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Ameliorative Role Of Quercetin Dihydrate In Diethylnitrosamine-Mediated Hepatic Carcinogenesis In Wistar Rats

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Abstract:
Objective: *Hepatocellular carcinoma is a well-known prevalent, asymptomatic disease worldwide. Quercetin dihydrate treats liver cancer and has antiproliferative, antioxidant, and hepatoprotective properties. The goal of the current study is to assess Quercetin dihydrate's (QDH) chemoprotective and therapeutic effects against hepatic cancer caused by diethylnitrosamine (DEN).*
Material and method: *To induce hepatocellular carcinoma, Wistar rats received 200 mg/kg of diethyl nitrosamine. Over 16 weeks, five groups were treated with QDH and a standard drug via oral gavage. The study evaluated QDH's anti-proliferative effects by analyzing serum biomarkers, hematological profiles, antioxidant and membrane-bound enzymes, and inflammatory markers. Histological and microscopic examinations were performed to validate the biochemical and therapeutic outcomes of QDH treatment.*
Results: *QDH exerts chemopreventive effects in DENA-induced hepatocellular carcinoma by enhancing antioxidant and membrane-bound enzyme activities, reducing inflammatory markers via the NF-κB pathway, and lowering serum hepatic markers. It restores liver architecture dose-dependently and inhibits the EGFR/ERK1/2 signaling pathway and related genes, highlighting its combined antioxidant and anti-inflammatory actions in combating hepatocellular carcinoma (HCC).*
Conclusion: *Our results confirm that QDH provides a protective effect against DEN-induced hepatocarcinogenesis and offers a potential strategy to enhance clinical outcomes in hepatocellular carcinoma treatment.*
Keywords: *Quercetin dihydrate, NF-κB pathway Hepatocellular carcinoma*

INTRODUCTION:

Primary liver cancer, especially HCC, is a major global health concern due to its high prevalence and cancer-related mortality (1). Hepatocellular carcinoma risk factors include hemochromatosis, alcohol use, hormonal exposure, hepatitis B/C infections, aflatoxin B1, and DEN, a potent carcinogen commonly used in experimental hepatocellular carcinoma (HCC) models. This substance, which poses a major risk to human health, is mostly prevalent in chemicals, fried meals, tobacco products, cosmetics, and pharmaceuticals (2). Treating hepatocellular carcinoma remains a significant challenge for the medical field, with chemoprotective agents offering an alternative approach to combat cancer. These agents, derived from natural, synthetic, or biological sources, work by inhibiting, counteracting, or halting the proliferation of cancer cells and are now recognized as innovative tools in the fight against various cancers. Numerous studies have explored natural biomolecules that target different stages of cancer development. These compounds modulate cell signaling pathways, control cell proliferation, trigger apoptosis, and protect against oxidative damage (3). In vivo research and reviews of the literature have shown that including flavonoids (triterpenoids) in daily meals can reduce the risk of cancer. At the 'preneoplastic' stage of HCC, a variety of genetic changes build up. Higher amounts of growth factors and inflammatory cytokines at this stage lead hepatocytes to undergo increased mitogenic activation. Key signaling pathways

that support angiogenesis, cell proliferation, and enhanced cell survival are activated as a result(4). In the pathophysiology of HCC, the EGFR signaling pathway is essential from the very beginning of the illness(5). Different ligands from the EGF family can activate EGFR, this can activate various downstream signaling pathways, such as the RAS/MAPK/ERK. A complex of adaptor proteins known as Grb2/Shc/Sos is activated when a growth factor binds to EGFR and phosphorylates it(6).whereas the two most prominent members of the flavanol group of flavonoids are quercetin dihydrate (QDH) and naringin (NA) (7). As a flavonoid, fruits and vegetables are among the finest natural food sources of QDH. Onions, tomatoes, kale, broccoli, and peppers are examples of vegetables, while apples, red grapes, cherries, and all berries—blackberries, raspberries, and cranberries—are examples of fruits(8). The effect of QDH on the EGFR/ERK1/2 signaling pathway in DENA-induced hepatocellular carcinoma in rats has not been previously studied. The study aimed to evaluate QDH chemopreventive and sorafenib hepatoprotective effects in rats with DENA-induced early hepatocellular carcinoma.

1. In vivo anticancer activity:

1.1. Materials:

Ascorbic acid, hydrogen peroxide, DPPH, ethanol, distilled water, phosphate buffer (7.4pH), chloroform, CCl4 ,ketamine were provided by Teerthanker Mahaveer College of Pharmacy. Tetramethylpyrazine was purchased from Sisco Research Laboratories Pvt. Ltd. Sorafenib was purchased from Pharmacy. DEN was purchased from Sigma Aldrich.

We bought the creatinine and GGT kit from J.B. Traders. The BCA protein assay kit was obtained from J.B. Traders, also the source of the AFP ELISA kit. We purchased Sizer DNA markers, Maxime RT-PCR PreMix Kits, and Easy-REDTM total RNA extraction from J.B. Traders. From J.B. Traders, we purchased primers, PCNA, Bcl2, and cfos. was the supplier of the b-actin primer. We purchased antibodies against ERK1/2 (42/44 kDa) and b-actin IgG from J.B. Traders.

1.2. DPPH radical scavenging activity assay:

In accordance with a described procedure, the radical scavenging ability of QDH and standard reference chemical, a-tocopherol (vitamin E), was evaluated using the DPPH assay (9). The EC50 value was obtained by plotting the percentage of inhibition versus the concentration. which was the concentration of QDH that resulted in 50% DPPH radical scavenging.

1.3. Animal:

The present study utilized 36 healthy Swiss albino rats (170–250 g, both sexes), housed in the Central Animal House Facility of the Department of Pharmacology, TMMC & RC, Moradabad. Animals were maintained under standardized environmental conditions: a controlled 12:12-hour light-dark cycle, ambient temperature of 25 ± 2 °C, and relative humidity of 45–55%. All animals were acclimatized for one week prior to experimentation and provided with a standard pellet diet and water ad libitum. The animals were procured from the Institutional Animal Experimental Unit, registered under CPCSEA (Registration No. 2175/PO/RcBiBt/S/22/CPCSEA).

1.4. Oral toxicity study:

The animals were divided into four groups (n = 3 per group): Group I received normal saline (control), while Groups II, III, and IV received QDH at 5, 30, and 50 mg/kg, respectively. Doses were calculated based on individual body weights recorded before administration to assess potential toxicological effects. No adverse effects were observed in any rats during the first 4 hours post-dosing. Following that, all rats were monitored for 72 hours (3 days) to check for any behavioral abnormalities, toxic effects of drugs, and other parameters, such as body weight, food intake, water consumption, temperature, breathing, skin color, urination, general appearance, eye color, fur, mucus membrane, respiratory, circulatory, autonomic, and nervous system, somatomotor activity, drowsiness, diarrhea, sedation, tremors, convulsions, lethargy, sleep and coma, salivation, convulsion (10) .

1.5. Experiment protocol:

There were five groups of rats total, each including six rats. Rats were given a tested medication and a cancer-causing substance (DEN).

