Received: 2 October 2021 | Revised: 9 February 2022 | Accepted: 1 April 2022

DOI: 10.1002/jbt.23078

RESEARCH ARTICLE

WILEY

Suppressive effects of thymoquinone on the initiation stage of diethylnitrosamine hepatocarcinogenesis in rats

Samar Ibrahim[1] | Sally A. Fahim[2] | Samer A. Tadros[3] | Osama A. Badary[4,5]

[1]Clinical Pharmacy Practice Department, Faculty of Pharmacy, Ahram Canadian University, 6th of October City, Egypt

[2]Department of Biochemistry, School of Pharmacy, Newgiza University, Giza, Egypt

[3]Department of Biochemistry, Faculty of Pharmacy, October University for Modern Sciences and Arts (MSA), 6th of October City, Egypt

[4]Clinical Pharmacy Practice Department, Faculty of Pharmacy, The British University in Egypt (BUE), Cairo, Egypt

[5]Clinical Pharmacy Department, Faculty of Pharmacy, Ain Shams University, Cairo, Egypt

Correspondence
Samar Ibrahim, Clinical Pharmacy Practice Department, Faculty of Pharmacy, Ahram Canadian University, 6th of October City, Egypt.
Email: samaribrahim370@gmail.com

Abstract

Hepatocellular carcinoma (HCC) is the fourth leading cause of cancer-related death globally. Chemoprevention is the most effective technique for reducing HCC incidence. Thymoquinone (TQ), the main bioactive constituent of *Nigella sativa*, exhibits anti-inflammatory and antineoplastic activities against various cancers. Therefore, TQ was tested as an inhibitor of the initial phase of diethylnitrosamine (DEN)-induced HCC in rats. Twenty-four male Wistar albino rats were randomly placed into four equal groups. Group 1 received saline and acted as the negative control; Group 2 received TQ; Group 3 received DEN; and Group 4 received TQ for 7 days and DEN on the 8th day. After 24 h of fasting, blood samples were taken from the slaughtered rats. Additionally, each rat's liver was dissected and separated into two halves for histological and biochemical investigation. DEN-induced hepatotoxicity was detected by elevated hepatic enzymes and HCC biomarkers reduced antioxidant and proapoptotic statuses. DEN administration caused a significant increase in the levels of glutathione, superoxide dismutase, malondialdehyde, caspase-3, alpha-fetoprotein (AFP), AFPL3, glypican 3, and the expression of BAX. However, DEN significantly decreased glutathione peroxidase, catalase, and CYP2E1 and the expression of BCl-2. Furthermore, it caused histological changes and showed a strong positive GSH *S*-transferase P expression in the hepatic parenchyma. Pretreatment with TQ prevented the histopathological and most of the biochemical changes and improved the antioxidant status. TQ supplementation appears to suppress the development of DEN-initiated liver cancer by reducing oxidative stress, activating the intrinsic mitotic apoptosis pathway, and retaining the antioxidant enzymes.

KEYWORDS

diethylnitrosamine, hepatocellular carcinoma, initiation stage, protective, thymoquinone

1 | INTRODUCTION

Hepatocellular carcinoma (HCC) is a fatal illness responsible for 75%–85% of primary liver malignancies. It is considered the fourth most common cause of cancer-related death.[1] There is an increase in chronic liver disorders and incidence of this neoplasia, with the trend predicted to continue in the future.[2] Hepatocarcinogenesis has been linked to several risk factors, including alcohol intake, obesity, iron overload, environmental contaminants, and dietary carcinogens.[3] Patients with hepatocarcinogenesis have a high mortality rate due to a lack of appropriate treatment and significant postsurgery recurrence rates. Only 30%–40% of HCC patients can be treated with curative methods, while most are treated with palliative methods.[4] Chemoprevention has been indicated as a

suitable method for reducing hepatocarcinogenesis-related morbidity and mortality. Natural medications have also recently been shown to have significant protective effects against this malignancy and have proven to be less hazardous than conventional therapy.[5]

Diethylnitrosamine (DEN) is commonly used to investigate HCC in experimental animal models influencing the initiation stage of carcinogenesis, probably through the induction of oxidative stress that attacks the cell membrane and DNA, resulting in liver damage with increased formation of harmful free radicals. Antioxidants have been used to prevent tissue damage in various clinical situations and experimental models.[6,7]

Thymoquinone (2-methyl-5-isopropyl-1,4-benzoquinone, TQ) is a natural compound derived from the black seeds of *Nigella sativa*.[8] For thousands of years, the black seeds of *Nigella sativa* have been used as a food preservative, spice, and traditional medicine for various conditions.[9,10] There is an increasing interest in the therapeutic effects of TQ due to its antioxidant, anti-inflammatory, chemotherapeutic, and hepatoprotective actions, among other pharmacological properties.[11–13] Additionally, it has anticancer properties.[14] Toxicity tests have shown that *N. sativa* oil and TQ are safe.[15]

Conclusively, these potentials encouraged us to perform this study to understand the possibility of mechanism-based protection from TQ supplementation against DEN-induced HCC onset.

2 | MATERIALS AND METHODS

2.1 | Chemicals

Both DEN and TQ were purchased from Sigma-Aldrich. TQ was freshly prepared by dissolving it in drinking water (5 mg/kg/day).[16] DEN was dissolved in normal saline at a 200 mg/kg dosage.[11] Biodiagnostic provided the assay kits, whereas primers were obtained from Bioline Inc.

2.2 | Animals

Twenty-four adult male Wistar rats (150–170 g) were obtained from the animal house of Ahram Canadian University. All experimental techniques followed the guidelines of the Ethical Committee of the Faculty of Pharmacy, Ahram Canadian University (P0221). Before starting the experiment, rats were placed in metallic cages with three rats per cage at room temperature for 1 week to acclimate. All animals were given ad libitum access to standard laboratory chow pellets and tap water.

2.3 | Experimental design

Animals were randomly divided into four groups of six each:

Group 1: As a negative control, the rats were fed a regular diet and received saline intraperitoneally (ip) on Day 8 of the experiment.

Group 2 (TQ group): Received TQ (5 mg/kg/day) in their drinking water for 7 days.

Group 3 (DEN control): Received a standard diet for 7 days, and on the 8th day, received DEN (200 mg/kg ip).

Group 4 (DEN + TQ): Received TQ (5 mg/kg/day) in their drinking water for 7 days and on the 8th day received DEN (200 mg/kg ip).

Blood samples were taken by retro-orbital venous plexus bleeding. After 24 h of fasting, rats were killed by decapitation. The liver from each rat was dissected, washed with normal saline, and cut into two parts. The first part was for histological examination, and the second part was used to prepare tissue homogenate for biochemical analysis.

