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Ameliorative Effects of Lycopene on Diethylnitrosamine-Induced Hepatocarcinogenesis in Male Rats

Ahmed Mohamed Mokhtar, Mohamed Fathy Elshafee, Ahmed Mohamed Mansour, El-Sayed Mohamed El-Sayed*

Pharmacology and Toxicology Department, Faculty of Pharmacy (Boys), Al-Azhar University, Nasr-City, Cairo, Egypt
*Corresponding author: elsayed200_1956@yahoo.com

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Abstract Hepatocellular carcinoma (HCC) is a common liver cancer-causing the high rate of mortality worldwide. The present study evaluates the possible protective role of lycopene during diethylnitrosamine (DEN)-induced HCC in rats for 16 weeks. A patch of 60 male rats was divided according to the required treatment into 6 groups. DEN was given to rats in drinking water (100 mg/L) to induce HCC. In the present study. The groups supplied with lycopene were administered with lycopene at 5, 10, and 20 mg/kg BW/orally/daily during the experiment. The DEN alone administered group induced alterations in all the investigated parameters. The lycopene pretreated DEN groups showed significant ameliorations in a dose-dependent manner against the deleterious alterations of DEN in the biological markers, oxidative stress, liver function, lipid profile, hematological parameters, serum inflammatory markers (TNF-α and IL-6), immunohistochemical markers, caspase-3 liver content and histopathological changes, where the lycopene dose of 20 mg/kg showed better ameliorative effects. In conclusion, the oral administration of lycopene provides considerable protective effects against DEN-induced HCC which suggested the potential efficacy of lycopene as an additional chemopreventive agent in the treatment of HCC.

Keywords: *lycopene, diethylnitrosamine, hepatocellular carcinoma, apoptosis, oxidative stress*

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1. Introduction

Hepatocellular carcinoma (HCC) is a common important form of liver cancer in both incidence and high rate of mortality worldwide [1,2].

Diethylnitrosamine (DEN) is a chemical used to induce HCC as previously reported [3,4]. When administered in the drinking water, DEN induces liver tumors in rats through induction of oxidative stress in the liver tissue and other organs that causes generation of free radicals as reactive oxygen species (ROS), which initiate peroxidative damage in the liver tissue [5].

The treatment of experimental animals with plant antioxidants like lycopene for a long period could augment the cellular endogenous antioxidants and defend against oxidative stress [6,7]. Lycopene is a red phytochemical pigment found in tomatoes that is believed to exert anti-inflammatory, antioxidant and hepatoprotective properties as well as anticancer activity [8,9]. The lycopene structure contains many double bonds that help in the scavenging activity against oxidative stress that causes tissue injuries [10]. In addition, lycopene can reverse the loss of antioxidant enzymes induced by exposure to some toxins revealing its protective and antioxidant roles against many toxins [11,12].

The present study aims to investigate the ameliorative effects of lycopene on hepatocarcinogenesis in diethylnitrosamine-induced male rats including oxidative stress, some biochemical, hematological, serum inflammatory markers (TNF-α and IL-6), immunohistochemical parameters and caspase-3 content as well as histopathological alterations with a view to its possible applications in the clinical field.

2. Materials and Methods

2.1. The Experimental Animals

The present study used 60 male Wistar albino rats (180-200 g), divided into 6 groups. The animals were obtained from El-Nile Company (Egypt) and were housed in the animal facility of Faculty of Pharmacy (Boys), Al-Azhar University, Nasr-City, Cairo, Egypt. They were supplied with standard food composed of 20% casein, 15% corn oil, 55% corn starch, 5% salt mixture, and 5%

vitaminized starch (Egyptian Company of Oils and Soap, Kafr-Elzayat, Egypt), and DEN water or tap water *ad libitum*. The animals were kept in standard conditions including room temperature (22°C±2°C) and relative humidity (55±3) with a 12-h light/dark cycle.

2.2. Ethical Approval

All rats were conducted following the ethical guidelines and complied with the guide for the care and use of laboratory animals. The study was approved and conducted under the supervision of the independent ethics committee of the Faculty of Pharmacy (Boys), Al-Azhar University, Nasr-City, Cairo, Egypt.

2.3. Chemicals

We used chemicals of analytical grade supplied from standard commercial suppliers.

2.3.1. Lycopene

Lycopene Redivivo (Lot ECBJ9005V, 36275-5G, Sigma Aldrich, St. Louis, USA) (5 g) was mixed in corn oil with vigorous stirring to the desired concentrations of lycopene/ml [13]. Lycopene was administered to the rats via oral route at doses of 5, 10, and 20 mg/kg BW/daily/orally.

The used doses of lycopene and the resulted control-like effects of corn oil alone treatments were verified by a pilot study conducted in our lab (data not shown) following the method of Rao and Shen [14].

2.3.2. Diethylnitrosamine (DEN) and Induction of Hepatocarcinogenesis

The DEN (Sigma Aldrich, St. Louis, USA) was prepared in dark bottles as a fresh solution (100 mg/L) and supplied to rats to induce hepatocarcinogenesis following a previous method [3].

2.4. Experimental Design

The rats were divided into six groups (10 rats each) as follows:

Group I (Control): Normal rats supplied with food and water for16 weeks.

Group II (Lycopene): Rats of this group were administered with freshly prepared lycopene at a dose of 20 mg/kg BW/daily/orally for the experimental period.

Group III (DEN-treated group): Rats were given DEN solution (100 mg/L) for 8 weeks to induce liver tumor followed by 8 weeks of tap water without DEN to allow the tumor to grow.

Group IV (Lycopene 5 mg-DEN-treated group): Rats of this group were pretreated with freshly prepared lycopene (5 mg/kg BW/daily/orally for 2 weeks pretreatment period and 16 weeks during the experiment) and administered by DEN as the same schedule mentioned in group III.

Group V (Lycopene 10 mg-DEN-treated group): Rats of this group were pretreated with freshly prepared lycopene (10 mg/kg BW/daily/orally for 2 weeks pretreatment period and 16 weeks during the experiment) and administered by DEN as the same schedule mentioned in group III.

Group VI (Lycopene 20 mg-DEN-treated group): Rats of this group were pretreated with freshly prepared lycopene (20 mg/kg BW/daily/orally for 2 weeks pretreatment period and 16 weeks during the experiment) and administered by DEN as the same schedule mentioned in group III.

2.5. Serum and Liver Tissue Sampling

Samples of blood and liver tissues were collected from anesthetized animals after 16 weeks. To prepare serum samples, blood was drained from the orbital plexus of the eye using capillary puncture, put into plain tubes and centrifuged (4000 rpm, 15 min). The prepared serum samples were preserved at -20ºC until future analysis. The liver samples were obtained from dissected animals, washed with isotonic saline, dried, and weighed immediately.

2.6. Estimation of Physical Characteristics

We estimate the relative liver mass, the percentage of body mass gain, the body mass gain%, the percentage of nodule incidence, and the nodular size.

2.7. Biochemical Study

Each liver tissue was homogenized in Tris-lysis buffer. The obtained homogenate samples were centrifuged (3000 rpm, 15 min) and then separated into aliquots and stored at -80°C until used.

The oxidative stress parameters (lipid peroxidation products, TBARS; catalase, CAT; superoxide dismutase, SOD; hepatic reduced glutathione, GSH) were determined using readymade kits (Biodiagnostic Co. Cairo, Egypt).

The serum liver function parameters (aspartate transaminase, AST; alanine transaminase ALT; alkaline phosphatase, ALP; total bilirubin, TBIL; total protein, TP; albumin as well as calculation of globulin and A/G ratio) and lipid profile (triglycerides, TG; total cholesterol, TC; high-density lipoprotein cholesterol, HDL-C; low-density lipoprotein cholesterol, LDL-C) were estimated using readymade kits (Elitech Co., France).