Group 1: Normal control

Group 2: Diethylnitrosamine control
Group 3: Diethylnitrosamine + sorafenib (10 mg/kg)
Group 4: Diethylnitrosamine + QDH (30 mg/kg)
Group 5: QDH + Diethylnitrosamine (30 mg/kg)
. Groups 2–5 Animals were administered a single I.P dose of DEN at 200 mg/kg body weight in buffer solution. Every rat's body weight was measured up until the next 16 weeks. The retro-orbital plexus was used to draw blood samples, after being taken out and weighed, liver tissue was rinsed with ice-cold saline and blotted dry with filter paper. For histological analysis, some of the liver was kept in a solution containing 10% formaldehyde.

1.6. Estimation of Biochemical Parameters:

Hepatic enzymes (AST, ALT, and ALP) were measured using assay kits from J.B. Traders (Gujarat, India), while non-hepatic markers (albumin, total protein, and total bilirubin) were assessed using diagnostic kits. All procedures adhered strictly to the manufacturers' protocols to ensure accuracy and reliability of biochemical evaluations.

1.6.1. Assessment of AFP:

AFP levels were estimated according to the standard method outlined by Anwar et al.,(11). Coated wells of the desired quantity were fastened into the holder. Standards, controls, and 25 µl of serum samples were added to respective wells, followed by 100 µl of zero buffer to ensure complete reaction, the samples were subsequently incubated at room temperature for 30 minutes.

1.7. Estimation of haematological parameters:

Hematological parameters (Hb, WBC, RBC) were measured using Verma et al.'s method (12):

1.7.1. Red blood cells:

Blood samples were diluted using a solution of 0.5 g mercuric chloride, 1 g sodium chloride, and 5 g sodium sulfate in 200 ml distilled water. Using an RBC pipette, blood was drawn up to the 0.5 ml mark, mixed immediately, and allowed to settle. A drop of diluted blood was placed in a Neubauer chamber, where it settled before being examined under a microscope. Red blood cells were then counted, and Results were reported as cell count per milliliter of blood. This method ensured accurate quantification of RBCs for hematological analysis.

1.7.2. White blood cells (WBC):

A mixture was prepared in a sterile test tube by combining 0.02 ml of blood with 0.38 ml of WBC diluting fluid, consisting of 1% gentian violet in GAA diluted with water, for white blood cell analysis. After allowing the cells to settle, a single drop of the diluted sample was placed in the Neubauer counting chamber and examined under a microscope.

1.7.3. Haemoglobin (Hb):

0.02 ml of Drabkin's reagent, prepared from potassium ferricyanide, potassium cyanide, and sodium bicarbonate in water, was mixed with blood. After 15 minutes of dilution, was measured at 540 nm wavelength, calibrated against a cyanomethemoglobin standard to determine hemoglobin concentration accurately. Results expressed in gl mol of hemoglobin.

1.8. Estimation of oxidative stress

Lipid peroxidation was measured by assessing MDA in liver tissue using Ohkawa et al.'s method (13) . To measure MDA, 0.2 ml tissue homogenate was mixed with 1.5 ml 20% acetic acid, 1.5 ml TBA, and 0.2 ml SDS, then diluted to 4 ml with distilled water. The mixture was heated at 90°C for 30 minutes and cooled.

1.9. Determination of enzymatic and non-enzymatic antioxidant Parameters:

To measure the several enzymatic antioxidant characteristics, including CAT, liver homogenates were used(14) SOD(14) (GPx) (15) No enzymatic antioxidant parameters, such as glutathione (GSH), were assessed or included in the analysis (16), Vitamin C (17) and (G6PD) (18) by commercially available kits. As previously mentioned, the amount of TBARS in liver homogenate was measured(19) In contrast, a pink color was produced at 532 nm when one MDA molecule reacted with two thiobarbituric acid molecules.

1.10. Estimation of proinflammatory cytokines:

Proinflammatory cytokines IL-1β, TNF-α, and IL-6 were quantified using ELISA kits from R & D Systems, Minneapolis. The assays were conducted precisely according to the manufacturer's protocols to ensure reliable and accurate results.

1.11. Estimation of membrane bound enzymes:

1.11.1. Na/K+ ATPase:

Na/K+ ATPase activity was measured using the previously described bonting method (20). A mixture of 1.0 ml buffer and 0.2 ml each of ATP, enzyme, EDTA, MgSO4, KCl, and NaCl was incubated at room temperature for 15 minutes. After incubation, 1 ml TCA was added to stop the reaction, and the sample was centrifuged.

2.11.2 Ca2+-ATPase:

Plasma membrane Ca^{2+}-ATPase levels were measured following Lotersztajn's method (21). Tissue homogenate was combined with 0.1 ml buffer, $CaCl_2$, ATP, and test sample, then incubated for 15 minutes at room temperature. After adding 1.0 ml of 10% TCA to stop the reaction, the mixture was analyzed.

2.12. Isolation of RNA and semi-quantitative RT-PCR:

Isol-RNA Lysis Reagent TM, which is based on phenol and guanidine, was used to isolate the total RNA using a total RNA extraction kit. Each experimental group's one microgram of total RNA was reverse-transcribed, and after that, the single-strand cDNA was amplified using a one-step PCR (Maxime RT PreMix kit) in accordance with the manufacturer's instructions. Following 30 minutes at 458C for the reverse transcription reaction, 5 minutes at 958C for RTase inactivation, 1 minute at 728C for an extension, and 5 minutes at 728C for the final extension for each primer, the PCR procedure began. Table 2 lists the size and specific primer sequences (23–26).A 1.5% agarose gel electrophoresis was used to resolve the PCR results, and ethidium bromide staining was applied (22). UVIBAND MAX software package was used to quantify the band intensity. The internal control, b-actin, was used to standardize the band measurements from densitometry.

TABLE 2 The specific primers sequences and PCR corresponding conditions:

Gene	Forward Primer	Reverse Primer	Size (bp)	Gene Function
β-actin	5'-CATCCACTATCGGCAATGAGC-3'	5'-GAACAGCACTGTGTTGGCATA-3'	187	Housekeeping gene used as a loading control in gene expression studies.
PCNA	5'-CCGTCGCAACTCCGCCACCAT-3'	5'-GATTCACGCCGCCCGAACTGA-3'	126	Proliferating cell nuclear antigen, a marker for cell proliferation.
Bcl-2	5'-CACCCACTGGCATCTTCTCCT-3'	5'-GTATGACGCTCCCCACACACA-3'	349	B-cell lymphoma 2, involved in regulating cell death (apoptosis).
c-fos	5'-GTCCTCGTTCCTCCAGTCCGA-3'	5'-TGCGATGGAAAGGCCAAGCCC-3'	520	Proto-oncogene that regulates cell proliferation, differentiation, and survival.