2.4 | Methods

2.4.1 | Determination of liver function

The serum was separated after blood collection by centrifugation at 3000*g* for 15 min at room temperature and kept at −80°C for biochemical analysis. Colorimetric diagnostic kits were used to measure serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) as directed by the manufacturer.[17] The total protein level was determined using commercially available kits.[18]

2.4.2 | HCC biomarkers

*Determination of alpha-fetoprotein (*AFP*) and AFP-L3*: The serum AFP and AFP-L3 levels were estimated quantitatively using an enzyme-linked immunosorbent assay by Elabscience (catalog no. E-EL-R0153) and LifeSpan BioSciences (catalog no. LS-F40468), respectively, according to the manufacturer's instructions.[19]

Western blotting of GPC3: Liver tissue samples were homogenized in a buffer (50 mM Tris pH 7.4, 10 mM NaF, 2 mM EDTA, 10 mM β-glycerol phosphate, 1 mM Na_3VO_4, 0.2% wt/vol sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail [Sigma-Aldrich; P8340]). Samples were centrifuged at 4°C for 15 min at 10,000*g*. Bradford Protein Assay Kit (Biobasic Inc.; Cat #BDE641) was used to determine the quantity of protein. Proteins were separated from whole-cell lysate in a 12% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (Bio-Rad Laboratories Inc.; Cat #161-0181) and then transferred to polyvinylidene fluoride membrane (Sigma-Aldrich; Cat #GE10600021). The blots were blocked in 5% nonfat milk and 0.1% Tween-20 in Tris-buffered saline for 2 h at room temperature before being incubated in a rabbit monoclonal antiserum against GPC3 (MyBioSource; Cat #MBS710143) and mouse anti-actin (1:1000; Cell Signaling; Cat #3700) at 4°C overnight. Finally, secondary antibodies were used to incubate blots with anti-mouse

immunoglobulin G labeled with horseradish peroxidase (Cell Signaling; Cat #7076). The protein bands were visualized using enhanced chemiluminescence (Clarity™ Western ECL Substrate; Bio-Rad; Cat #170-5060). A charge-coupled device camera-based imager was used to capture the chemiluminescent signals. On the ChemiDoc MP imager, image analysis software was used to compare the band intensity of the target proteins to the control sample beta-actin (by protein normalization).

2.4.3 | Estimation of hepatic antioxidant capacity

For tissue homogenate preparation, the liver was homogenized in an ice-cold phosphate buffer (20% wt/vol homogenate). The resulting homogenates were then centrifuged at 3000 rpm for 15 min. Finally, the supernatants were obtained for estimating the oxidative stress parameters.

Reduced glutathione (GSH) level was estimated by 5,5′-dithiobis (2-nitrobenzoic acid), and the product was detected colorimetrically at 405 nm (Beckman DU-64 UV/VIS spectrophotometer).[20] Superoxide dismutase (SOD) level was determined spectrophotometrically by suppressing superoxide-driven NADH oxidation.[21] The rate of breakdown of H_2O_2 at 510 nm was used to measure catalase (CAT) activity in tissue homogenate.[22] Glutathione peroxidase (GPx) activity was measured according to the method of Gross.[23] Tissue content of malondialdehyde (MDA), lipid peroxidation (LPO) marker, and MDA was measured using a spectrophotometric measurement of thiobarbituric acid at 535 nm.[24]

2.4.4 | Liver histopathology and immunohistochemical study

Liver specimens were fixed in 10% neutral formalin, dried in alcohol, cleaned in xylene, and paraffin-embedded. Cross-sections of 4 μm were stained on a glass slide with hematoxylin and eosin, and optical light microscopy was used to examine it.[25] Immunohistochemical staining was used to detect glutathione *S*-transferase P (GST-P) protein expression in liver tissue samples. It was performed by the streptavidin–biotin–peroxidase complex method. First, the anti-GST-P antibody (Biogenex) was incubated overnight at 47°C on histology slides, washed with phosphate-buffered saline (PBS), followed by the biotinylated anti-rabbit antibody for 10 min, and streptavidin–alkaline phosphatase for 5 min. After that, horseradish peroxidase-conjugated with streptavidin counterstaining was performed by hematoxylin, where the liver tissue appeared blue. Finally, control experiments were carried out by replacing the primary antibody with PBS according to the method of Mancini.[26] On the slides, the number of GST-P stained foci was counted.

2.4.5 | Molecular investigation of BCl-2, BAX, caspase-3, and CYP2E1 genes

According to the manufacturer's instructions, total RNA was isolated using Direct-zol RNA Miniprep Plus (Cat #R2072; Zymo Research Corp). The SuperScript IV One-Step RT-PCR Kit (Cat #12594100; Thermo Fisher Scientific) was used to perform quantitative real-time polymerase chain reaction (RT-PCR). Thermal cycling conditions were: incubation at 94°C for 15 min then 94°C for 15 s (40 cycles) followed by 60°C for 30 s and 70°C for 30 s. All complementary DNAs were generated in duplicate and contained BCL-2, BAX, miR-1-3p, CYP2E1, and caspase-3 that had previously been prepared (Table 1). The data were expressed as relative quantification (RQ) of each target gene and calculated and normalized to a housekeeping gene using the $\Delta\Delta C_t$ method. RQ was estimated by taking $2^{-\Delta\Delta C_t}$.

2.5 | Statistical analysis

The Kolmogorov–Smirnov and Shapiro–Wilk tests were used to determine the normality of the data ($p > 0.05$), and therefore, we used one-way analysis of variance (parametric test). Due to the homogeneity of the variances, we used the Tukey honestly significant difference as a post hoc test. Continuous variables were expressed as mean ± SD. Statistical analyses were executed using IBM SPSS Statistics software version 26. $p < 0.05$ was used as the cutoff value for statistical significance, and GraphPad Prism Software version 9.1.1 was used to plot the graphs.

TABLE 1 Primers' sequence of all studied genes

	Forward sequence	Reverse sequence
Bax	ACTCCCATTCTTCCACCTTTG	CCCTGTTGCTGTAGCCATATT
BCL2	AGTTCGGTGGGGTCATGTGTG	CCAGGTATGCACCCAGAGTG
Caspase-3	ATGGAGAACAACAAAACCTCAGT	TTGCTCCCATGTATGGTCTTTAC
GAPDH	TGGATTTGGACGCATTGGTC	TTTGCACTGGTACGTGTTGAT
CYP2E1	GTCTTTAACCAAGTTGGCAA	CCAATCAGAAAGGTAGGGTC

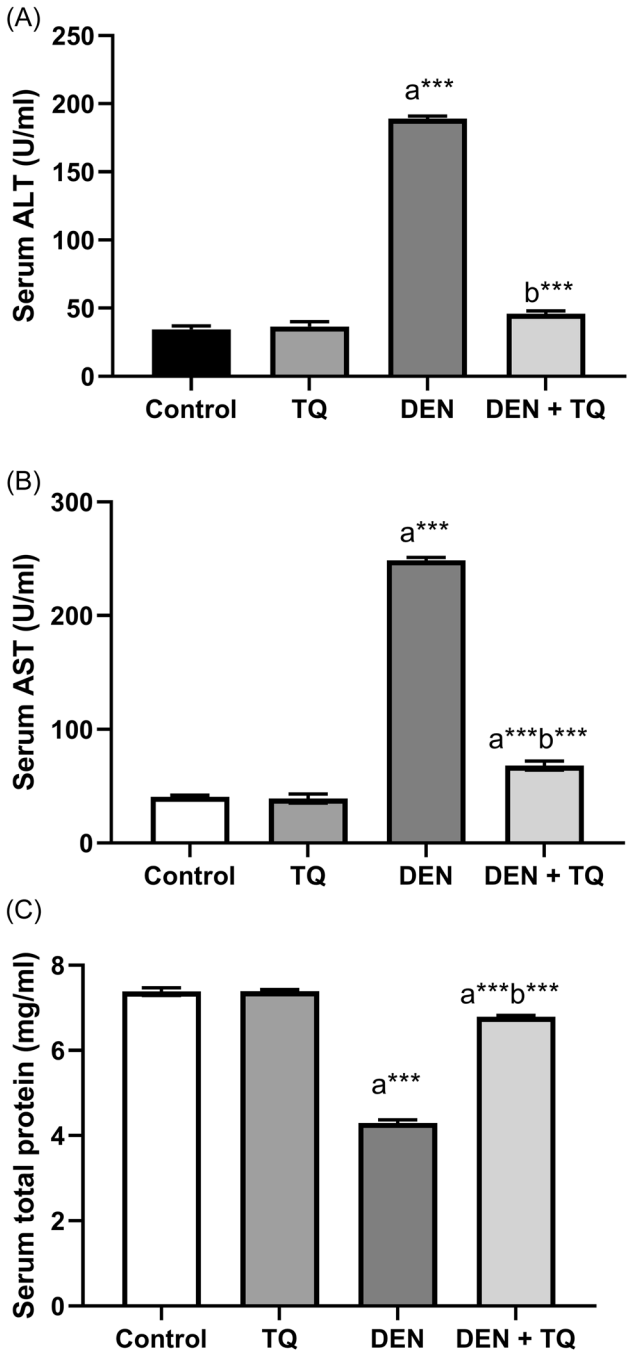

FIGURE 1 Effects of DEN, TQ, and their combination on serum liver function, ALT (A), AST (B), and total proteins (C). [a]Statistically significant from negative controls. [b]Statistically significant from DEN control. *$p < 0.05$ significant differences. ***$p < 0.0001$ significant differences. ALT, alanine aminotransferase; AST, aspartate aminotransferase; DEN, diethylnitrosamine; TQ, thymoquinone