2.8. Estimation of Liver Caspase-3 Content

The caspase-3 content in liver tissues was evaluated using ELISA kits (CUSABIO, Fannin St., Houston, USA) using methods outlined in the diagnostic kit.

2.9. Hematological Measurements

The hematological parameters (Red blood cell, RBC count; hemoglobin, Hb concentration; hematocrit, Hct percentage; platelet count; white blood cell, WBC count) were measured in the blood samples (CBC analyzer, sk9000, U.S).

2.10. Inflammatory Markers

The tumor necrosis factor-alpha (TNF-α) was estimated in the blood serum of rats by ELISA technique using a kit purchased from BT LAB, Jiaxing, Zhejiang, China, according to the manufacturer's instructions provided with the TNF-

α assay kit (Catalog No: E0764Ra). The interleukin-6 (IL-6) was estimated in the blood serum of rats by ELISA technique using a kit purchased from BT LAB, Jiaxing, Zhejiang, China, according to the manufacturer's instructions provided with the IL-6 assay kit (Catalog No: E0135Ra).

2.11. Immunohistochemical Assay

The Avidin-Biotin immunoperoxidase complex technique (ABC) [15] was used in the present study to determine the expression of cytokines (TNF-α, IL-6) and α-fetoprotein tumor markers (Zhong Shan Golden Bridge Biological Technology Co., Ltd., Beijing, China). The colored images were processed and the relative expression level (% control) was quantitated by measuring the color intensity value using ImageJ 1.46r software (Wayne Rasband, National Institutes of Health, Bethesda, USA) to produce maps showing only the biomarker's stained areas.

2.12. The Histopathological Study

The liver tissue samples were fixed, processed, sectioned, and stained according to the methods of Bancroft and Gamble [16].

2.13. Statistical Analysis

Data are presented as mean ± SEM. Multiple comparisons were performed using one-way analysis of variance (ANOVA) followed by Tukey–Kramer for post-hoc analysis. Statistical significance was accepted at a level of $p<0.05$, as appropriate. Data analysis was performed using GraphPad Instat software (version 3.06). Graphs were sketched using GraphPad Prism (ISI® software, USA, version 5).

3. Results

3.1. The Effects of Lycopene on the Physical Parameters of Different Groups at the End of 16 Weeks are Represented in Table 1

The lycopene alone, lycopene 10 mg-DEN, and lycopene 20 mg-DEN treated groups showed insignificant changes in body mass gain, body mass gain%, liver mass & the relative liver mass when compared to control group. On the contrary, the DEN alone treated group and the lycopene 5 mg-DEN-treated group recorded significantly reduced body mass gain and body mass gain % in contrast to a significantly increased ($p<0.05$) liver mass and the relative liver mass as compared to the control group.

However, the lycopene 10 mg-DEN and lycopene 20 mg-DEN treated groups showed significantly increased body mass gain, body mass gain%, in contrast to significantly reduced ($p<0.05$) liver mass and relative liver mass when compared to the group treated with DEN alone.

Moreover, the lycopene 10 mg-DEN and lycopene 20 mg-DEN treated groups (compared to the lycopene 5 mg-DEN treated group) recorded significantly increased body mass gain and body mass gain%, in contrast to significantly reduced liver mass and the relative liver mass.

3.2. The Effects of Lycopene on the Morphometric Parameters of the Liver Tissues of Different Groups at the End of 16 Weeks are Represented in Table 1 and Figure 1

The morphometric parameters of the liver tissues of the control and lycopene alone treated group revealed a smooth liver with a normal appearance at the end of 16 weeks. On the other hand, the morphology of livers in the DEN, lycopene 5 mg-DEN, lycopene 10 mg-DEN, and lycopene 20 mg-DEN treated groups showed enlarged liver with several foci and nodules of HCC.

There was a 100% incidence of nodules in DEN, lycopene 5 mg-DEN, lycopene 10 mg-DEN, and lycopene 20 mg-DEN treated groups. All lycopene DEN treated groups recorded significantly reduced total nodules and nodules of all sizes when compared to the group treated with DEN alone. Additionally, the lycopene 20 mg-DEN-treated group recorded significantly reduced total nodules and nodules of all sizes when compared to the lycopene 5 mg-DEN or the lycopene 10 mg-DEN-treated groups. Also, the lycopene 10 mg-DEN treated group recorded significantly reduced total nodules and nodules of all sizes when compared to the lycopene 5 mg-DEN treated group.

3.3. The Effects of Lycopene on the Oxidative Stress in the Liver Tissues of Different Groups at the End of 16 Weeks are Represented in Table 2

The lycopene alone treated group showed insignificant changes in the liver oxidative stress parameters of GSH, SOD, CAT, and TBARS when compared to the control group. On the contrary, the DEN alone, lycopene 5 mg-DEN, lycopene 10 mg-DEN, and lycopene 20 mg-DEN treated groups recorded significantly increased TBARS, in contrast to significantly reduced SOD, CAT, and GSH when compared to the control group.

Moreover, all lycopene DEN-treated groups showed significantly reduced TBARS in the liver and significantly increased levels of GSH, SOD, and CAT in the liver when compared to the group treated with DEN alone. Also, the lycopene 20 mg-DEN-treated group (compared to the lycopene 5 mg-DEN or the lycopene 10 mg-DEN-treated groups) and the lycopene 10 mg-DEN treated group (compared to lycopene the 5 mg-DEN treated group) recorded significantly reduced TBARS and significantly increased levels of SOD, CAT, and GSH in the liver.

3.4. The Effects of Lycopene on the Tissue Caspase-3 content Apoptotic Marker in the Liver Tissue of Different Groups at the End of 16 Weeks Are Represented in Figure 2

The lycopene alone treated group showed insignificant changes in the caspase-3 content in the liver when compared to the control group. On the contrary, the

DEN and all lycopene DEN treated groups recorded significantly increased liver caspase-3 content when compared to the control group.

Additionally, all lycopene DEN-treated groups recorded significantly decreased liver caspase-3 content when compared to the group treated with DEN alone. Moreover, the lycopene 20 mg-DEN treated group (compared to the lycopene 5 mg-DEN or the lycopene 10 mg-DEN treated groups) and the lycopene 10 mg-DEN-treated group (compared to the lycopene 5 mg-DEN-treated group) recorded significantly reduced liver caspase-3 content in the liver.

Table 1. The effects of lycopene on the physical and morphometric parameters in the liver of different groups at the end of 16 weeks

Parameters	Control	Lycopene	DEN	L (5 mg)-DEN	L (10 mg)-DEN	L (20 mg)-DEN
Body mass gain (g)	131.7±11.9	131.3±15.4	85.8±1.35^{a}	94.2±1.68^{a}	119.2±10.8^{b,c}	127.5±9.75^{b,c}
Body mass gain (g)%	57.8±5.45	57.5±6.71	36.6±1.07^{a}	41.7±0.94^{a}	53.3±4.87^{b,c}	55.9±4.49^{b,c}
Liver mass (g)	10.2±0.43	10.1±0.55	30.7±1.57^{a}	17.7±1.10^{a,b}	13.1±0.42^{b,c}	12.0±2.98^{b,c}
Relative liver mass (g)	4.47±0.19	4.44±0.25	13.4±0.62^{a}	7.79±0.49^{a,b}	5.77±0.17^{b,c}	5.34±1.34^{b,c}
Nodule incidence %	0	0	100	100	100	100
Average no. of nodules	0	0	68.3±2.58^{a}	53.9±2.59^{a,b}	45±2.80^{a,b,c}	20.7±1.30^{a,b,c,d}
Nodular diameter <1 mm	0	0	56.8±1.48^{a}	51.3±2.95^{a,b}	43±2.80^{a,b,c}	18.3±1.12^{a,b,c,d}
Nodular diameter 1-3 mm	0	0	32.7±0.54^{a}	25.7±0.94^{a,b}	22.2±0.87^{a,b,c}	15.7±1.00^{a,b,c,d}
Nodular diameter >3 mm	0	0	11.0±1.44^{a}	8.1±1.02^{a,b}	5.1±0.44^{a,b,c}	3.4±0.34^{a,b,c,d}

Results are presented as mean ± SEM; a, Significantly different from control at P<0.05; b, Significantly different from DEN at P<0.05; c, Significantly different from L (5 mg)-DEN at P<0.05; d, Significantly different from L (10 mg)-DEN at P<0.05; DEN, diethylnitrosamine; L, lycopene.