2.13 . (SDS-PAGE) and Western blot analysis:

Liver tissues were homogenized using Invitrogen's lysis buffer (FNN0071) for protein extraction, followed by the addition of Roche's complete protease inhibitor cocktail to preserve protein integrity. Total cell extracts were prepared and subjected to immunoblotting procedures. Primary antibodies were applied at a 1:1000 dilution to detect target proteins, following previously established immunoblotting protocols (23) Blots were incubated with HRP-conjugated anti-rabbit secondary antibody diluted 1:5000. Immune-reactive bands were detected using Ultra TMB-Blotting Solution, and membrane images and band densities were captured and analyzed using Bio-Rad.

2.14 . Histological study of liver tissue:

Liver tissue was preserved by storing it in a 10% formalin solution. Eosin and hematoxylin were used to stain the 5µm sections that were embedded in paraffin, deparaffinized, rehydrated for the histological investigation. With a light microscope, the slides were visible.

2.15 Statistical analysis

Results were presented as mean ± SEM. Statistical evaluation involved one-way ANOVA followed by Dunnett's test using GraphPad Prism software.

2. RESULT:

3.1 Oral toxicity study:

According to OECD guideline 423, the hazardous impact of QDH was assessed using a test medication limit dose of 50 mg/kg. No hazardous symptoms or deaths were seen following QDH was administered orally at doses of 5, 30, and 50 mg/kg. Observations of the control and treated drug groups' overall behaviour were conducted for the first four hours and continued for 72 hours. The animals showed no signs of drug-related changes in their skin and eyes, water intake, behaviour, food intake, temperature, or breathing. Consequently, the QDH was deemed safe up to a dose level of 100 mg/kg, and an LD50 of >100 mg/kg was employed. Group IVth (animals treated with test dose of 50 mg/kg) showed signs of lethargy and sleepiness despite the signs of drowsiness. Following the QDH's introduction, Table 1 shows the parameters for the oral toxicity research (10).

Table 1. Observed Behavioral and Physical Parameters in Acute Toxicity Study of Experimental Animals:

Oral toxicity study (behavioral observations)					
Observation	**Control group**	**5 mg/kg**	**30 mg/kg**	**50 mg/kg**	**100 mg/kg**
Weight of Body	+	-	-	-	++
Intake Food	+	+	+	+	+
Consumption of water	+	+	+	+	+
Temperature	+	+	+	+	+
Breathing	+	+	+	+	+
Urination	+	+	+	+	+
General appearance	+	+	+	+	+++
skin colour	-	-	-	-	-
Eyes colour	-	-	-	-	-
Fur	-	-	-	-	-
Mucus membrane	-	-	-	-	-
Respiratory	-	-	-	-	-
Circulatory	-	-	-	-	-
Autonomic and nervous system	-	-	-	-	-
Somatomotor activity	-	-	-	-	-
Drowsiness	--	--	--	--	--
Diarrhea	---	---	---	---	---
Sedation	--	--	--	--	--
Tremors	--	--	--	--	--
Convulsions	--	--	--	--	--
Salivation	---	---	---	---	---
Lethargy	---	---	---	---	++++
Sleep and Coma	---	---	---	---	---

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Here + = Normal, - = No change, ++ = change, +++ = Lethargy, ~ = Not Present, ~~ = No, ++++ = Yes

3.2 Antioxidant Potential of QDH Determined by DPPH Free Radical Scavenging Assay: QDH was more effective than vitamin E at scavenging free radicals for DPPH. As shown in Figure, the EC50 values for ascorbic acid and QDH are 11.25 and 24.34 mg/mL, respectively.

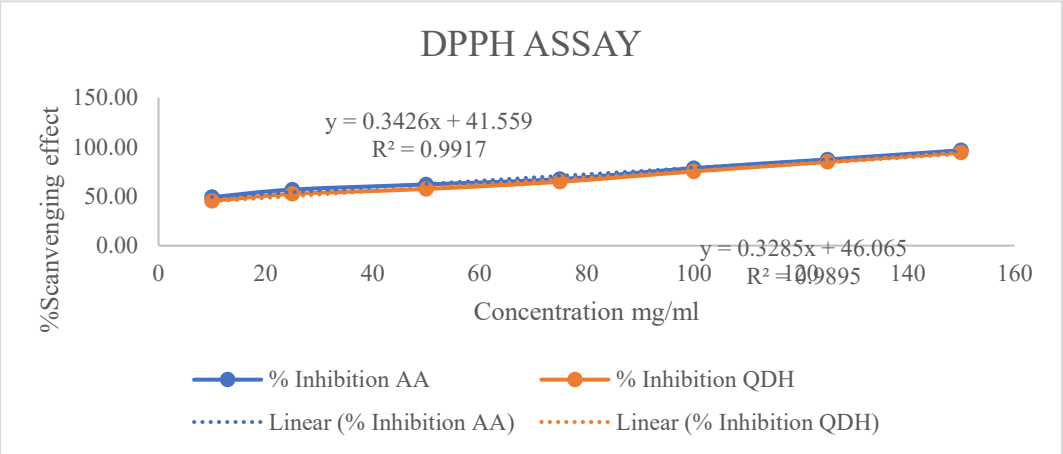

Figure. 1: antioxidant activity of various QDH and ascorbic acid concentrations. As a percentage of the scavenging action, the antioxidant impact is represented by the mean value of 6 SD.

3.3 Effect of QDH Administration on Body and Liver Weight:

Figure 2 shows that rats in the DEN group had significantly lower body weight compared to the normal control group ($p < 0.001$). Oral administration of QDH to DEN-treated rats increased their body weight in a dose-dependent manner ($p < 0.001$). Additionally, rats with DEN-induced hepatocarcinogenesis exhibited significantly higher liver weights than both the normal and QDH-treated groups, liver enlargement caused by DEN.

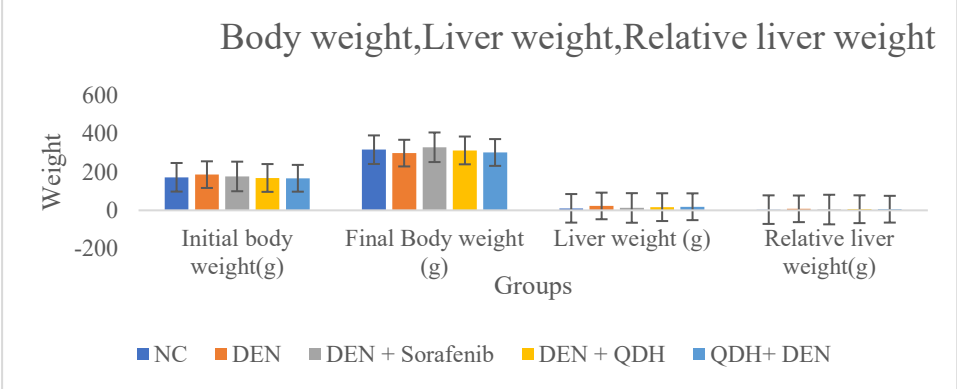

Figure. 2: Impact of Quercetin Dihydrate on DEN-Induced Alterations in Body and Liver Weights

3.4 Effect of QDH Treatment on Gross Liver Morphology in Experimental Rats:

The total number of rats, liver nodules, nodule incidence, and average number of nodules per liver are all shown in Figure 3. Hepatic nodules were exclusively observed in the DEN control group, indicating tumor development, while no nodules were detected in the normal control group, confirming the absence of pathological changes. Treatment with QDH significantly reduced the number of hepatic nodules in DEN-induced rats compared to the untreated DEN group, indicating QDH's potential protective effect against liver nodule formation caused by DEN.