3 | RESULTS

3.1 | Determination of liver functions

The effect of DEN TQ and their combination on serum liver function, ALT (Figure 1A), AST (Figure 1B), and total proteins (Figure 1C) is shown in Figure 1. Compared with the negative control group, DEN led to a significant elevation of ALT and AST and a significant decrease in total proteins by 448%, 514%, and 42%, respectively. TQ administration alone had no significant difference compared with the negative control group. Compared with the DEN group, pretreatment with TQ prevented liver enzymes and serum protein changes.

3.2 | Determination of HCC biomarkers; AFP, AFPL3, and GPC3

As shown in Figure 2, DEN administration produced a significant increase in serum AFP (Figure 2A), AFP-L3 (Figure 2B), and GPC3 (Figure 2C) levels by 318%, 251%, and 246%, respectively, compared with the negative control group. These elevations were attenuated after TQ administration to the DEN intoxicated rats by 71%, 62%, and 58% for AFP, AFP-L3, and GPC3, respectively, compared with DEN-intoxicated rats.

3.3 | LPO and antioxidant activity

Figure 3 illustrates the effect of DEN on the activity of the antioxidant enzymes GSH (Figure 3A), CAT (Figure 3B), GPx (Figure 3C), SOD (Figure 3D), and LPO levels measured as MDA (Figure 3E). The injection of DEN significantly decreased hepatic activities of GSH, CAT, GPx, and SOD by 40%, 78%, 72%, and 40% compared with the negative control group. However, the tissue content of MDA and CYP2E1 were significantly increased by 324% and 166%, respectively. It was found that TQ greatly enhanced the hepatic contents of GSH, CAT, GPx, and SOD by 43%, 211%, 189%, and 50% when administered 1 week before DEN injection. However, MDA tissue content and CYP2E1 were significantly decreased by 69% and 37%, respectively, compared with the DEN group (Figure 3).

3.4 | Effects of DEN and/or TQ on apoptotic biomarkers Bcl-2, BAX, and caspase-3 gene expression

Figure 4 depicts the antiapoptotic BCL-2 (Figure 4A), proapoptotic BAX (Figure 4B), BAX:BCL-2 ratio (Figure 4C), and caspase-3 (Figure 4D) gene expression. DEN therapy enhanced the gene expression level of the antiapoptotic protein BCL-2 by 24%. Interestingly, it lowered the gene expression levels of the proapoptotic proteins BAX and caspase-3 by 49% and 50%, respectively, compared with the negative control group. However, TQ before DEN injection significantly lowered BCL-2 expression and elevated the BAX, caspase-3 gene expression by 12%, 71%, and 81%, respectively, compared with DEN-intoxicated rats. Compared with the DEN-injected group, the BAX:BCL-2 ratio was significantly increased by 49% at TQ administration before the DEN injection.

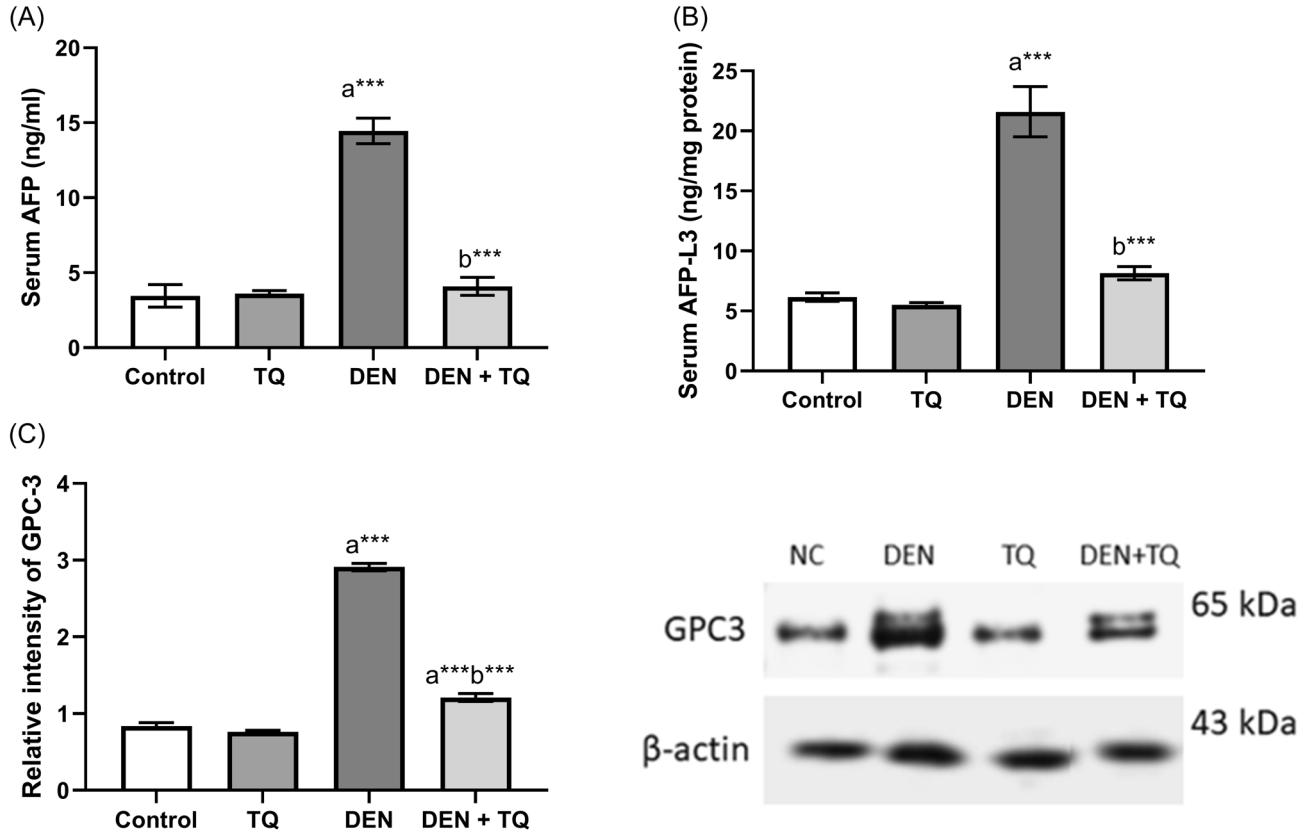

FIGURE 2 Effects of DEN, TQ, and their combination on liver biomarkers, AFP (A), AFP-L3 (B), and GPC3 (FC). [a]Statistically significant from negative controls (NC). [b]Statistically significant from DEN control. *$p < 0.05$ significant differences. ***$p < 0.0001$ significant differences. AFP, alpha-fetoprotein; DEN, diethylnitrosamine; TQ, thymoquinone

3.5 | Liver histopathology

The histological alterations in liver tissue samples caused by DEN in normal and TQ-supplemented rats are shown in Figure 5. Microscopic examination of the liver from the negative control (Figure 5A) and TQ control (Figure 5B) revealed normal histology of the hepatic parenchyma where the polygonal hepatocytes were arranged in hepatic cords radiating from the central vein toward the portal area.

