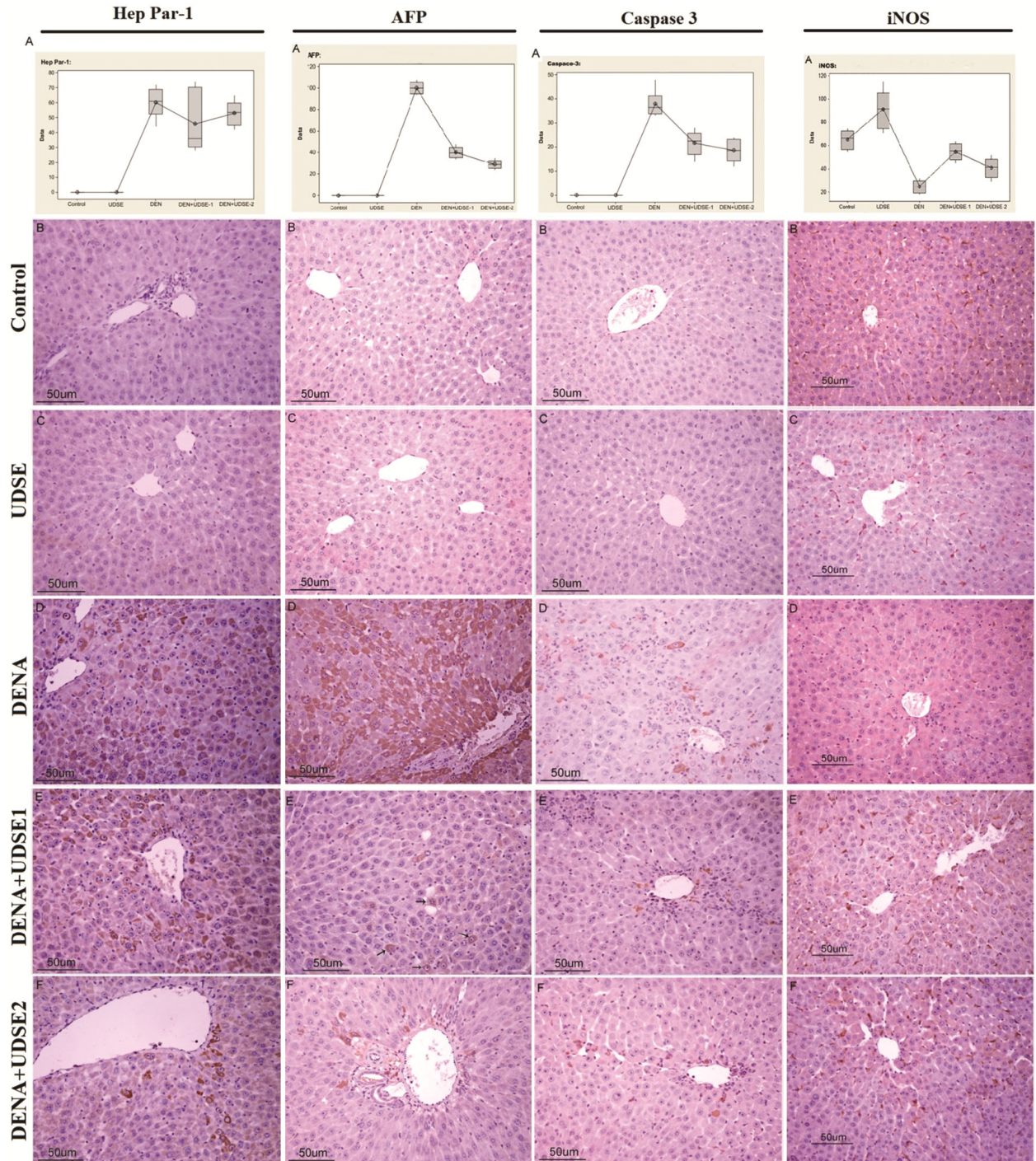
Fig. 6 — Hep Par-1 immunolocalisation in cross-sections of the rat liver. (A) Graph of mean Hep Par-1 positive cells from all groups. (B) Control group, (C) UDSE group, (D) DENA group, (E) DENA+UDSE-1 group and (F) DENA+UDSE-2 group, liver, Hep Par-1, IHC, Bar=50 μm. AFP immunolocalisation in cross-sections of the rat liver. (A) Graph of mean Hep Par-1 positive cells from all groups. (B) Control group, (C) UDSE group, (D) DENA group, (E) DENA+UDSE-1 group and (F) DENA+UDSE-2 group, liver, AFP, IHC, Bar=50 μm. Caspase 3 immunolocalisation in cross-sections of the rat liver. (A) Graph of mean Hep Par-1 positive cells from all groups. (B) Control group, (C) UDSE group, (D) DENA group, (E) DENA+UDSE-1 group and (F) DENA+UDSE-2 group, liver, caspase 3, IHC, Bar=50 μm. iNOS immunolocalisation in cross-sections of the rat liver. (A) Graph of mean Hep Par-1 positive cells from all groups. (B) Control group, (C) UDSE group, (D) DENA group, (E) DENA+UDSE-1 group and (F) DENA+UDSE-2 group, liver, iNOS, IHC, Bar=50 μm
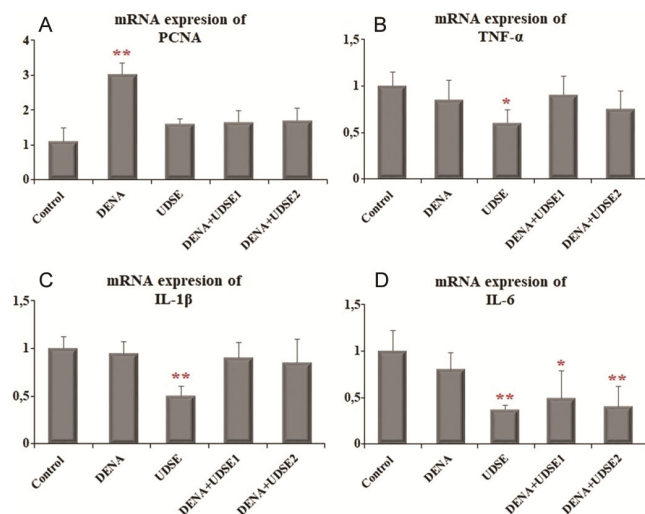
Fig. 7 — Proliferation marker and pro-inflamatory cytokine gene expression levels. (A) *PCNA* expression in liver tissue of rats in different experimental groups. Data represent mean and standard deviation. ** indicates significance level, *P* <0.01. Pro-inflammatory cytokine expression levels in liver tissue of rats. (B) TNF-α; *(C)* IL-1β; and (D) IL-6 gene expression in liver tissue in experimental groups. Data represent mean and standard deviation. *and** indicate significance levels, *P* <0.05 and *P* <0.01, respectively