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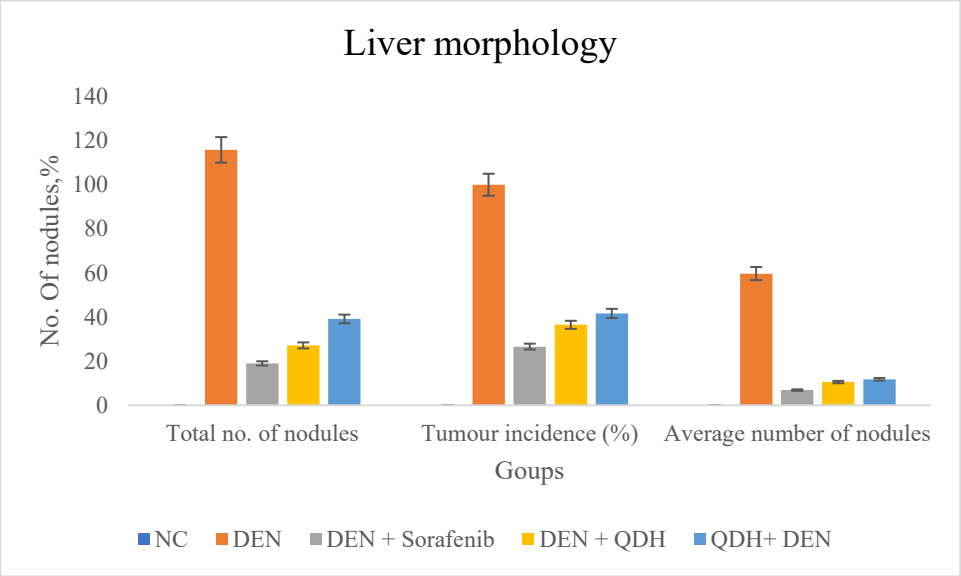

Figure. 3: Effect of QDH on Liver Nodule Incidence and Burden in DEN-Induced Rats

3.5 Effect of QDH on hepatic parameter:

Figure 4 shows that liver markers (AST, ALP, ALT, AFP) were elevated in the DEN control group compared to normal controls. QDH treatment significantly reduced these hepatic serum marker levels in DEN-induced liver cancer rats in a dose-dependent manner.

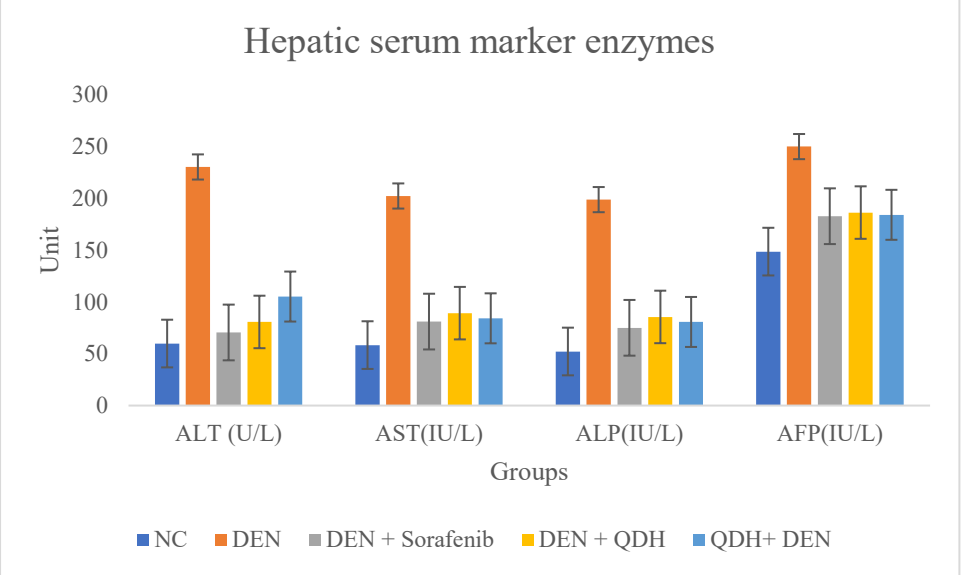

Figure. 4: QDH Treatment Attenuates Elevated Hepatic Biomarkers in DEN-Treated Rats

3.6 Impact of QDH on non-hepatic parameter of serum marker Enzymes:

Figure 5 shows that DEN control rats had reduced A/G ratio, total protein, albumin, and total bilirubin levels, indicated impaired liver function and weakened immune defense. The low levels of albumin and total protein further reflected poor liver health. However, DEN-induced rats treated with different doses of QDH showed a significant dose-dependent increase in total protein, albumin, and total bilirubin. These improvements suggest a restoration of liver function. The elevated levels of non-hepatic markers in QDH-treated rats demonstrate the compound's potential in reversing liver damage and improving overall hepatic performance in DEN-induced hepatocellular carcinoma.

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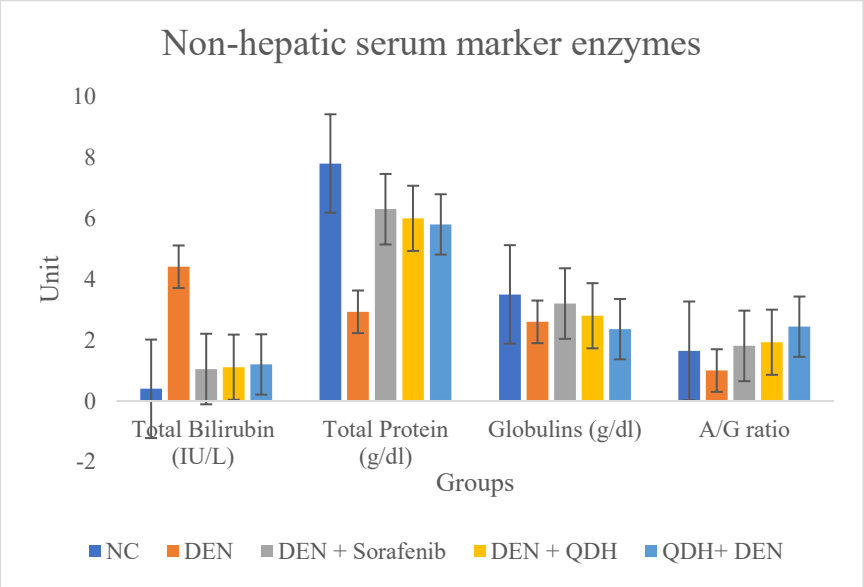

Figure. 5: Impact of QDH on Non-Hepatic Serum Parameters in DEN-Induced Hepatocellular Carcinoma

3.7 Impact of QDH on haematological factors:

Figure 6 illustrates the results of DEN and QDH on hematological markers (Hb, RBC, and WBC). DEN significantly reduced Hb and RBC levels while increasing WBC counts. QDH treatment effectively ($p < 0.001$) restored these hematological parameters in DEN-induced hepatocarcinogenesis rats. Additionally, sorafenib treatment markedly ($p < 0.001$) normalized the hematological profile, bringing values close to those observed in the normal control group.