Several histopathological changes were detected in the affected hepatic parenchyma, considering the DEN group. Additionally, the multifocal areas of mononuclear inflammatory cell infiltration were commonly noticed in the hepatic parenchyma accompanied by necrosis and karyorrhectic debris (Figure 5C). However, TQ before DEN injection showed a few mononuclear inflammatory cell infiltration in the hepatic lobules. Additionally, moderate vacuolated hepatocytes were noticed in some circumstances (Figure 5D).

3.6 | Immunohistochemical study

Immunohistochemical investigations of the expression of GST-P are shown in Figure 6. GST-P was not detected in the negative control and TQ control groups. GST-P staining was strongly positive in the DEN-treated group only. However, TQ treatment before DEN injection decreased the GST-P-positive foci in hepatic tissue.

4 | DISCUSSION

This study aims to understand how TQ pretreatment affects hepatic damage caused by DEN and investigate the mechanisms that could be involved. One of the best indications of hepatocellular injury is the release of ALT and ALP into the bloodstream and a decrease in total protein.[27] After DEN administration in this study, a significant increase in serum indicators of liver function and a reduction in total protein levels were observed. Several models of DEN-induced hepatocellular degeneration have previously shown higher levels of serum indicators of hepatocellular damage.[28,29] This impact could be secondary to DEN-induced hepatocyte membrane LPO.[30]

This investigation demonstrated that DEN increased MDA while decreasing GSH, GPx, SOD, and CAT in liver tissue samples, indicating that the liver's antioxidant defense mechanism was completely disrupted. Since membrane lipids are more vulnerable to reactive oxygen species (ROS), the LPO state has been employed as a diagnostic marker of oxidative stress.[31] The interaction of ROS with essential macromolecules such as DNA, DNA repair mechanisms, and other enzymes play a key role in tumor promotion.[32]

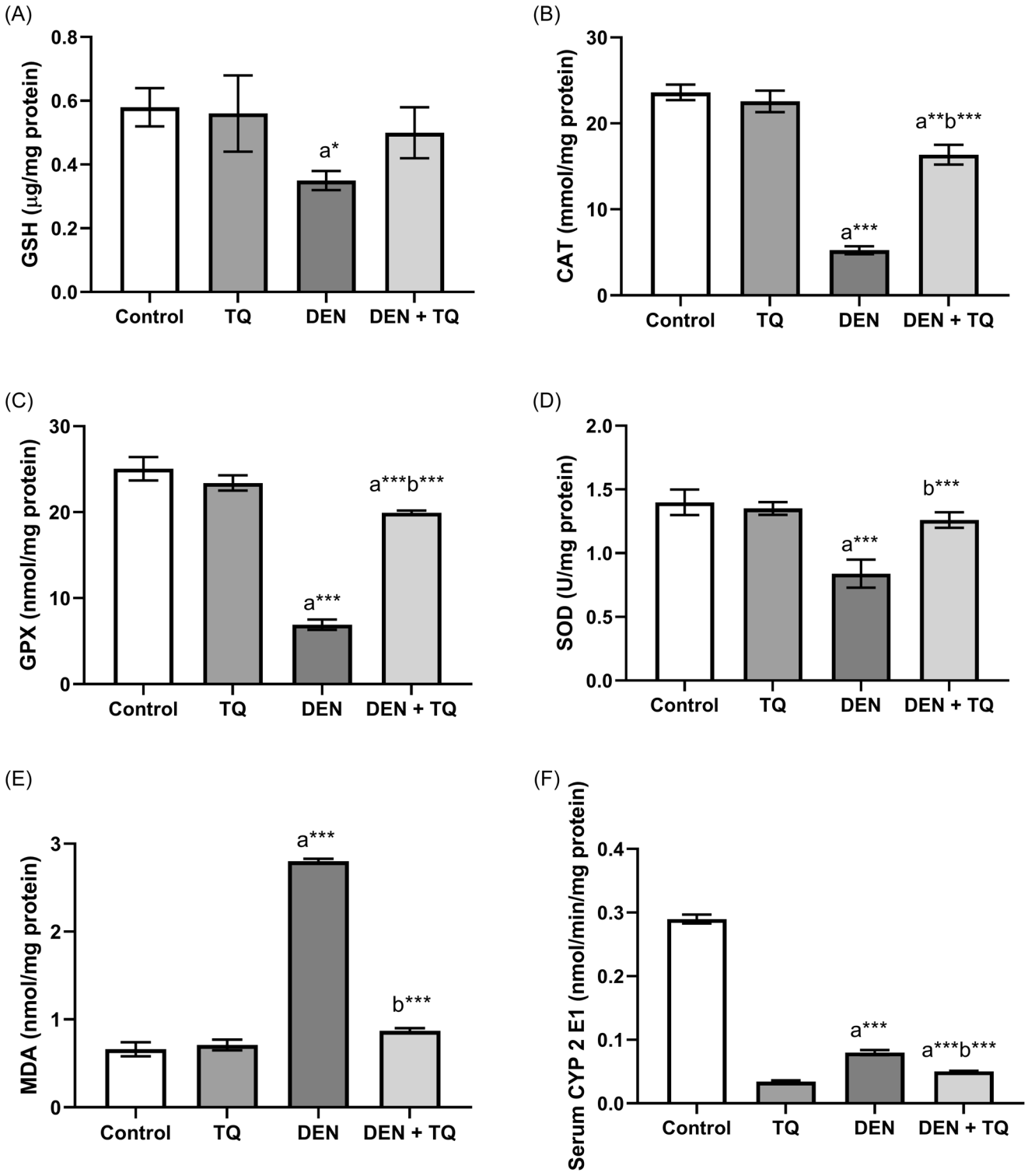

FIGURE 3 Effects of DEN, TQ, and their combination on the activity of the antioxidant enzymes GSH (A), CAT (B), GPx (C), and SOD (D), levels of lipid peroxidation measured as MDA (E) and CYP2E1 (F). [a]Statistically significant from negative controls. [b]Statistically significant from DEN control. $^{*}p < 0.05$ significant differences. $^{***}p < 0.0001$ significant differences. CAT, catalase; DEN, diethylnitrosamine; GPx, glutathione peroxidase; GSH, glutathione; MDA, malondialdehyde; SOD, superoxide dismutase; TQ, thymoquinone