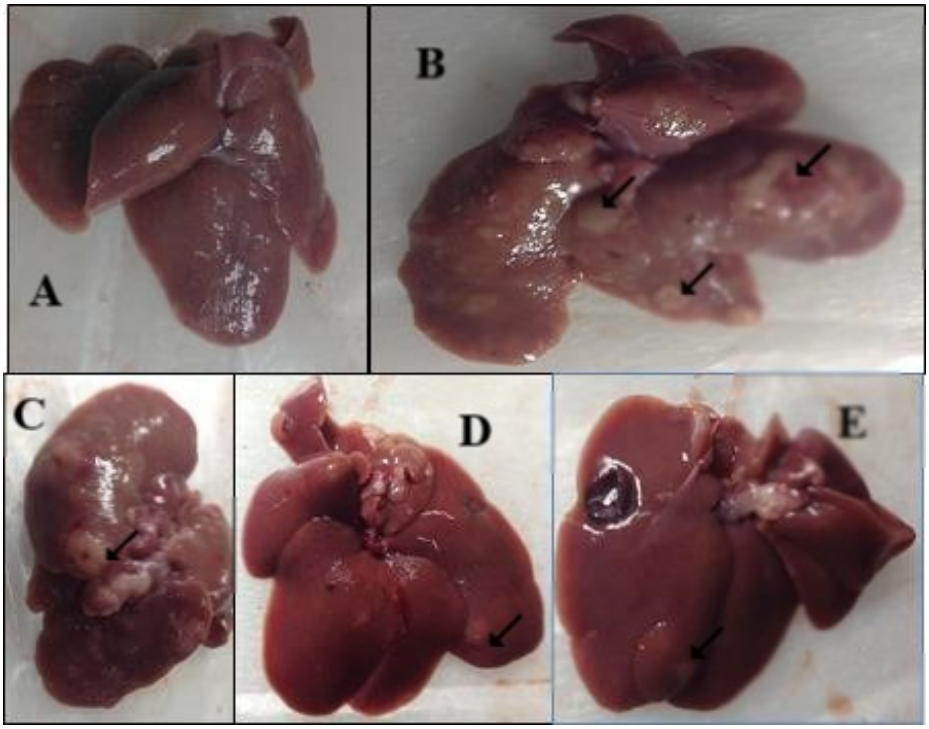

Figure 1. The effects of lycopene on the macroscopic gross appearance of livers at the end of 16 weeks of different groups. Arrows indicate nodules. A, control; B, diethylnitrosamine (DEN)-treated group; C, lycopene 5 mg-DEN-treated group; D, lycopene 10 mg-DEN-treated group; E, lycopene 20 mg-DEN-treated group

Table 2. The effects of lycopene on the oxidative stress parameters in the liver of different groups at the end of 16 weeks

Parameters	Control	Lycopene	DEN	L (5 mg)-DEN	L (10 mg)-DEN	L (20 mg)-DEN
TBARS (nmol/g tissue)	195.2±1.32	196.2±1.20	329.1±3.30^{a}	301.6±0.68^{a,b}	260.9±2.51^{a,b,c}	209±2.60^{a,b,c,d}
SOD (U/mg tissue)	69.1±1.61	68.0±1.37	34.7±0.46^{a}	43.2±0.61^{a,b}	52.3±1.76^{a,b,c}	61.8±0.61^{a,b,c,d}
CAT (U/mg tissue)	1.58±0.01	1.59±0.01	0.69±0.01^{a}	0.81±0.02^{a}	0.97±0.02^{a,b,c}	1.25±0.13^{a,b,c,d}
GSH (nmol/g tissue)	3.03±0.02	2.97±0.02	1.59±0.01^{a}	1.92±0.02^{a,b}	2.04±0.06^{a,b}	2.56±0.11^{a,b,c,d}

Results are presented as mean ± SEM; a, Significantly different from control at P<0.05; b, Significantly different from DEN at P<0.05; c, Significantly different from L (5 mg)-DEN at P<0.05; d, Significantly different from L (10 mg)-DEN at P<0.05; DEN, diethylnitrosamine; L, lycopene;TBARS, thiobarbituric acid reactive substances; SOD, superoxide dismutase; CAT, catalase; GSH, reduced glutathione.

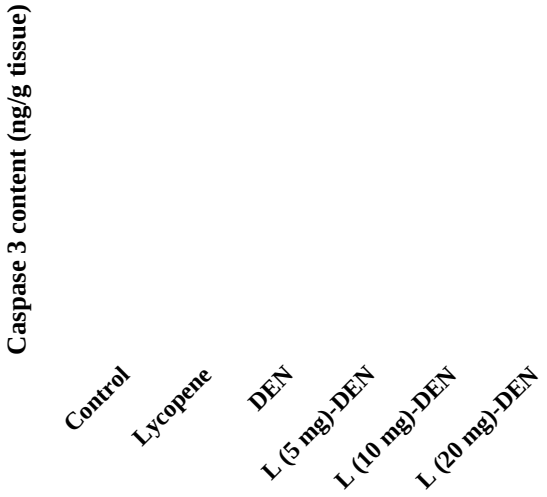

Figure 2. The effects of lycopene on the hepatic caspase 3 content of different groups at the end of 16 weeks. a, Significantly different from control at P<0.05; b, Significantly different from DEN at P<0.05; c, Significantly different from L (5 mg)-DEN at P<0.05; d, Significantly different from L (10 mg)-DEN at P<0.05; DEN, diethylnitrosamine; L, lycopene

3.5. The Effects of Lycopene on the Serum Liver Functions of Different Groups at the End of 16 Weeks Are Represented in Table 3

The lycopene alone treated group showed insignificant changes in serum liver function parameters (AST, ALT, ALP, TBIL, TP, globulin, albumin, and A/G ratio) when compared to the control group. On the contrary, the DEN, lycopene the 5 mg-DEN, the lycopene 10 mg-DEN, and the lycopene 20 mg-DEN treated groups recorded significantly increased levels of serum AST, ALT, ALP, TBIL, TP, and globulin in contrast to significantly reduced levels of serum albumin and A/G ratio when compared to the control group.

In addition, all lycopene DEN-treated groups recorded significantly reduced levels of serum AST, ALT, ALP, TBIL, TP, and globulin in contrast to significantly increased levels of serum albumin and A/G ratio when compared to the group treated with DEN alone. Also, the lycopene 20 mg-DEN treated group (compared to the lycopene 5 mg-DEN-treated or the lycopene 10 mg-DEN-treated groups) and the lycopene 10 mg-DEN treated group (compared to the lycopene 5 mg-DEN-treated group) recorded significantly reduced levels of serum AST, ALT, ALP, TBIL, globulin, and TP, in contrast, to significantly increased serum albumin and A/G ratio.

3.6. The Effects of Lycopene on the Serum Lipid Parameters and Their Risk Ratios of Different Groups at the End of 16 Weeks Are Represented in Table 4

The lycopene alone treated group was similar to the control group in serum lipid parameters and their risk ratios. On the contrary, the DEN and all lycopene DEN-treated groups recorded significantly increased values of serum TG, TC, LDL-C, as well as their risks, in contrast, to significantly reduced values of serum HDL-C when compared to the control group.