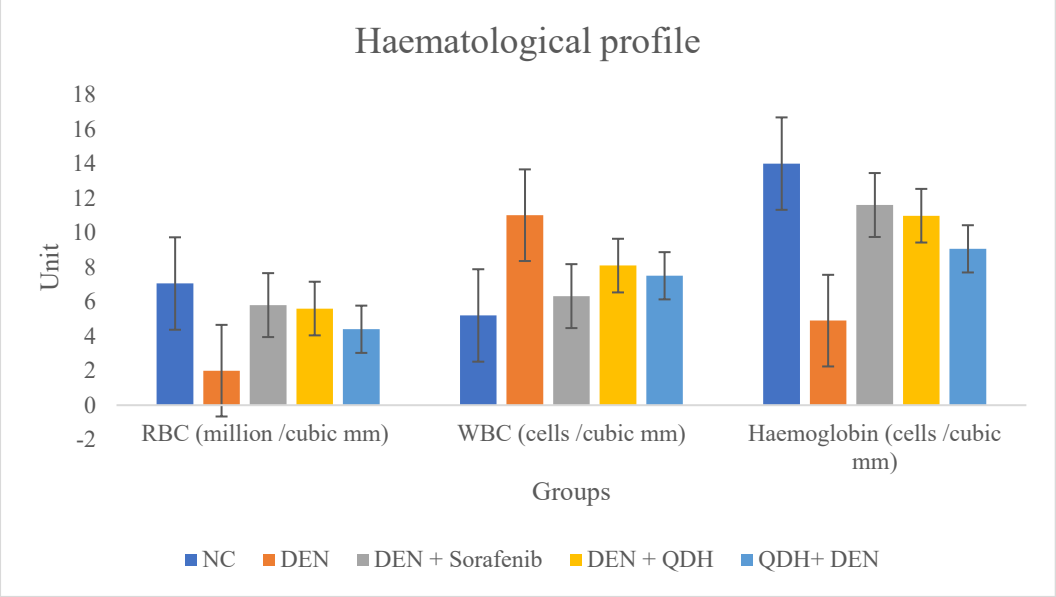

Figure. 6: Impact of QDH on Hematological Parameters in DEN-Induced Hepatocarcinogenesis

3.8 Antioxidant parameters:

Figure 7 shows that antioxidant markers—SOD, Catalase, GPx, GSH, G6PD, and vitamin C—were significantly reduced in DEN group rats ($p < 0.001$) and significantly elevated in a dose-dependent manner in QDH-treated groups ($p < 0.001$). These findings were consistent with standard pharmacological treatment. QDH notably regulated DEN-induced hepatocarcinogenesis by enhancing both enzymatic and non-enzymatic antioxidant levels, demonstrating its strong protective antioxidant potential.

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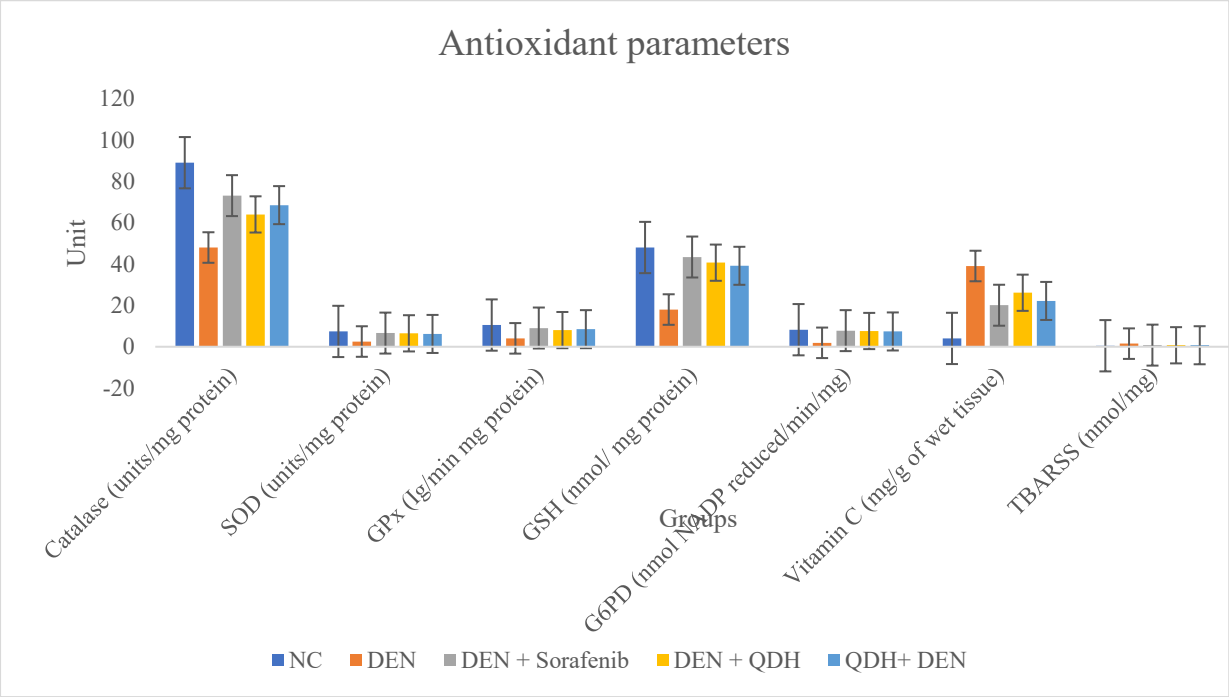

Figure. 7: Impact of Quercetin Dihydrate on Antioxidant Parameters in a DEN-Induced Hepatocarcinoma Model

3.9 Impact of lipid peroxidation activity:

DEN increased LPO levels significantly, measured in terms of MDA. LPO showed in fig 8. a dose-dependent decrease after DHQ administration, confirming the medication's antioxidant qualities. Rats in the silymarin group were able to prevent lipid peroxides, which are brought on by DEN in the liver tissue.