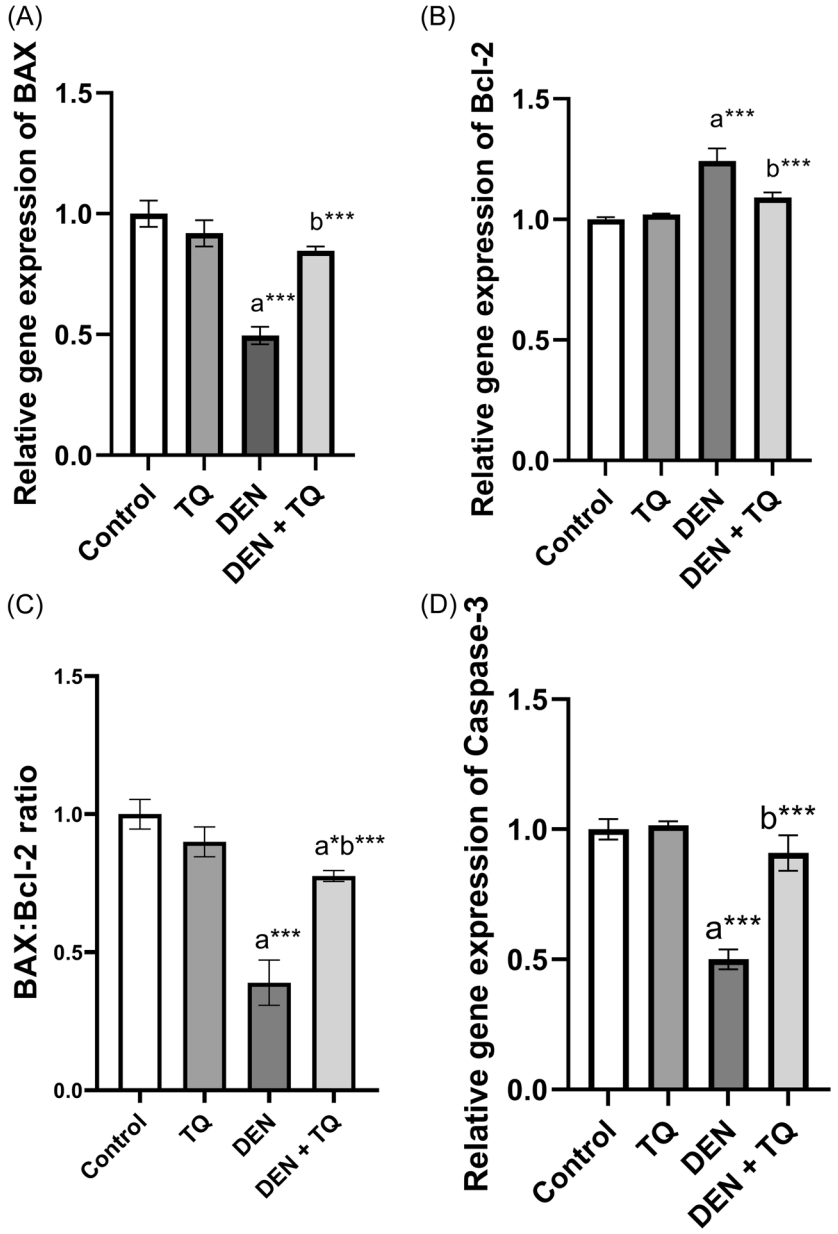

FIGURE 4 Effects of DEN, TQ, and their combination on the gene expression of antiapoptotic Bcl-2 (A), proapoptotic BAX (B), BAX: Bcl-2 ratio (C), and caspase-3 (D) proteins. [a]Statistically significant from negative controls. [b]Statistically significant from DEN control. $^{*}p < 0.05$ significant differences. $^{***}p < 0.0001$. DEN, diethylnitrosamine; TQ, thymoquinone

When ROS production surpasses the cell's antioxidant capacity, oxidative damage results[29]; in this regard, the loss in enzyme activities could be attributed to the decrease in their production or their overuse in scavenging the free radicals produced. Many DEN-induced hepatocellular cancer models have shown increased ROS production and reduced antioxidant enzymes in liver tissue samples.[30,33] This also agrees with Erbaş et al.,[34] who found that the administration of DEN led to an increase in the levels of LPO.

TQ showed strong antioxidant activity by reversing the MDA increase and decrease in GSH, CAT, SOD, and GPx produced by DEN in liver tissue. Also, TQ acts by overcoming the exogenous antioxidants scavengers of ROS and preventing additional peroxidative damage to the hepatocytes,[35–38] possibly due to the TQ treatment's recovery of MDA levels and the enzymic and nonenzymic antioxidants.[39,40] Therefore, TQ treatment prevented the increase in hepatic enzymes and a drop in total protein levels, which could protect against DEN-induced liver damage. Additionally, this benefit could be related to TQ antioxidant activity, which reduces ROS generation and lipid peroxidation, resulting in the stability of hepatocyte membranes and a reduction in liver enzyme leakage.

Cytochrome P450s are the main enzymes in the development and treatment of cancer.[41] CYP2E1 is a cytochrome P450 monooxygenase activated during drug metabolism, creating electrophilic metabolites and oxidative stress.[42] Additionally, it is involved in the metabolic activation of many low molecular mass procarcinogens.[43] In this study, the liver of DEN-treated rats produced an overexpression of the CYP2E1 gene compared with the control group. DEN is metabolized by CYP2E1 to its active ethyl radical metabolite, which can interact with DNA causing mutation and carcinogenesis. TQ pretreated animals showed significant downregulation in the