induced carcinogenesis. Unfortunately, DENA treatment did not induce TNF-α, IL-1β, and IL-6 expression and inflammation in the liver tissues of rats (Fig. 7B-D). However, treatment of rats with UDSE decreased the expression of TNF-α, IL-1β, and IL-6 cytokines in the liver tissue (Fig. 7B-D). Results indicate the anti-inflammatory properties of UDSE. It should be noted that TNF-α and IL-1β expression did not decrease significantly in the DENA+UDSE-1 and DENA+UDSE-2 groups (Fig. 7B and C). Only IL-6 was down-regulated considerably in DENA+UDSE-1 and DENA+UDSE-2 groups, indicating that the anti-inflammatory effect on UDSE is preliminary to IL-6 cytokine (Fig. 7D).

Discussion

A change in the activities of alanine aminotransferase (ALT), aspartate-transaminase (AST), and lactate dehydrogenase (LDH) enzymes in some tissues and organs in the body is essential in interpreting cell proliferation and damage[22]. In experimental HCC studies performed with DENA, increased serum ALT, AST, and LDH enzyme levels have been reported. In our study, AST, ALT, and LDH levels were found to be higher in rats in the DENA group compared to the rats in the control

group. The increase in these enzyme activities has been interpreted as the deterioration of the membrane integrity of the cells with the increase in free radical production in the liver tissue and, therefore, the passage of these enzymes into the systemic circulation[23]. It has been reported that UDSE, used against CCl4-induced liver damage in rats, significantly reduces lipid peroxidation and liver enzymes AST and ALT activities and positively regulates AST, ALT, and LDH activities[24]. Our study noted that these enzymes were at lower levels in the UDSE-treated groups than in the control and DENA groups. This shows that DEN causes damage to the liver, and UDSE administration strongly prevents liver damage.

SOD, GSH, CAT, GSH-Px, TAS, TOS, and MDA values were analyzed to interpret the antioxidant capacity, oxidative stress, some antioxidant enzymes, and lipid peroxidation in liver damage caused by DENA. Some previous studies noted that DENA suppressed antioxidant capacity and increased TOS and MDA levels in blood and liver tissue[25]. In our study, the application of DENA decreased TAS, GSH, CAT, SOD, and GSH-Px levels and increased TOS and MDA levels in both liver and serum samples.