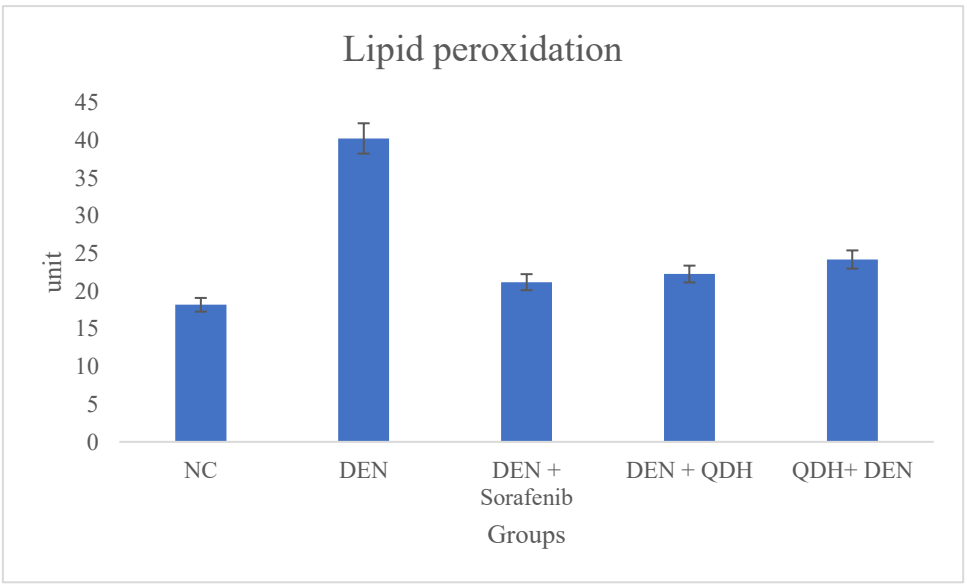

Figure. 8: Effect of QDH on Lipid Peroxidation Levels in DEN-Induced Hepatocarcinogenesis

3.10 Effect of QDH, sorafenib on renal function:

Figure 9 shows serum creatinine and urea levels in all groups. DEN significantly increased these markers ($p < 0.05$), indicating renal dysfunction. Treatment with QDH and sorafenib reduced these levels toward normal. No significant differences were found between treated and control groups, suggesting both QDH and sorafenib offer protective effects against DEN-induced kidney damage.

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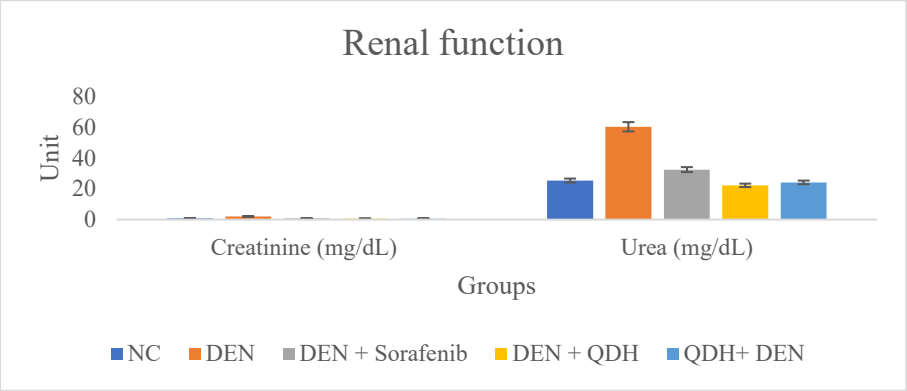

Figure. 9: Effect of QDH and Sorafenib on Renal Function Markers in DEN-Induced Rats

3.11 Proinflammatory cytokines and inflammatory mediators:

Figure 10 highlights proinflammatory cytokines TNF-α, IL-6, and IL-1β. DEN-induced rats showed significantly elevated levels of these markers compared to the normal control group. QDH administration led to a significant ($p < 0.001$) and dose-dependent reduction in TNF-α levels by the end of the study. Similar reductions were observed in IL-6 levels, which were elevated in DEN-induced rats but significantly decreased with QDH and sorafenib treatment. IL-1β levels were also notably higher in DEN-induced hepatocarcinogenesis but were significantly ($p < 0.001$) restored toward normal levels following QDH and sorafenib administration. These findings demonstrate QDH's strong anti-inflammatory effects in DEN-induced liver cancer models.

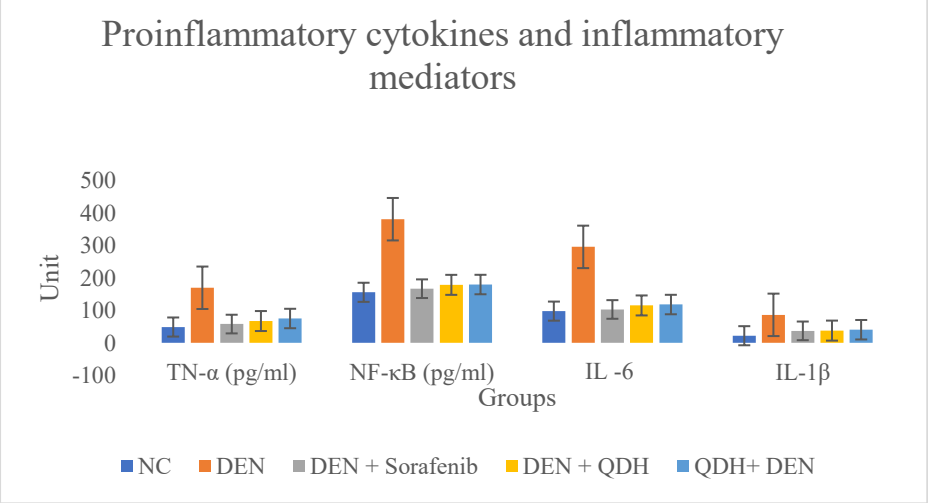

Figure. 10: Effect of QDH and Sorafenib on Proinflammatory Cytokines in DEN-Induced Hepatocarcinogenesis.

3.12 Membrane bound enzymes:

QDH and sorafenib restored ATPase levels in DEN-induced rats.

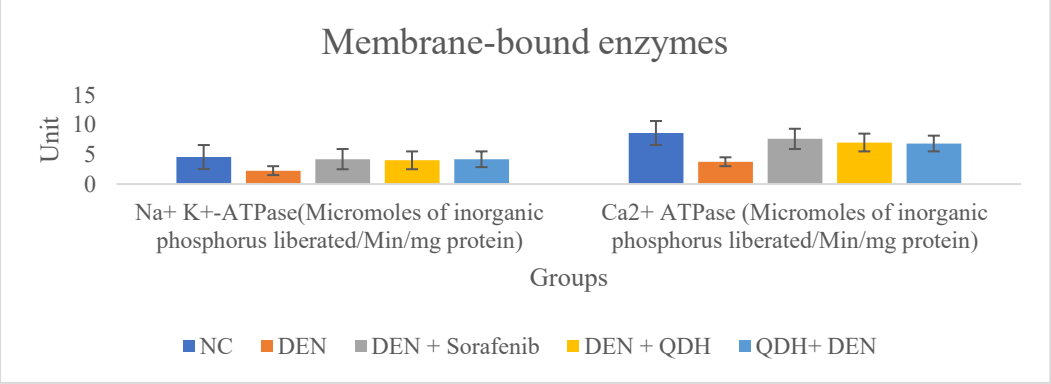

Figure. 11: Effect of QDH and Sorafenib on Membrane-Bound ATPase Activity in DEN-Induced Rats

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3.13 Effect of QDH, or Sorafenib on EGFR/ERK activation:

Figure 12 presents Western blot results showing the levels of phosphorylated EGFR (Tyr 1068) and ERK1/2 (Tyr 204) in liver tissue lysates from various rat groups, demonstrating differences in protein activation across treatments. According to the findings, DENA treatment considerably ($P<0.05$) raised the phosphorylated levels of EGFR to roughly 1.67 times higher than those in the control group. When DENA was administered, phospho-ERK1/2 increased significantly ($P<0.05$), by around 3.1 times the amount in the control group. QDH and sorafenib significantly inhibited DENA-induced EGFR and ERK1/2 activation. QDH offered stronger protection against EGFR phosphorylation, normalizing P-EGFR levels to control values.