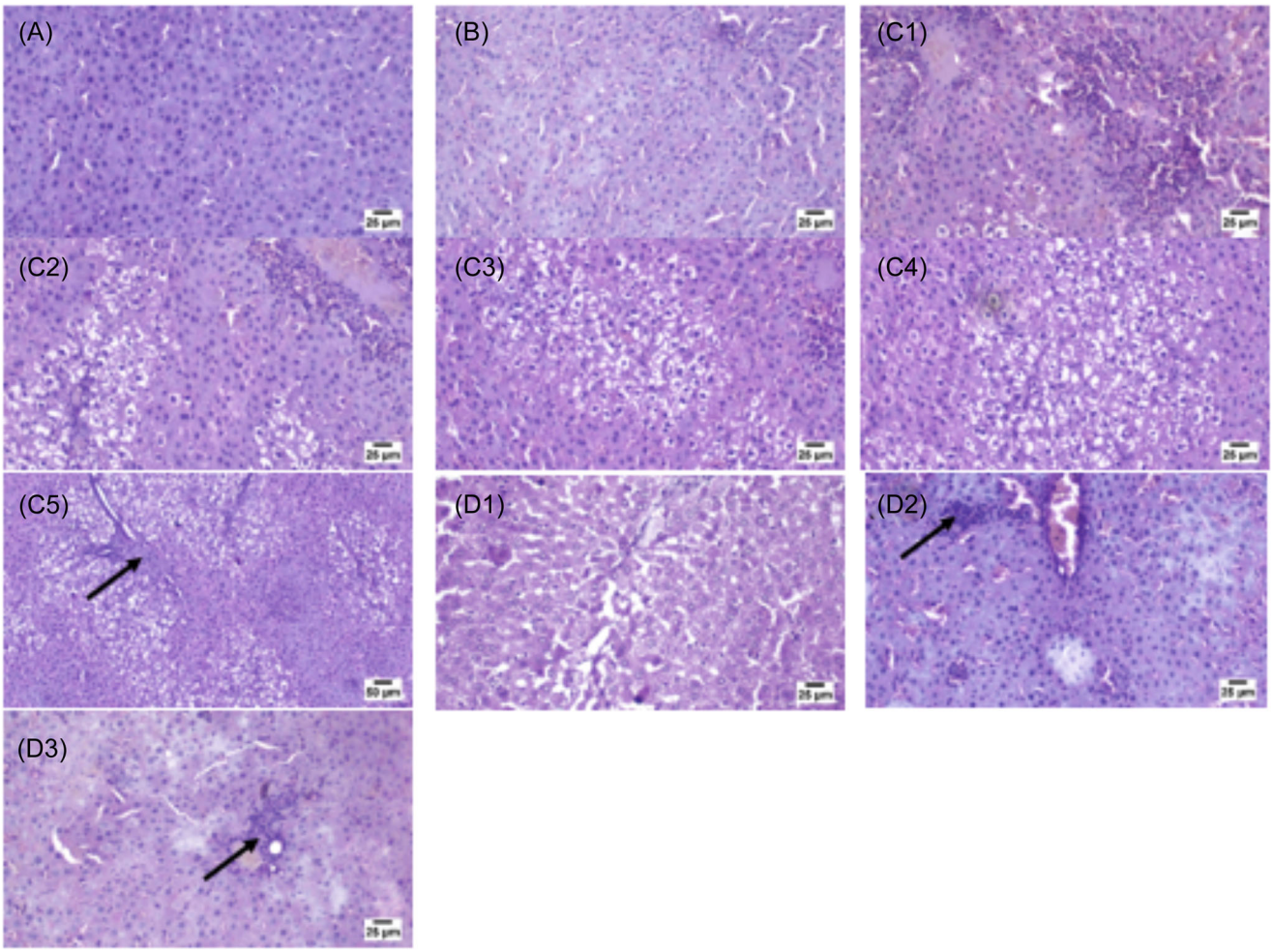

FIGURE 5 Effects of DEN, TQ, and their combination on histopathological changes in liver tissues. Liver from control (A) and TQ control (B) revealed normal histology of the hepatic parenchyma in which the polygonal hepatocytes were arranged in hepatic cords radiating from the central vein toward the portal area. (C1–C4) Several histopathological changes were detected in the affected hepatic parenchyma in the DEN group. Multifocal areas of mononuclear inflammatory cells infiltration were commonly noticed in the hepatic parenchyma, followed by necrosis, portal fibroplasia, and karyorrhectic debris. (D1–D3) Liver from rat treated with TQ to DEN few numbers of mononuclear inflammatory cells infiltration in the hepatic lobules. Additionally, mild vacuolated hepatocytes were noticed in some circumstances. DEN, diethylnitrosamine; TQ, thymoquinone

CYP2E1 gene expression compared with the DEN-treated group. Also, TQ was reported to interact with the CYP450 isozymes acting as a potent inhibitor of CYP isoforms.[44,45]

Histopathological investigations of the liver confirmed the biochemical findings. Histopathological injury in liver tissue caused by DEN indicated oxidative stress and hepatocellular damage resulting in HCC formation. This result agreed with Santos et al.,[46] who reported that DEN-induced HCC has a histological and genetic resemblance to human tumors. In contrast, hepatocytes in liver sections of rats pretreated with TQ restored normal organization and architecture with signs of recovery. Therefore, the result suggests that TQ provided significant protection, indicated by a significant decrease in hepatic vacuolation and inflammatory infiltrates. It is speculated that the antioxidant activity via the lowering of ROS and LPO formation is mostly responsible for this protective effect.

When overexpressed, the homologous BCL-2 proteins can prolong cell life by inhibiting apoptosis, whereas proapoptotic proteins (BAX and caspases) can function as cell death inducers.[47] Additionally, apoptosis dysfunction makes cancer treatment more difficult and helps tumorigenesis progress.[48] Our results show that DEN inhibits apoptosis by significantly increasing BCL-2 and decreasing BAX and caspase-3. This effect was reported to promote cancer cell proliferation and increase cell survival.[48] Furthermore, TQ pretreated rats revealed significant elevation of BAX and caspase-3 genes and reduction in BCL-2 compared with DEN-treated animals. These results agreed with Abd El-Ghany et al.,[49] who reported that TQ triggers apoptotic signaling pathways. Additionally, the overexpression of the BAX gene in TQ pretreated rats enhanced cytochrome release from mitochondria leading to caspase-3 cleavage and inducing apoptosis.[50]

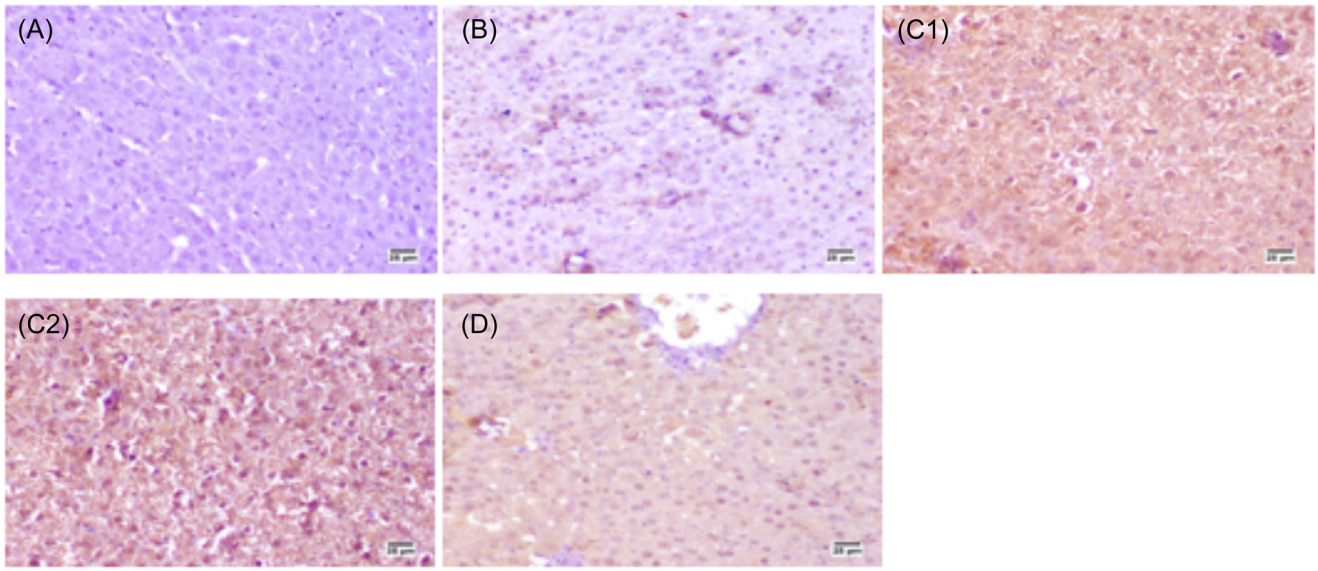

FIGURE 6 Immunohistochemistry of GST-P in rat liver. Liver from negative control (A) revealed negative expression of GST-P in the hepatic parenchyma. Likewise, examination of TQ groups' liver (B) revealed negative to a mild expression of GST-P in the hepatic tissue. On the contrary, DEN control (C1, C2) exhibited a strong positive expression of GST-P in the hepatic parenchyma. Diffuse expression of GST-P was also detected in several hepatic lobules of the DEN control group. (D) Moderate expression of GST-P was found in DEN + TQ group. DEN, diethylnitrosamine; GST-P, glutathione *S*-transferase P; TQ, thymoquinone

AFP is a biomarker used to diagnose and identify HCC.[51] AFP-L3, an isoform of AFP specific to malignant tumors used to assess liver cancer risk. AFP-L3 can detect the development of HCC earlier than AFP and aid in diagnosing AFP-negative HCC.[52] GPC3 has been postulated as a potential marker for distinguishing between benign and malignant liver disorders. GPC3 is highly expressed in HCC, with an expression pattern that differs according to the degree of cell differentiation.[53] Compared with normal untreated control rats, our study showed that DEN injection significantly increased AFP, AFP-L3, and GPC3 levels. This finding is consistent with previous research by Kadasa et al.[54] and Shahin et al.,[55] who found a higher level of AFP in DEN-intoxicated rats than in normal rats. Contrary to DEN-treated rats, TQ pretreatment considerably reduced AFP, AFP-L3, and GPC3 levels, possibly due to TQ's antiproliferative activity, linked to the suppression of malignant tumor transformation. It was also reported that TQ pre-and posttreatment dramatically reduced the level of AFP in DEN-induced HCC.[55]

Glutathione *S*-transferases (GSTs) are a multigene family of enzymes that catalyze the conjugation of the reduced form of GSH to xenobiotic substrates for detoxification. GSTs protect the cells against cytotoxicity and carcinogenic chemicals, while GST-P is a prominent tumor marker for hepatocarcinogenesis.[56] In our study, immunohistochemistry demonstrated that GST-P is overexpressed in rats' hepatic foci and tumors during DEN-induced carcinogenesis. Similarly, Satoh and Hatayama[57] discovered that the enzyme level in preneoplastic foci was 150–250 times higher than in normal cells after immunochemical and stereological examinations. Furthermore, in a DEN-induced liver cancer model, TQ administration reduced the number of liver tumors and GST-P-positive hepatocytes, indicating that TQ can suppress tumor formation.