UD is a medicinal plant with anti-inflammatory, antimicrobial, antioxidant, hepatoprotective, antidiabetic, and hypoglycemic properties and is widely used in alternative medicine today[12]. Various studies have emphasized the powerful antioxidant properties of UD. It has been reported that UDSE increases GSH and CAT activities/levels against CCl4-induced liver damage in rats[26]. It was reported that UDSE, used against doxorubicin-induced liver damage in rats, significantly increased SOD, CAT, and GSH-Px activities[27]. A study reported that the application of UDSE and CCl4 to rats increased TAS levels and decreased TOS levels compared to the CCl4 group[28]. In our study, the application of UDSE and DENA increased antioxidant capacity and suppressed oxidative stress (TOS, MDA). These results were compatible with the authors results mentioned in the literature[24].

It has been reported that there is an increase in AFP and CEA markers in the liver in cases of HCC induced by DENA[29]. The serum levels of cancer markers CA 15-3, CA 125-II, CA 19-9, CEA, and AFP were noted in people diagnosed with HCC[30].

Although AFP and CEA values in serum samples in the DENA+UDSE groups were lower than in the

DEN group, the results were insignificant. However, CA 15-3, CA 19-9, and CA 125-II activities were significantly lower in the DENA+UDSE1 and 2 groups compared to the DENA group, indicating that UDSE significantly suppressed the inducing effect of DENA on cancer markers.

The previous studies determined that apoptotic caspase-3 was high, but antiapoptotic total Bcl-2 was low in HCCs induced by DENA in rats[31]. In this study, it was revealed that DENA significantly increased caspase-3 levels in serum samples. However, in the DENA+UDSE-1 and -2 groups, it was shown that UDSE significantly suppressed DENA-induced caspase-3 levels and partially restored BcL-2 levels.

Many investigators have recorded different lesions in liver damage and hepatocellular carcinoma processes induced by DENA administration at different doses and durations in rats. In these studies, nodular tumoral lesions, preneoplastic foci, and trabecular structures hydropic degeneration in hepatocytes, areas of multifocal necrosis, inflammatory cell infiltration, focal hemorrhagic areas, macrovesicular adiposity, hyperplasia in bile ducts, fibrosis, cells with clear cytoplasm, hyperchromasia in hepatocytes, hyperplasia in Kupffer cells and atypia in hepatocytes were noted[32]. In our study, although nodular tumoral lesions were not observed in the livers of the rats in the DENA group, partial enlargement of the livers, mass at the edges, and a diffusely fine granular appearance on the surfaces were detected. Histopathologically, areas consisting of dysplastic hepatocytes characterized by large and small cell changes, which are accepted as histological precursors of hepatocellular carcinomas, were widely seen. The absence of nodular lesions and tumoral cell proliferation in the livers of rats in this study may be related to the DENA dose and experiment period. In addition, the histologically observed foci of dysplastic hepatocytes[15], hydropic degeneration, inflammatory cell infiltration, macrovesicular steatosis, hyperplasia in the bile ducts, fibrosis, and hyperplasia in Kupffer cells were similar to the findings of the researchers cited in the literature[32].

A previous study reported that up to 95% of Hep-Par-1 immunoreactions were detected in HCCs immunohistochemically[33]. This study observed intense intracytoplasmic staining for Hep-Par-1, primarily in hepatocytes around the vena central is in the DENA group. However, the number of Hep Par-1 positive cells was found to be lower in the groups of DENA and UDSE-administered.

It has been reported that DENA increases the expression of AFP in DENA-induced liver damage and hepatocellular carcinomas in rats[34]. This study noted that dysplastic hepatocytes in the DENA group were mainly concentrated around the vena centralis (periacinar). Similarly, AFP expression was more intense, especially in the DENA group periacinar hepatocytes. However, lower staining was observed in the groups given DENA and UDSE compared to the DENA group. These results showed that DENA activated AFP gene expression in hepatocytes, but administration of UDSE and DENA inhibited AFP expression by preventing hepatocellular damage.

Caspase-3, which plays a vital role in extracellular and intracellular apoptotic signalling pathways, shows a positive reaction in apoptosis cells[35]. Therefore, caspase-3 is essential in detecting and evaluating cells undergoing apoptosis and in the solid morphological recognition of apoptotic cells. A study stated that the apoptotic factor caspase-3 activity increased in DENA-induced HCCs[36]. In this study, intense intracytoplasmic staining was detected in hepatocytes in the DENA group. However, lower intensity staining was seen in the DENA and UDSE groups compared to the DENA group. In addition, it was determined that there were relatively fewer positive cells in the DENA+ UDSE-2 (ether oil extract) group than in the DENA+UDSE-1 (ethanol oil extract) group. In our study, increased expression of caspase-3 in rats administered DENA showed that DENA induced apoptosis. However, the decrease in caspase-3 positive cells in the groups treated with DENA and UDSE suggested that UDSE suppressed caspase-3 expression and had an antiapoptotic effect.