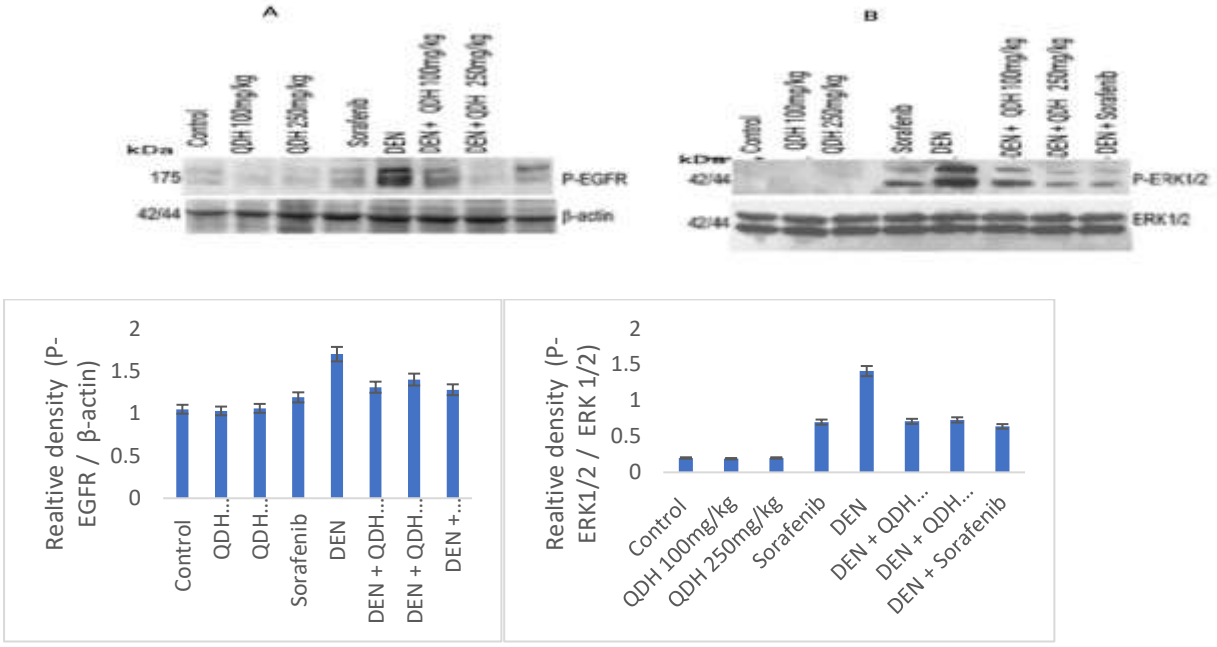

Figure 12: shows Western blot analysis of P-EGFR (Tyr1068) (A) and P-ERK1/2 (Tyr204) (B) expression in liver samples from various rat groups (mean ± SE, n=3). Band densities were quantified using Quantity One software, normalized to β-actin for P-EGFR and ERK1/2 for P-ERK1/2. Statistical significance was marked as * (vs. control) and # (vs. induction) at $p < 0.05$.

3.14 Effect of QDH ,or Sorafenib on the gene expression levels of c-fos, PCNA, and Bcl2

In this study, the expression levels of PCNA, c-fos, and Bcl2 genes were evaluated in experimental rat groups subjected to DENA induction, as well as various treatments including QDH at different doses and Sorafenib. The DENA group showed significant up-regulation in gene expression: PCNA (4-fold), c-fos (2.14-fold), and Bcl2 (1.78-fold) compared to the control group ($P < 0.05$), indicating liver damage and tumor promotion. Sorafenib did not significantly affect Bcl2 levels when co-administered with QDH. However, PCNA expression significantly increased in the Sorafenib group and decreased in the QDH group. All protective treatment groups, including QDH (three doses) and Sorafenib, exhibited significant down-regulation of PCNA, c-fos, and Bcl2 when compared to the DENA-induced group, suggesting protective effects against gene overexpression. Specifically, QDH at 250 mg/kg restored normal PCNA and Bcl2 levels, while QDH at 250 mg/kg restored only Bcl2. Sorafenib normalized Bcl2 and c-fos expression levels. However, one protective group showed significant down-regulation of PCNA and Bcl2 despite having normal c-fos expression compared to controls. Overall, both QDH and Sorafenib demonstrated protective effects by significantly reducing the overexpression of oncogenic and proliferative markers. The most notable effect was observed in the protected group, which effectively down-regulated PCNA, c-fos, and Bcl2 gene expressions compared to the DENA group. PCR analysis of three samples from various experimental rat groups for c-fos (520 bp), PCNA (126 bp), Bcl2 (349 bp), and internal control b-actin (187 bp) is shown in figure 13.