Moreover, all lycopene DEN treated groups showed significantly reduced levels of serum TG, TC, LDL-C, and their risks in contrast to significantly increased levels of HDL-C when compared to the group treated with DEN alone. In addition, the lycopene 20 mg-DEN treated group (compared to the lycopene 5 mg-DEN or the lycopene 10 mg-DEN-treated groups) and the lycopene 10 mg-DEN-treated group (compared to the lycopene 5 mg-DEN treated group) recorded significantly reduced levels of serum TG, TC, LDL-C, and their risks in contrast to a significantly increased level of serum HDL-C.

Table 3. The effects of lycopene on the serum liver functions parameters of different groups at the end of 16 weeks

Parameters	Control	Lycopene	DEN	L (5 mg)-DEN	L (10 mg)-DEN	L (20 mg)-DEN
AST (U/L)	64.2±1.52	63.7±1.43	264.5±6.39^{a}	130.3±2.23^{a,b}	127.9±2.31^{a,b}	103.7±2.90^{a,b,c,d}
ALT (U/L)	64.1±1.28	62.4±1.64	185.2±2.72^{a}	133.7±2.56^{a,b}	126.3±2.30^{a,b}	101.7±2.85^{a,b,c,d}
ALP (U/L)	110.5±2.06	108.9±5.71	302.3±5.31^{a}	241.0±7.43^{a,b}	207.4±15.9^{a,b}	171.9±7.03^{a,b,c,d}
TBIL (mg/dL)	0.40±0.04	0.40±0.05	2.02±0.04^{a}	1.13±0.06^{a,b}	0.88±0.02^{a,b}	0.71±0.01^{a,b,c,d}
TP (g/dL)	6.16±0.07	6.10±0.08	9.84±0.18^{a}	8.83±0.20^{a,b}	8.28±0.13^{a,b}	7.26±0.09^{a,b,c,d}
Albumin (g/dL)	4.06±0.01	4.08±0.02	2.87±0.02^{a}	3.44±0.15^{a,b}	3.69±0.05^{a,b}	3.87±0.02^{a,b,c,d}
Globulin (g/dL)	1.53±0.01	1.52±0.02	2.45±0.04^{a}	2.20±0.05^{a,b}	2.06±0.03^{a,b}	1.81±0.02^{a,b,c,d}
A/G ratio (g/dL)	2.61±0.03	2.63±0.03	1.63±0.03^{a}	1.82±0.04^{a,b}	1.94±0.02^{a,b}	2.21±0.03^{a,b,c,d}

Results are presented as the mean ± SEM; a, Significantly different from control at P<0.05; b, Significantly different from DEN at P<0.05; c, Significantly different from L (5 mg)-DEN at P<0.05; d, Significantly different from L (10 mg)-DEN at P<0.05; DEN, diethylnitrosamine; L, lycopene; AST, aspartate transaminase; ALT, alanine transaminase; ALP, alkaline phosphatase; TBIL, total bilirubin; TP, total protein; A/G, albumin-globulin ratio.

Table 4. The effects of lycopene on the serum lipid parameters of different groups at the end of 16 weeks

Parameters	Control	Lycopene	DEN	L (5 mg)-DEN	L (10 mg)-DEN	L (20 mg)-DEN
TG (mg/dL)	80.7±0.19	83.7±1.59	170.0±2.98^{a}	140.2±1.08^{a,b}	129.5±1.84^{a,b}	95.4±1.22^{a,b,c,d}
TC (mg/dL)	97.7±0.15	95.6±1.18	185.±6.44^{a}	160.6±3.71^{a,b}	134.1±1.71^{a,b}	112.1±6.88^{a,b,c,d}
HDL-C (mg/dL)	64.6±0.18	64.0±2.22	31.1±0.43^{a}	46.0±3.23^{a,b}	53.1±0.52^{a,b}	58.6±1.34^{a,b,c,d}
LDL-C (mg/dL)	16.6±0.21	16.9±0.31	143.±6.88^{a}	89.1±5.54^{a,b}	60.3±1.77^{a,b}	47.3±9.28^{a,b,c,d}
TG/HDL-C risk ratio (mg/dL)	1.24±0.00	1.31±0.03	5.45±0.03^{a}	3.12±0.21^{a,b}	2.43±0.03^{a,b}	1.63±0.05^{a,b,c,d}
TC/HDL-C risk ratio (mg/dL)	1.51±0.00	1.50±0.04	5.94±0.12^{a}	3.55±0.22^{a,b}	2.52±0.02^{a,b}	1.91±0.10^{a,b,c,d}
LDL-C/HDL-C risk ratio (mg/dL)	0.25±0.00	0.26±0.00	4.59±0.16^{a}	1.96±0.13^{a,b}	1.13±0.03^{a,b}	0.80±0.14^{a,b,c,d}

Results are presented as mean ± SEM; a, Significantly different from control at P<0.05; b, Significantly different from DEN at P<0.05; c, Significantly different from L (5 mg)-DEN at P<0.05; d, Significantly different from L (10 mg)-DEN at P<0.05; DEN, diethylnitrosamine; L, lycopene; TG, triglycerides; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

Table 5. The effects of lycopene on the hematological parameters of different groups at the end of 16 weeks

Parameters	Control	Lycopene	DEN	L (5 mg)-DEN	L (10 mg)-DEN	L (20 mg)-DEN
RBC count (X106 corpuscle/mm3)	8.59±0.01	8.60±0.02	5.65±0.08^{a}	6±0.14^{a,b}	6.51±0.13^{a,b,c}	7.58±0.15^{a,b,c,d}
Hb concentration (g/dL)	15.6±0.02	15.6±0.03	10.1±0.18^{a}	11.0±0.25^{a,b}	11.7±0.22^{a,b,c}	12.9±0.18^{a,b,c,d}
Hct (%)	47.0±0.03	47.1±0.06	31.1±0.68^{a}	36.4±0.83^{a,b}	40.0±1.02^{a,b,c}	43.0±0.91^{a,b,c,d}
Platelet count (103 platelet/mm3)	937.1±0.82	937.2±1.10	491.8±11.6^{a}	582.5±8.29^{a,b}	647.5±6.18^{a,b,c}	722.5±6.18^{a,b,c,d}
WBC count (103 cell/mm3)	9.72±0.00	9.73±0.01	5.43±0.07^{a}	6.03±0.11^{a,b}	7.10±0.08^{a,b,c}	8.07±0.16^{a,b,c,d}

Results are presented as mean ± SEM; a, Significantly different from control at P<0.05; b, Significantly different from DEN at P<0.05; c, Significantly different from L (5 mg)-DEN at P<0.05; d, Significantly different from L (10 mg)-DEN at P<0.05; DEN, diethylnitrosamine; L, lycopene; RBC, red blood corpuscle; Hb, hemoglobin; Hct, hematocrit; WBC, white blood cell count.

3.7. The Effects of Lycopene on the Hematological Parameters of Different Groups at the End of 16 Weeks Are Represented in Table 5

The lycopene alone treated group was similar to the control in the measured hematological parameters. On the other hand, the DEN, the lycopene 5 mg-DEN, the lycopene 10 mg-DEN, and the lycopene 20 mg-DEN treated groups recorded significantly reduced RBC count, Hb concentration, Hct percentage, platelet count, and WBC count when compared to the control group.

The lycopene 5 mg-DEN, the lycopene 10 mg-DEN, and the lycopene 20 mg-DEN treated groups showed significant increase in all measured hematological parameters when compared to the group treated with DEN alone. Moreover, the lycopene 20 mg-DEN treated group (compared to the lycopene 5 mg-DEN or the lycopene 10 mg-DEN-treated groups) and the lycopene 10 mg-DEN-treated group (compared to the lycopene 5 mg-DEN treated groups) recorded significant increase in all measured hematological parameters.