In a previous study, it was stated that nitric oxide synthase (NOS) expression at high levels (for example, generated by activated macrophages) might be cytostatic or cytotoxic to tumor cells. At the same time, low-level activity may conversely promote tumor growth. In our study, iNOS immunoreaction was observed intra-cytoplasmically in *Kupffer* cells and fixed liver macrophages in all the groups. The strongest immunopositivity was found in the UDSE group, the weakest reaction was in the DENA group, and moderate immunopositivity was found in the DENA+UDSE group. However, the immunoreaction in UDSE-1 was more potent than in the UDSE-2

group. These findings evaluated that iNOS expression was suppressed in the DENA group, while UDSE application stimulated iNOS production and had an antitumoral effect. In addition, immunohistochemical results showed a parallel with histopathological and biochemical findings[37].

Cell proliferation is one of the most important characteristics of cancer[38]. The proliferative activity of cells or tissue is an important prognostic marker in cancer initiation, development, and treatments. Proliferating cell nuclear antigen (*PCNA*), Ki67, and minichromosome maintenance (MCM) proteins are proliferation markers, and they are commonly used to evaluate cell or tumor growth[39]. *PCNA* is functional in DNA replication, increases polymerase efficiency in the long strand of DNA, and is widely used as a proliferation marker in molecular cancer studies[40]. In the present study, *PCNA* gene expression was evaluated for this purpose. Results indicated that DENA treatment increased *PCNA* gene expression in the liver, but DENA+UDSE-1 and DENA+UDSE-2 treatment suppressed *PCNA* gene expression in the liver. The results suggest that UDSE protects liver DENA-induced carcinogenesis.

Cytokines are pivotal in the initiation, maintenance, and progression of tumors and are the pleiotropic hormones of the immune system[41,42]. Neoplastic cells and tumour-associated macrophages secrete cytokines such as IL-1, IL-6, and TNF-α and promote tumor development[43,44]. Studies have shown that UD leaf extracts suppress pro-inflammatory cytokines. A previous study reported that *U. dioica* leaf extract suppresses pro-inflammatory through the transcription factor NF-κB[45]. The study evaluated the effect of UDSE on pro-inflammatory cytokines IL-1β, IL-6, and TNF-α in DENA-induced carcinogenesis by RT-qPCR. Results showed that UDSE also suppresses pro-inflammatory cytokine expression IL-1β, IL-6, and TNF-α in the liver of UDSE-treated groups (Fig. 7). This finding indicated that inhibition of pro-inflammation by UDSE seed extracts might be similar to leaf extract, which could be NF-κB inactivation. However, in this study, DENA treatment did not induce pro-inflammatory cytokine expression.

Although it has been reported in some studies that activation of DENA in hepatocytes leads to ethylation of DNA, causing hepatocyte DNA damage and causing TNF-α and IL-6release by *Kupffer* cells, this study showed that DENA decreased the TNF- α, IL-1β, and IL-6 expression. This result indicates that the DENA application did not induce *Kupffer* cells, and DENA-induced liver injury is independent of pro-inflammation. In addition, this study showed that IL-6 mRNA expression in the UDSE, DENA+UDSE-1 and DENA+UDSE-2 groups was significantly lower than in control. IL-6 has pleiotropic effects, and its primary function is inflammation through the acute-phase protein production in the liver[46]. Our data suggest that the anti-inflammatory effect of UDSE is mainly on the down-regulation of IL-6.

Conclusion

This study observed that DENA administration caused the formation of dysplastic hepatocytes, which are thought to be the histological procurers of hepatocellular carcinomas in the liver. Still, these lesions remained at minimal levels in the UDSE-administered groups together with DENA. Also, it was observed that AST, ALT, LDH activities, TOS and MDA levels were high biochemically together with DENA application, but TAS, GSH, CAT, SOD, and GSH-Px levels were low. In addition, it was observed that UDSE application increased antioxidant capacity and suppressed oxidative stress, thus preventing liver damage. Furthermore, it was determined that DEN application significantly increased the tumor markers CA 15-3, CA 19-9, and CA 125-II, especially apoptotic caspase-3 levels, but UDSE application reduced these increases.

The immunohistochemical evaluation showed a significant reaction in Hep par-1, AFP, and caspase-3 stainings in the DENA group, while it showed weak immunoreaction in iNOS staining. In addition, the UDSE application significantly decreased the expression of these antibodies and increased the expression of iNOS. These results suggested that UDSE may prevent tissue damage with its potent antioxidant capacity and have significant antitumor activity in DENA-induced hepatocellular carcinogenesis. According to the literature, since this study is the first to investigate the effect of UDSE in the process of DENA-induced liver damage or hepatocellular carcinogenesis in rats, we believe that the results obtained from this study will guide future studies.

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Conflicts of interest

All authors declare no conflicts of interest.

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