In conclusion, according to this study's findings, pretreatment with TQ protects against DEN-induced hepatic injury and carcinogenesis by decreasing HCC biomarkers, oxidative stress, and lipid peroxidation, increasing antioxidant enzyme activity, and triggering the apoptotic signaling pathway.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data used to support the findings of this study are available from the corresponding author upon request.

ORCID

Samar Ibrahim http://orcid.org/0000-0003-0044-9310

REFERENCES

[1] H. Sung, J. Ferlay, R. L. Siegel, M. Laversanne, I. Soerjomataram, A. Jemal, F. Bray, *CA Cancer J. Clin.* **2021**, *71*, 209. https://doi.org/10.3322/caac.21660

[2] A. M. Moon, A. G. Singal, E. B. Tapper, *Clin. Gastroenterol. Hepatol.* **2020**, *18*, 2650. https://doi.org/10.1016/j.cgh.2019.07.060

[3] T. Akinyemiju, S. Abera, M. Ahmed, N. Alam, M. A. Alemayohu, C. Allen, R. Al-Raddadi, N. Alvis-Guzman, Y. Amoako, A. Artaman, T. A. Ayele, A. Barac, I. Bensenor, A. Berhane, Z. Bhutta, J. Castillo-Rivas, A. Chitheer, J. Y. Choi, B. Cowie, L. Dandona, R. Dandona, S. Dey, D. Dicker, H. Phuc, D. U. Ekwueme, M. S. Zaki, F. Fischer, T. Fürst, J. Hancock, S. I. Hay, P. Hotez, S. H. Jee, A. Kasaeian, Y. Khader, Y. H. Khang, A. Kumar, M. Kutz, H. Larson, A. Lopez,

R. Lunevicius, R. Malekzadeh, C. McAlinden, T. Meier, W. Mendoza, A. Mokdad, M. Moradi-Lakeh, G. Nagel, Q. Nguyen, G. Nguyen, F. Ogbo, G. Patton, D. M. Pereira, F. Pourmalek, M. Qorbani, A. Radfar, G. Roshandel, J. A. Salomon, J. Sanabria, B. Sartorius, M. Satpathy, M. Sawhney, S. Sepanlou, K. Shackelford, H. Shore, J. Sun, D. T. Mengistu, R. Topór-Mądry, B. Tran, K. N. Ukwaja, V. Vlassov, S. E. Vollset, T. Vos, T. Wakayo, E. Weiderpass, A. Werdecker, N. Yonemoto, M. Younis, C. Yu, Z. Zaidi, L. Zhu, C. Murray, M. Naghavi, C. Fitzmaurice, Global Burden of Disease Liver Cancer Collaboration, *JAMA Oncol.* **2017**, *3*, 1683. https://doi.org/10.1001/jamaoncol.2017.3055

[4] P. Intaraprasong, S. Siramolpiwat, R. K. Vilaichone, *Asian Pac. J. Cancer Prev.* **2016**, *17*, 3697.

[5] J. Bruix, M. Sherman, *Hepatology* **2011**, *53*, 1020. https://doi.org/10.1002/hep.24199

[6] X. Zhang, H. Yu, *Iran. J. Pharm. Res.* **2016**, *15*, 491.

[7] A. Fahmi, N. Hassanen, M. Abdur-Rahman, E. Shams-Eldin, *Biomarkers* **2019**, *24*, 436. https://doi.org/10.1080/1354750x.2019.1606280

[8] N. K. Mohammed, M. Y. Abd Manap, C. P. Tan, B. J. Muhialdin, A. M. Alhelli, A. S. Meor Hussin, *Evidence Complementary Altern. Med.* **2016**, *2016*, 6273817. https://doi.org/10.1155/2016/6273817

[9] R. N. Chopra, S. L. Nayar, I. C. Chopra, L. V. Asolkar, K. K. Kakkar, O. J. Chakre, B. S. Varma, *Glossary of Indian Medicinal Plants; [With] Supplement*, Council of Scientific & Industrial Research, New Delhi **1956**.

[10] A. ElKhoely, H. F. Hafez, A. M. Ashmawy, O. A. Badary, A. Abdelaziz, A. Mostafa, S. A. Shouman, *J. Nat. Med.* **2015**, *69*, 313. https://doi.org/10.1007/s11418-015-0895-7

[11] M. M. Sayed-Ahmed, A. M. Aleisa, S. S. Al-Rejaie, A. A. Al-Yahya, O. A. Al-Shabanah, M. M. Hafez, M. N. Nagi, *Oxid. Med. Cell. Longevity* **2010**, *3*, 254. https://doi.org/10.4161/oxim.3.4.12714

[12] E. Entok, M. C. Ustuner, C. Ozbayer, N. Tekin, F. Akyuz, B. Yangi, H. Kurt, I. Degirmenci, H. V. Gunes, *Mol. Biol. Rep.* **2014**, *41*, 2827. https://doi.org/10.1007/s11033-014-3137-2

[13] O. A. Badary, R. A. Taha, A. M. Gamal el-Din, M. H. Abdel-Wahab, *Drug Chem. Toxicol.* **2003**, *26*, 87. https://doi.org/10.1081/dct-120020404

[14] M. Asaduzzaman Khan, M. Tania, S. Fu, J. Fu, *Oncotarget* **2017**, *8*, 51907. https://doi.org/10.18632/oncotarget.17206

[15] B. H. Ali, G. Blunden, *Phytother. Res.* **2003**, *17*, 299. https://doi.org/10.1002/ptr.1309

[16] A. Ahmad, S. Alqahtani, B. L. Jan, M. Raish, A. K. Rabba, K. M. Alkharfy, *Saudi Pharm. J.* **2020**, *28*, 403. https://doi.org/10.1016/j.jsps.2020.01.022

[17] R. J. Henry, N. Chiamori, O. J. Golub, S. Berkman, *Am. J. Clin. Pathol.* **1960**, *34*, 381. https://doi.org/10.1093/ajcp/34.4_ts.381

[18] A. Hiller, J. Plazin, D. D. Van Slyke, *J. Biol. Chem.* **1948**, *176*, 1401.

[19] M. Uotila, E. Ruoslahti, E. Engvall, *J. Immunol. Methods* **1981**, *42*, 11. https://doi.org/10.1016/0022-1759(81)90219-2

[20] E. Beutler, O. Duron, B. M. Kelly, *J. Lab. Clin. Med.* **1963**, *61*, 882.

[21] M. Nishikimi, N. Appaji, K. Yagi, *Biochem. Biophys. Res. Commun.* **1972**, *46*, 849. https://doi.org/10.1016/s0006-291x(72)80218-3

[22] H. Aebi, *Methods Enzymol.* **1984**, *105*, 121. https://doi.org/10.1016/s0076-6879(84)05016-3

[23] R. T. Gross, R. Bracci, N. Rudolph, E. Schroeder, J. A. Kochen, *Blood* **1967**, *29*, 481.

[24] Y. J. Garcia, A. J. Rodríguez-Malaver, N. Peñaloza, *J. Neurosci. Methods* **2005**, *144*, 127. https://doi.org/10.1016/j.jneumeth.2004.10.018

[25] J. D. Bancroft, A. Stevens, *Theory and Practice of Histological Techniques*, 4th ed., Churchill Livingstone, New York **1996**.