3.8. The Effects of Lycopene on the Immunohistochemical Localization of the Inflammatory Cytokines (TNF-α, IL-6) and α-fetoprotein Tumor Marker Protein Expressions in the Liver Tissue of Different Groups at the End of 16 Weeks Are Represented in Figure 3 – Figure 5

The normal control group and the lycopene alone treated group showed a weak expression of TNF-α, IL-6, and α-fetoprotein. On the other hand, the periportal and peri-venular areas of hepatocytes in rat livers subjected to DEN and all lycopene DEN treated groups revealed diffuse upregulation of cytoplasmic reactivity of TNF-α, IL-6, and α-fetoprotein expressions. Interestingly, all lycopene DEN-treated groups exhibited decreased expression of TNF-α, IL-6, and α-fetoprotein compared with DEN only treated group.

Semi-quantitative analysis of immunohistochemical staining expressed as relative intensity of expression of TNF-α, IL-6, and α-fetoprotein protein revealed that DEN group significantly (p<0.05) increased the hepatic expression of TNF-α, IL-6, and α-fetoprotein compared with normal control group. In contrast, the expression of TNF-α, IL-6, and α-fetoprotein in all lycopene DEN-treated groups were significantly (p<0.05) downregulated compared with the group treated with DEN alone. Moreover, the lycopene 20 mg-DEN treated group (compared to the lycopene 5 mg-DEN or the lycopene 10 mg-DEN treated groups) and the lycopene 10 mg-DEN-treated group (compared to the lycopene 5 mg-DEN-treated group) recorded significantly (p<0.05) downregulated TNF-α, IL-6, and α-fetoprotein expressions in the liver.

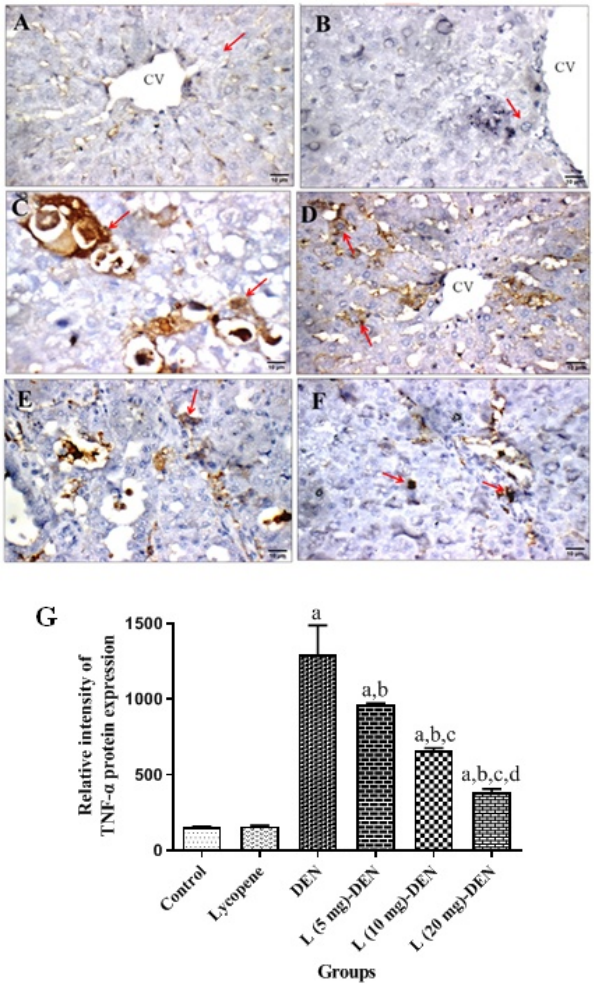

Figure 3. The effects of lycopene on the immunohistochemical localization of tumor necrosis factor-α (TNF-α) protein expression of different groups at the end of 16 weeks. **A**, higher power view of the control liver showing negative reactivity for TNF-α in the peri-venular area (red arrow); **B**, lycopene 20 mg-only treated liver tissue view showing negative cytoplasmic reactivity for TNF-α in the peri-venular area (red arrow); **C**, diethylnitrosamine (DEN)-treated liver tissue showing severe cytoplasmic reactivity for TNF-α (red arrow); **D**, lycopene 5 mg-DEN-treated liver tissue showing marked cytoplasmic reactivity for TNF-α in the peri-venular area (red arrow); **E**, lycopene 10 mg-DEN-treated group showing moderately cytoplasmic reactivity for TNF-α (red arrow); **F**, lycopene 20 mg-DEN-treated liver tissue showing weak cytoplasmic reactivity for TNF-α (red arrow); cv, central vein. **G,** show a semi-quantitative analysis of the relative intensity of tumor necrosis factor-α (TNF-α) protein expression of different groups at the end of 16 weeks. The relative expression level (% control) was quantitated by measuring the intensity value using image J software (NIH). Columns not sharing common letters are significantly different (P<0.05). DEN, diethylnitrosamine; L, lycopene

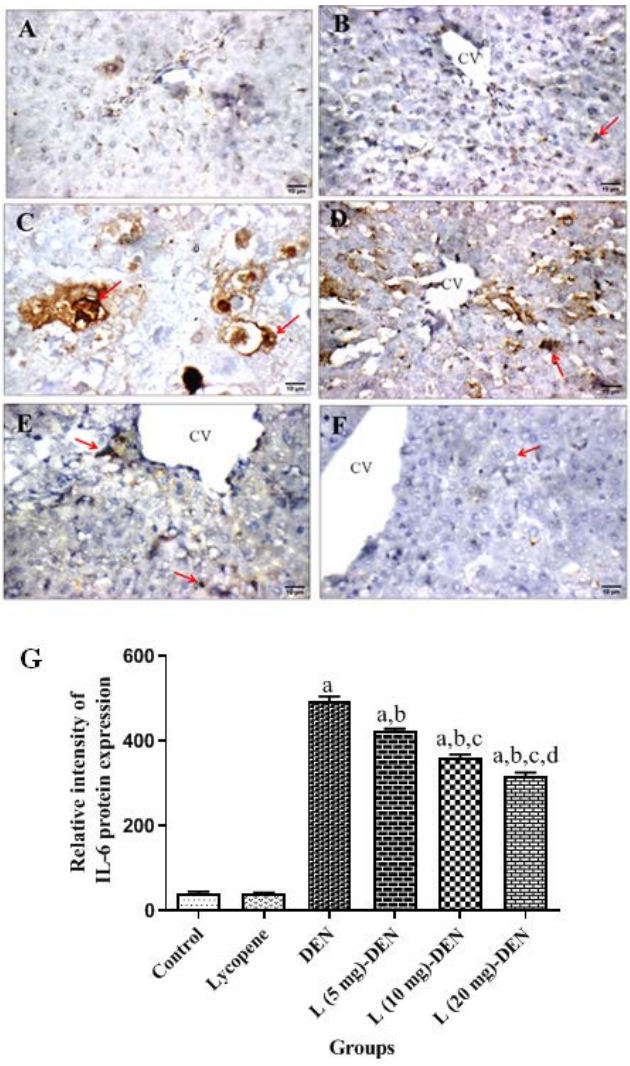

Figure 4. The effects of lycopene on the immunohistochemical localization of interleukin-6 (IL-6) protein expression of different groups at the end of 16 weeks. A, higher power view of the control liver showing negative reactivity for IL-6 in the periportal area (red arrow); B, lycopene 20 mg-only treated liver tissue view showing negative cytoplasmic reactivity for IL-6 in the peri-venular area (red arrow); C, diethylnitrosamine (DEN)-treated liver tissue showing marked cytoplasmic reactivity for IL-6 (red arrow); D, lycopene 5 mg-DEN-treated liver tissue showing moderate cytoplasmic reactivity for IL-6 in the peri-venular area (red arrow); E, lycopene 10 mg-DEN-treated group showing weak cytoplasmic reactivity for IL-6 (red arrow); F, lycopene 20 mg-DEN-treated liver tissue showing negative cytoplasmic reactivity for TNF-α (red arrow); cv, central vein. G, show a semi-quantitative analysis of the relative intensity of interleukin-6 (IL-6) protein expression of different groups at the end of 16 weeks. The relative expression level (% control) was quantitated by measuring the intensity value using image J software (NIH). Columns not sharing common letters are significantly different (P<0.05). DEN, diethylnitrosamine; L, lycopene

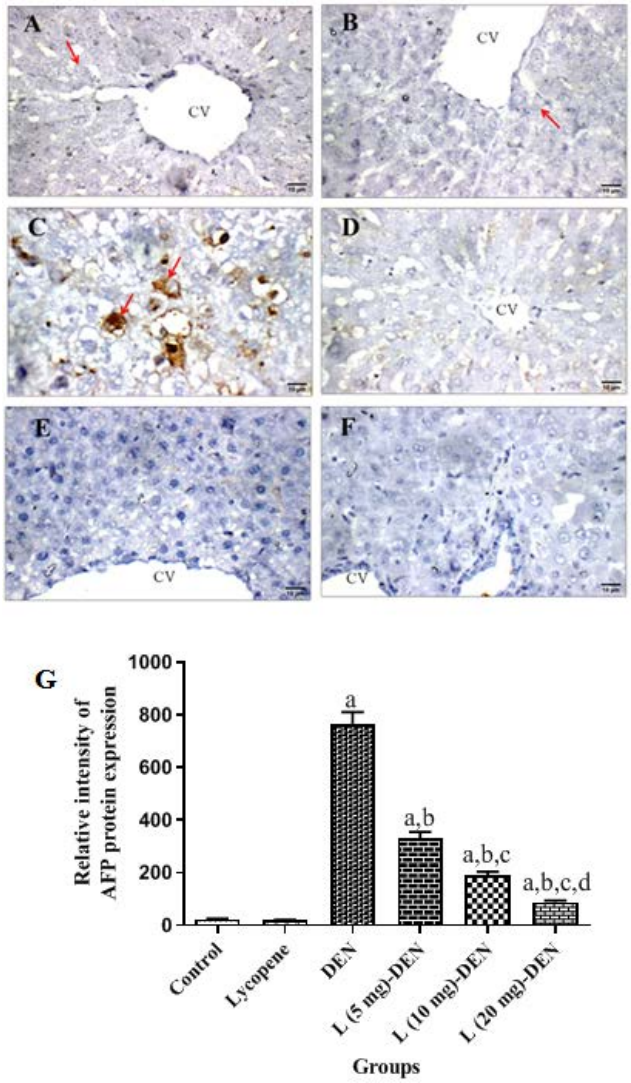

Figure 5. The effects of lycopene on the immunohistochemical localization of α-fetoprotein (AFP) protein expression of different groups at the end of 16 weeks. A, higher power view of the control liver showing negative reactivity for AFP in the peri-venular area (red arrow); B, lycopene 20 mg-only treated liver tissue view showing negative cytoplasmic reactivity for AFP in the peri-venular area (red arrow); C, diethylnitrosamine (DEN)-treated liver tissue showing marked cytoplasmic reactivity for AFP (red arrow); D, lycopene 5 mg-DEN-treated liver tissue; E, lycopene 10 mg-DEN-treated group; F, lycopene 20 mg-DEN-treated liver tissue; cv, central vein. G, show a semi-quantitative analysis of the relative intensity of α-fetoprotein (AFP) protein expression of different groups at the end of 16 weeks. The relative expression level (% control) was quantitated by measuring the intensity value using image J software (NIH). Columns not sharing common letters are significantly different (P<0.05). DEN, diethylnitrosamine; L, lycopene

Table 6. The effects of lycopene on the TNF-α and IL-6 levels in the serum of different groups at the end of 16 weeks

Parameters	Control	Lycopene	DEN	L (5 mg)-DEN	L (10 mg)-DEN	L (20 mg)-DEN
TNF-α (pg/ml)	41.8±5.3	39.8±8.5	104.0±12.1[a]	83.8±6.3[a,b]	72.9±5.7[a,b]	59.7±4.3[b,c,d]
IL-6 (pg/ml)	26.4±3.4	24.7±4.0	57.4±7.2[a]	50.5±4.6[a,b]	44.8±3.4[a,b]	36.6±4.2[b,c,d]

Results are presented as mean ± SEM; a, Significantly different from control at P<0.05; b, Significantly different from DEN at P<0.05; c, Significantly different from L (5 mg)-DEN at P<0.05; d, Significantly different from L (10 mg)-DEN at P<0.05; DEN, diethylnitrosamine; L, lycopene; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6.

Table 7. The effects of lycopene on the liver histopathological characters of different groups at the end of 16 weeks

Parameters	Control	Lycopene	DEN	L (5 mg)-DEN	L (10 mg)-DEN	L (20 mg)-DEN
CV	0	0	++	+	+	+
Hepatocytes	0	0	+	+	+	+
PT	0	0	++	+	+	+
Cirrhosis	0	0	++	+	+	+
HCC	0	0	++	+	0	0

DEN, diethylnitrosamine; L, lycopene
• Central vein (CV):
0: Within normal +: Dilated ++: Markedly dilated
• Hepatocytes:
0: Within normal +: Single cell necrosis ++: Confluent or diffuse necrosis
• Portal tract (PT):
0: Within normal +: Expanded ++: Expanded with inflammatory infiltrate
• Cirrhosis:
0: No cirrhosis +: Cirrhosis with no dysplasia ++: Cirrhosis with dysplasia
• HCC (Hepatocellular carcinoma):
0: No HCC +: Low-grade HCC ++: High-grade HCC