[26] G. Mancini, A. O. Carbonara, J. F. Heremans, *Immunochemistry* **1965**, *2*, 235. https://doi.org/10.1016/0019-2791(65)90004-2

[27] F. Bulle, P. Mavier, E. S. Zafrani, A. M. Preaux, M. C. Lescs, S. Siegrist, D. Dhumeaux, G. Guellaën, *Hepatology* **1990**, *11*, 545. https://doi.org/10.1002/hep.1840110404

[28] J. Song, W. Ding, B. Liu, D. Liu, Z. Xia, L. Zhang, L. Cui, Y. Luo, X. Jia, L. Feng, *Mol. Med. Rep.* **2020**, *22*, 697. https://doi.org/10.3892/mmr.2020.11135

[29] R. Tolba, T. Kraus, C. Liedtke, M. Schwarz, R. Weiskirchen, *Lab. Anim.* **2015**, *49*, 59. https://doi.org/10.1177/0023677215570086

[30] P. Velu, A. Vijayalakshmi, P. Iyappan, D. Indumathi, *Biomed. Pharmacother.* **2016**, *84*, 430. https://doi.org/10.1016/j.biopha.2016.09.060

[31] L.-J. Su, J.-H. Zhang, H. Gomez, R. Murugan, X. Hong, D. Xu, F. Jiang, Z. Y. Peng, *Oxid. Med. Cell. Longevity* **2019**, *2019*, 5080843. https://doi.org/10.1155/2019/5080843

[32] U. S. Srinivas, B. W. Q. Tan, B. A. Vellayappan, A. D. Jeyasekharan, *Redox Biol.* **2019**, *25*, 101084. https://doi.org/10.1016/j.redox.2018.101084

[33] D. Huang, H. Li, Q. He, W. Yuan, Z. Chen, H. Yang, *Water, Air, Soil Pollut.* **2018**, *229*, 81. https://doi.org/10.1007/s11270-018-3739-8

[34] D. Erbaş, A. Ekmekçi, A. Aricioğlu, M. Koz, M. Ulküer, *Prostaglandins, Leukotrienes Essent. Fatty Acids* **1993**, *49*, 805. https://doi.org/10.1016/0952-3278(93)90029-v

[35] M. Ismail, G. Al-Naqeep, K. W. Chan, *Free Radical Biol. Med.* **2010**, *48*, 664. https://doi.org/10.1016/j.freeradbiomed.2009.12.002

[36] M. N. Nagi, H. A. Almakki, *Phytother. Res.* **2009**, *23*, 1295. https://doi.org/10.1002/ptr.2766

[37] M. N. Nagi, K. Alam, O. A. Badary, O. A. Al-Shabanah, H. A. Al-Sawaf, A. M. Al-Bekairi, *Biochem. Mol. Biol. Int.* **1999**, *47*, 153. https://doi.org/10.1080/15216549900201153

[38] O. A. Badary, A. B. Abdel-Naim, M. H. Abdel-Wahab, F. M. Hamada, *Toxicology* **2000**, *143*(3), 219. https://doi.org/10.1016/s0300-483x(99)00179-1

[39] S. M. Hosseini, E. Taghiabadi, K. Abnous, A. T. Hariri, H. Pourbakhsh, H. Hosseinzadeh, *Iran. J. Basic Med. Sci.* **2017**, *20*, 927. https://doi.org/10.22038/IJBMS.2017.9116

[40] Y. S. Abulfadl, N. N. El-Maraghy, A. A. E. Ahmed, S. Nofal, O. A. Badary, *Neurol. Res.* **2018**, *40*, 324. https://doi.org/10.1080/01616412.2018.1441776

[41] I. Elfaki, R. Mir, F. M. Almutairi, F. Duhier, *Asian Pac. J. Cancer Prev.* **2018**, *19*, 2057. https://doi.org/10.22034/APJCP.2018.19.8.2057

[42] Z. Wang, Z. Li, Y. Ye, L. Xie, W. Li, *Oxid. Med. Cell. Longevity* **2016**, *2016*, 7891574. https://doi.org/10.1155/2016/7891574

[43] D. T. Trafalis, E. S. Panteli, A. Grivas, C. Tsigris, P. N. Karamanakos, *Expert Opin. Drug Metab. Toxicol.* **2010**, *6*, 307. https://doi.org/10.1517/17425250903540238

[44] O. A. Badary, M. F. Abd-Ellah, M. A. El-Mahdy, S. A. Salama, F. M. Hamada, *Food Chem. Toxicol.* **2007**, *45*, 88. https://doi.org/10.1016/j.fct.2006.08.004

[45] A. A. Albassam, A. Ahad, A. Alsultan, F. I. Al-Jenoobi, *Saudi Pharm. J.* **2018**, *26*, 673. https://doi.org/10.1016/j.jsps.2018.02.024

[46] N. P. Santos, I. C. Pereira, M. J. Pires, C. Lopes, R. Andrade, M. M. Oliveira, A. Colaço, F. Peixoto, P. A. Oliveira, *In Vivo* **2012**, *26*, 921.

[47] J. Kale, E. J. Osterlund, D. W. Andrews, *Cell Death Differ.* **2018**, *25*, 65. https://doi.org/10.1038/cdd.2017.186

[48] C. M. Pfeffer, A. T. K. Singh, *Int. J. Mol. Sci.* **2018**, *19*, 448. https://doi.org/10.3390/ijms19020448

[49] R. M. Abd El-Ghany, N. M. Sharaf, L. A. Kassem, L. G. Mahran, O. A. Heikal, *Drug Discoveries Ther.* **2009**, *3*, 296.

[50] A. E. Ashour, A. R. Abd-Allah, H. M. Korashy, S. M. Attia, A. Z. Alzahrani, Q. Saquib, S. A. Bakheet, H. E. Abdel-Hamied, S. Jamal, A. K. Rishi, *Mol. Cell. Biochem.* **2014**, *389*, 85. https://doi.org/10.1007/s11010-013-1930-1

[51] J. Zhang, G. Chen, P. Zhang, J. Zhang, X. Li, D. Gan, X. Cao, M. Han, H. Du, Y. Ye, *PLoS ONE* **2020**, *15*, e0228857. https://doi.org/10.1371/journal.pone.0228857
[52] Z. Zhang, Y. Zhang, Y. Wang, L. Xu, W. Xu, *Onco. Targets Ther.* **2015**, *9*, 123. https://doi.org/10.2147/OTT.S90732
[53] M. S. Mohamed, *Al-Azhar Int. Med. J.* **2020**, *1*, 21. https://doi.org/10.21608/aimj.2020.21007.1021
[54] N. M. Kadasa, H. Abdallah, M. Afifi, S. Gowayed, *Asian Pac. J. Cancer Prev.* **2015**, *16*, 103. https://doi.org/10.7314/apjcp.2015.16.1.103
[55] Y. R. Shahin, N. M. Elguindy, A. Abdel Bary, M. Balbaa, *Environ. Toxicol.* **2018**, *33*, 885. https://doi.org/10.1002/tox.22574
[56] A. Chatterjee, S. Gupta, *Cancer Lett.* **2018**, *433*, 33. https://doi.org/10.1016/j.canlet.2018.06.028
[57] K. Satoh, I. Hatayama, *Carcinogenesis* **2002**, *23*, 1193. https://doi.org/10.1093/carcin/23.7.1193

How to cite this article: S. Ibrahim, S. A. Fahim, S. A. Tadros, O. A. Badary, *J. Biochem. Mol. Toxicol*. **2022**, e23078. https://doi.org/10.1002/jbt.23078