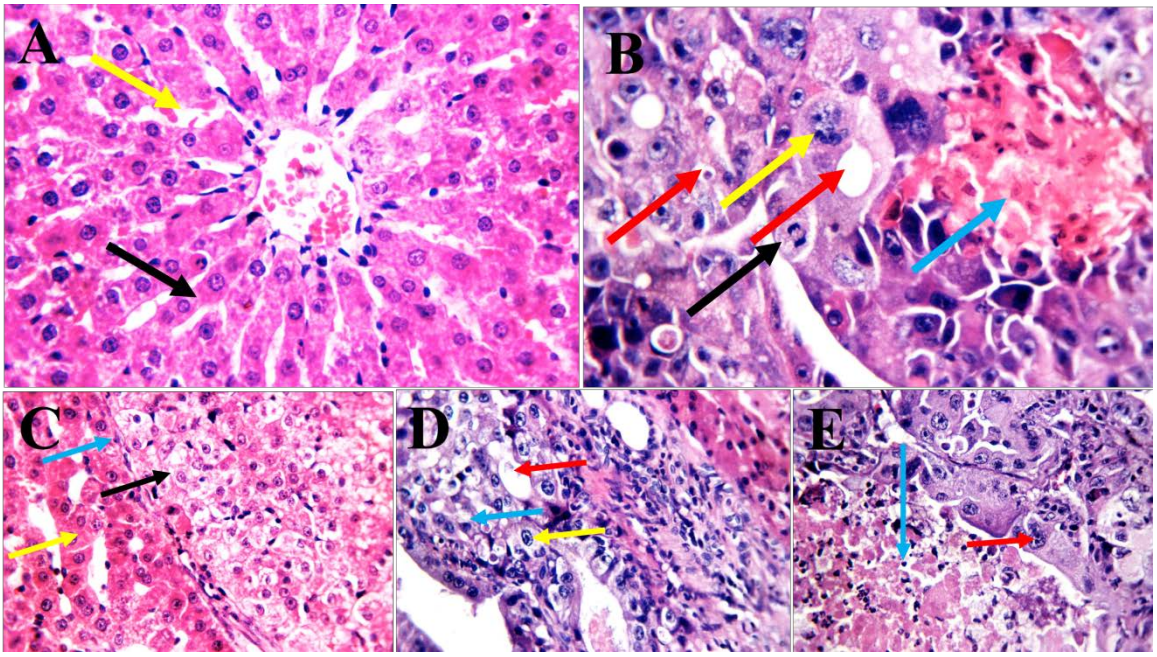

Figure 6. The effects of lycopene on the microscopic histological appearance of livers at the end of 16 weeks of different groups. A, higher power view of the control liver showing hepatocytes arranged in single-cell plates (black arrow) with intervening blood sinusoids (yellow arrow); B, diethylnitrosamine (DEN)-treated liver tissue showing moderately pleomorphic cells with prominent nucleoli (yellow arrow), arranged in trabecular and sinusoidal patterns (red arrows), scattered abnormal mitotic figures (black arrow) with an area of necrosis (blue arrow); C, lycopene 5 mg-DEN-treated liver tissue showing an area of average liver tissue (yellow arrow), and a nodule (black arrow) showing adenomatous hyperplastic changes with no infiltrating edges (blue arrow); D, lycopene 10 mg-DEN-treated group showing moderately pleomorphic cells with prominent nucleoli (yellow arrow), arranged in trabecular (blue arrow) and sinusoidal patterns (red arrow); E, lycopene 20 mg-DEN-treated liver tissue showing markedly pleomorphic cells with prominent nucleoli (red arrow) with an area of necrosis (blue arrow). All slides were stained with H&E and the magnification power is X 400

3.9. The Effects of Lycopene on the TNF-α and IL-6 Levels in the Serum of Different Groups at the End of 16 Weeks Are Represented in Table 6

The lycopene alone treated group showed insignificant changes in the TNF-α and IL-6 serum levels in when compared to the control group. On the contrary, the DEN alone, the lycopene 5 mg-DEN, and the lycopene 10 mg-DEN groups recorded significant increase in the TNF-α and IL-6 serum levels when compared to the control group.

In addition, the lycopene 20 mg-DEN-treated group showed significant decrease in the TNF-α and IL-6 serum levels when compared to the DEN alone group. Finally, there was no significant change between the lycopene 5 mg-DEN, and the lycopene 10 mg-DEN treated groups in the TNF-α and IL-6 serum levels.

3.10. The Effects of Lycopene on the Liver Tissue Histopathology of Different Groups at the End of 16 Weeks Are Represented in Table 7 and Figure 6

The histological morphology of the livers showed the normal liver tissue in the control and lycopene only treated groups. The DEN alone treated group and the groups treated with lycopene and DEN showed severe necrotic changes to the character with karyopyknosis, acute cellular swelling in hepatocytes, karyomegaly, apoptosis, sinusoidal and portal congestion, periportal cell

infiltration, intra-, and extracellular bile pigment accumulations, and Kupffer cell hyperplasia.

The lycopene 5 mg-DEN, the lycopene 10 mg-DEN, and the lycopene 20 mg-DEN treated groups showed ameliorative morphological effects when compared to the group treated with DEN alone.

4. Discussion

The HCC occurs predominantly in patients with chronic liver disease and cirrhosis [17]. DEN can induce liver cancer in experimental animals. The model used in the present study was described previously [3]. This model could mimic human HCC and allow studying hepatocarcinogenesis and screening of the potential anti-tumor agents on various phases of liver cancer. A tumor induction begins with cellular transformation, growth of cells (hyperproliferation), formation of nodules, and metastatic lesions [18,19].

The present study recorded body weight loss and an increase in liver mass as the major characteristics of HCC that agree with [20]. The present study also recorded significantly reduced body mass gain and significantly increased liver mass and relative liver mass as compared to the control which might be due to the development of liver tumors [2,21]. The formation of nodules is a good sign of liver cancer and there is a correlation between the size of the hyperplastic nodules and the occurrence of HCC [3,22] as evidenced by the present data.

The present results reveal that the supplementation of DEN-treated rats with Lycopene cause significant amelioration in all physical and morphometric characters in a dose-dependent manner (Lycopene dose of 20 mg was the best treatment) as compared to DEN alone treated group. That agrees with a previous study [9] who observed that lycopene decreased liver preneoplastic foci in DEN-treated rats. In addition, some studies [23,24] have demonstrated that in DEN-induced experimental hepatocarcinogenesis, lycopene inhibits the development of hepatocellular carcinoma and provide a protective effect by inhibiting proliferation by interrupting intercellular communication by affecting cell surface receptors, especially in cancer cells. Additional studies show that pretreatment with lycopene is effective against hepatic tumor induction, growth, and also lycopene is effective when administered in combination with toxic substances [22,25].

The liver is a common target for several toxicants [26]. The present study recorded significantly increased TBARS, in contrast to significantly reduced GSH, SOD, and CAT in all DEN treated groups when compared to the control group. DEN metabolism occurs in the liver that causes oxidative stress, generates ROS, and induced carcinogenic effects [27]. The oxidative stress state injures tissues and damages the biological systems as well as cell membranes leading to carcinogenesis steps (i.e. Initiation, promotion, and progression) [3,9]. The cells in biological systems contain endogenous natural enzymatic antioxidants like SOD and CAT and non-enzymatic antioxidants mainly GSH used in defenses against oxidative stress [28]. The present results are consistent with previous work that reported that the treatment of rats with DEN leads to depletion of the previously mentioned endogenous antioxidants [3].

The GSH defends against oxidative stress by maintaining the normal reduced state of cells [29]. GSH can scavenge ROS and other radicals by conjugation with GPx and GST, so it is used as a marker for evaluating the anticarcinogenic potential, and counteracting a variety of carcinogens [22,30]. The depletion in GSH level after DEN administration may be due to inhibition of GSH synthetase or due to its diffusion through damaged cell membranes [31].

The SOD is the only enzyme known to utilize free radicals as a substrate and dismutase the superoxide radicals to hydrogen peroxide and oxygen molecules. However, CAT is a supporting enzyme that transforms hydrogen peroxide to water thereby protecting against ROS, and could be used as an index of increased hydrogen peroxide production [32,33]. The decrease in CAT activity in response to the overproduction of H_2O_2 might be interpreted by the prospect of oxidative alteration of proteins which can have a substantial physiological impact [3,34].

The current study also recorded that the supplementation of DEN treated rats with lycopene causes significantly reduced liver TBARS, in contrast, to significantly increased liver SOD, CAT, GSH antioxidants in a dose-dependent manner (lycopene dose of 20 mg was the best treatment) as compared to the group treated with DEN alone. This indicates that lycopene has potent antioxidant effects as it can scavenge free radicals and prevent lipid peroxidation [12,35]. Our data are consistent with previous studies which recorded that the dietary supplement of watermelon juice (a rich natural source of lycopene) can inhibit lipid peroxidation in the liver and effectively protect against hepatocellular injury induced by toxicity [6,36]. In addition, similar researches indicate the ability of lycopene to protect the liver against oxidative damage [11,37,38,39].

The increased caspase-3 content in the liver tissue indicates an apoptosis-induction process [40]. Caspase-3 is an important apoptotic marker enzyme in the apoptosis process. It acts as an executioner enzyme that altered many types of proteins in the cell and promotes apoptosis [41,42]. The present results record significantly increased caspase-3 content in the liver of all groups treated with DEN compared with the control which indicates the induction of apoptosis.

The lycopene DEN treated groups in the present study recorded significantly inhibited tissue caspase-3 content in a dose-dependent manner (lycopene dose of 20 mg was the best treatment) which reveals the anti-apoptotic property of lycopene. These data are in approval with Hassan et al. [7] who found that caspase-3 content is elevated in DEN-induced hepatocellular carcinoma in rats. On the other hand, supplementation of animals with lycopene led to a reduction in the liver caspase-3 content in a dose-dependent manner in comparison with rat groups treated with DEN alone and did not receive the previous supplementation suggesting the anti-apoptotic and protective effects of lycopene as consistent with previous studies [43,44].

The hepato-specific enzymes were increased at hepatocellular damage [3]. The liver function parameters in the present study recorded significantly increased liver

enzymes, serum total protein, and globulin concentration in contrast to significantly reduced albumin & albumin-globulin ratio in all DEN treated groups when compared to the control. The measured liver function parameters in the present study are generally accepted as an index of liver damage [4,45]. The increase in serum AST & ALT indicates the impairment of the cell membranes of the liver and leakage of these enzymes to the blood [3]. In addition, the elevation in ALP activity and TBIL may be due to the large bile duct obstruction, intrahepatic cholestasis, or infiltrative release from the tissues to the blood [46]. The serum proteins are synthesized in the liver. These proteins serve to maintain the osmotic pressure and water balance as well as transport of materials [47]. The present results are consistent with previous work that reported the change in serum protein in carcinogenic rats and attribute this to the impairment of the integrity of the cell membranes of the liver, kidney, and other tissues [3].

The present study shows that lycopene inhibited hepatic oxidative stress caused by DEN can ameliorate the biomarkers of liver injury and dysfunction in a dose-dependent manner (lycopene dose of 20 mg was the best treatment). Administration of lycopene (all groups) recorded significantly reduced transaminases, ALP, and TBIL in comparison with the DEN alone treated group. Lycopene also ameliorated the lowering effect of DEN on serum contents of TP and ALB. The present results agree with Bayramoglu et al. [6] who reported improvements in liver function parameters following treatment with lycopene in rats exposed to liver injury. The current finding together with available data suggest a protective impact of lycopene against liver cell damage induced by DEN, possibly due to its inhibitory effect on lipid peroxidation and its stimulatory effect on the antioxidant parameters, which led to stabilization of hepatocyte membrane and enhancement of liver synthetic function [48,49]. In addition, Cui et al. [9] reported that lipoproteins of lycopene can protect nucleic acids and proteins from oxidative stress that could lead to cancer. Moreover, the hepatoprotective effect of lycopene could be due to its ability to scavenge the free radicals and counteract its bad effects [10,11].

The effect of DEN on lipid profile, as shown in the present work displayed significantly increased lipid fractions of TC, TG, and LDL-C, in contrast, to significantly reduced levels of HDL-C when compared to the control as previously reported [4,50].

The effect of DEN on lipid profile, as shown in the present work displayed significantly increased lipid fractions of TC, TG, and LDL-C, in contrast, to significantly reduced levels of HDL-C when compared to the control as previously reported [4,50]. The present results indicated that lycopene exerted a lowering effect on DEN-induced hyperlipidemia in a dose-dependent manner. The obtained results displayed a significant decrease of TG, TC, and LDL-C; and a significant increase in HDL-C in serum of rats treated with lycopene especially the dose of 20 mg when compared to the group treated with DEN alone. The lipid-modulating effect of lycopene has previously been reported [51,52]. In addition, Inoue et al. [53] detected the ability of lycopene to significantly reduce serum TC and TG. Furthermore, the current finding of decreased level of LDL-C by lycopene is consistent with published data exhibiting a reduction in LDL-C levels following consumption of tomato products containing lycopene [54]. Interestingly, the observed beneficial effect of lycopene on lipid metabolism raised its clinical efficacy as it could be used in the protection against cardiovascular diseases including atherosclerosis [11,54]. In this context, available data showed that lycopene may lower circulating cholesterol levels via increasing its fecal excretion and hence reducing its intestinal absorption. Also, it has been reported that lycopene may inhibit the synthesis of cholesterol and can protect LDL-C against oxidation thus preventing its contribution to the generation of atherosclerosis and may reduce the risk of cardiovascular diseases [51,53].

The current data recorded significantly reduced all measured hematological parameters in all DEN treated groups. These findings are consistent with previous studies [4,50] that attributed this reduction to the disturbance of the hematopoietic system and impairment of the cell membrane permeability which leading to osmotic swelling and cell hemolysis. Interestingly, the administration of lycopene to DEN treated groups attenuated the detrimental effects of DEN on hematological parameters in a dose-dependent manner that could be due to increased antioxidants that led to reduced oxidative stress on hematopoietic cells production or maturation systems [6,55].

Regarding the immunohistochemical localization of the pro-inflammatory cytokines (TNF-α, IL-6) and α-fetoprotein tumor (AFP) marker protein expressions, the present study revealed upregulation of cytoplasmic reactivity of TNF-α, IL-6, and α-fetoprotein expressions in rat livers subjected to DEN. Cytokines play an important role in regulating immunity and include pro-inflammatory cytokines (TNF-α, IL-6, and IL-Iβ) [56]. The inflammatory status due to DEN administration was confirmed in the present study by higher circulatory and tissue levels of TNF-α and IL-6 in the rats which hint that the inflammatory reaction appears after rats exposed to DEN as agreed with many previous studies [57,58]. The AFP was the most widely used tumor biomarker available for the early detection of HCC [59]. It has long been recognized that the exposure of rats to certain carcinogens like DEN caused an increase of the circulating AFP levels associated with HCC [20]. The upregulation of the AFP gene expression in DEN-administered rats might be due to the necrosis of hepatocytes that regulate the AFP synthesis on the cellular level [60]. Although the mechanism of this regulation was not fully understood, the possible explanation for the initiation of AFP synthesis by neoplastic hepatocytes included either increased transcription of AFP gene or post-translational modification affecting AFP production [61].

Oxidative and inflammatory insults are intimately connected with each other in multistage carcinogenesis. Hence, it is expected that an agent with anti-inflammatory property will inhibit oxidative stress and vice versa [62]. In agreement with the present study, Kim et al. [63] recorded the lycopene capability to scavenge free radicals in different systems and chelating metal ions, indicating that it can act as a natural antioxidant through many potential pathways and may be a useful therapeutic agent for treating radical-related pathological damage. Therefore, lycopene may be further developed as a potent antioxidant

and anti-inflammatory agent in functional health food [64,65]. In agreement with the present AFP results, lycopene has been extensively studied for its antioxidant and cancer-preventing properties [66]. In contrast to many other food phytonutrients whose effects have only been studied in animals, lycopene from tomatoes has been repeatedly studied in humans and found to be protective against several cancers, which now include colorectal, prostate, breast, lung, liver, and pancreatic cancers [67,68] as agreeing with the present study.

Finally, the histopathological analysis of the liver tissue in the present study shows that the groups of animals treated with DEN recorded marked cancerous features which were ameliorated in the DEN treated groups supplemented with lycopene as consistent with a previous study [22].

5. Conclusion

The data of the present study proved that oral lycopene supplementation ameliorated all the investigated parameters in animals exposed to DEN. Moreover, lycopene was able to mitigate liver tissue damage induced by DEN through amelioration of the morphometric, histopathological, immunohistochemical, liver function, lipid profile, and hematological parameters. These findings suggested the potential efficacy of lycopene as an additional chemopreventive agent in the treatment of hepatocellular carcinoma by mitigating the apoptotic, oxidative stress and inflammatory effects as well as stimulation of carcinogen detoxification.

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Conflict of Interest

The authors declare that they do not have any conflict of interest.

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