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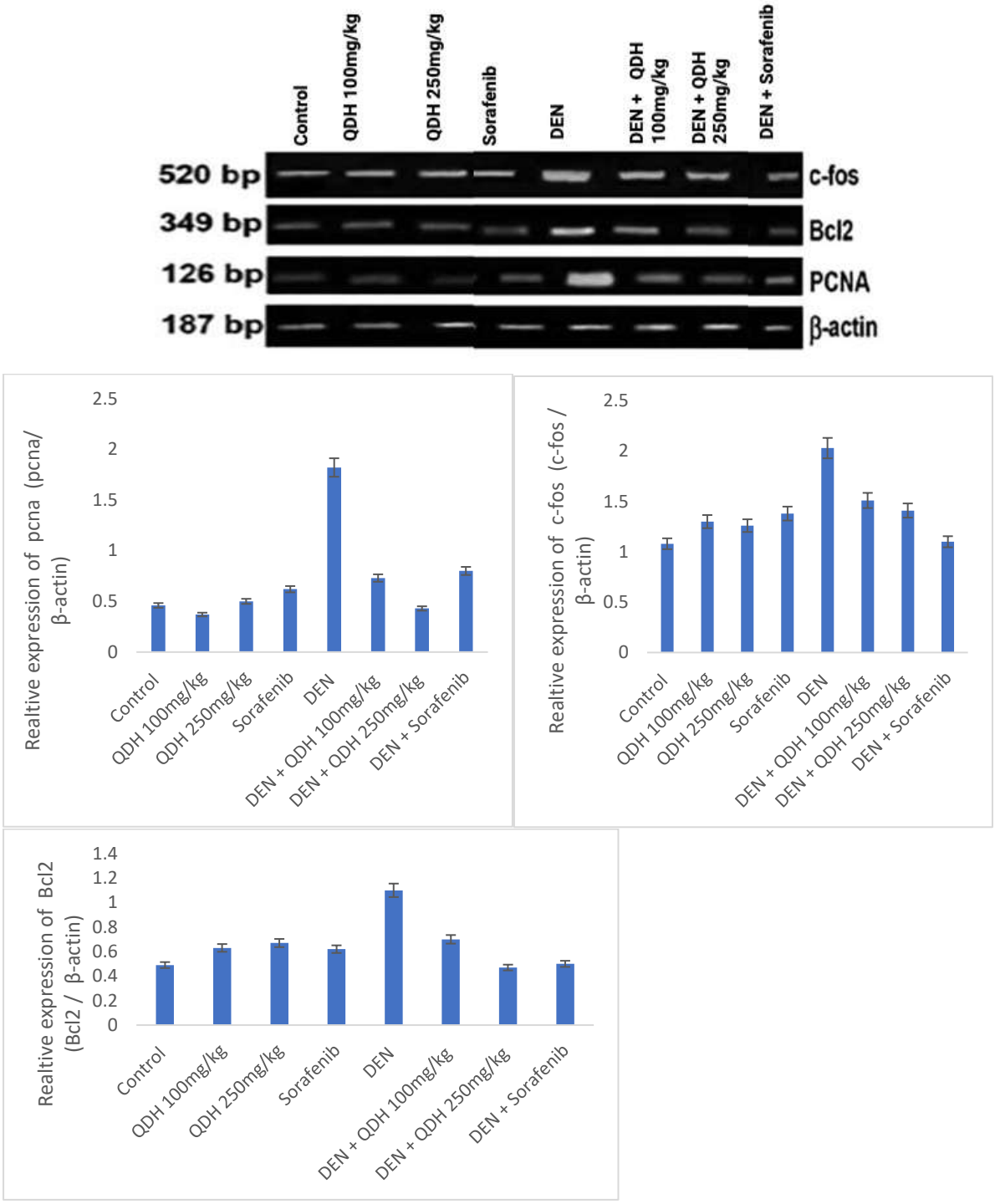

Figure 13: Presents histograms showing the relative expression levels of PCNA (A), c-fos (B), and Bcl2 (C) across three samples (mean ± SE), analyzed using Quantity One software and normalized to β-actin. Significant differences ($P < 0.05$) are marked: (*) indicates differences from the control group, while (#) denotes differences from the induction group, highlighting the effects of treatments on gene expression.

3.15 Histology of hepatic tissue:

Normal rat liver showed healthy hepatocytes; DEN-induced cancer caused necrosis, irregular cells, and stellate cell proliferation. Histological analysis revealed notable improvements in hepatic tissue in the DEN + Sorafenib group, including restored cytoplasm, reduced necrosis, and improved hepatocyte structure. Similarly, QDH treatment demonstrated dose-dependent protective effects in liver-induced rats by reducing inflammatory cell infiltration, decreasing binucleated cells, normalizing cytoplasmic

morphology, and restoring hepatic architecture. These findings confirm the hepatoprotective potential of both Sorafenib and QDH in mitigating liver damage and preserving tissue integrity show in fig 14.

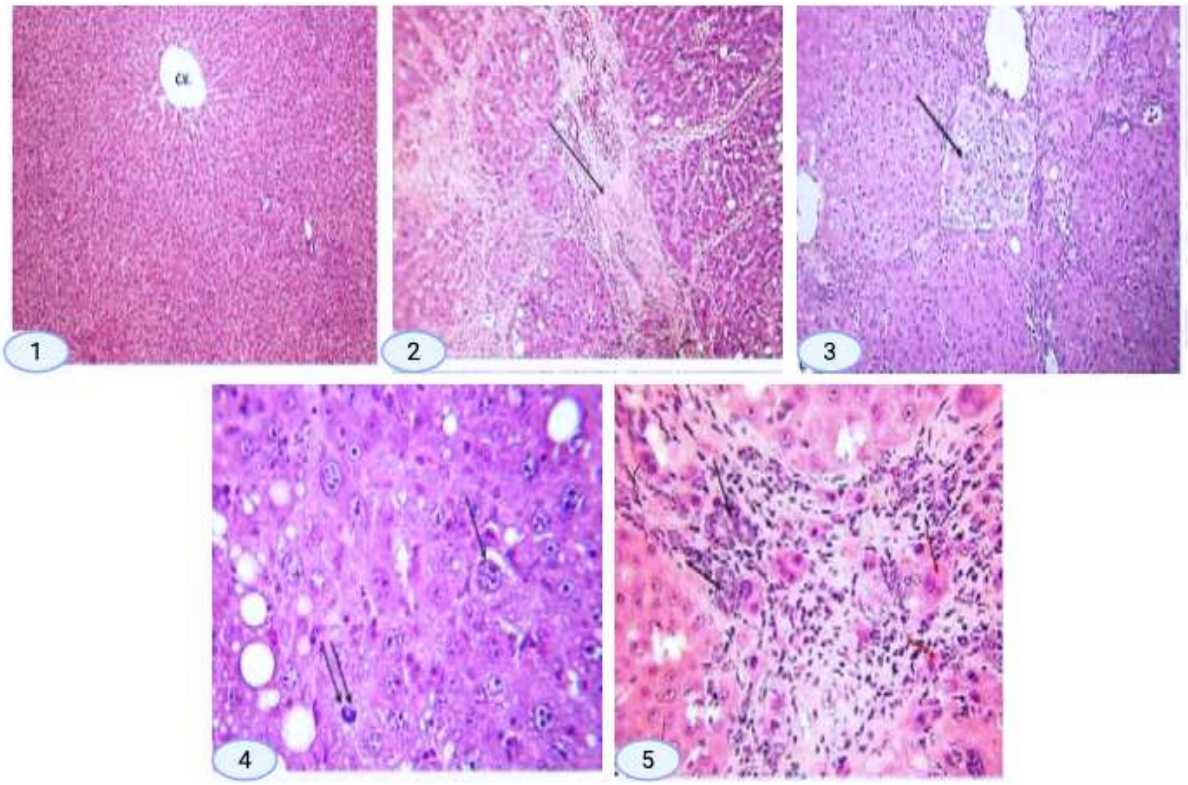

Figure 14: Hematoxylin and eosin-stained liver sections from various experimental groups. 1: Liver tissue from the control group (magnification x100) displayed normal hepatic structure, with healthy hepatocytes featuring granular cytoplasm and uniformly sized, small nuclei organized around the central vein (C.V.).2: Tissue from the DENA-treated group exhibited signs of liver cirrhosis, including disrupted lobular architecture, pronounced fibrous septa (indicated by arrow), and noticeable fatty changes (x100).3: A prominent dysplastic nodule was observed within cirrhotic areas (x100).4: The dysplastic region consisted of large, atypical hepatocytes with enlarged nuclei (single arrow) and abnormal mitotic activity (double arrows) (x400).4 (continued): The portal area showed bile duct proliferation (black arrow), lymphocyte infiltration, and signs of hepatocyte degeneration (red arrow) (x400).5: Liver section from the DENA and sorafenib-treated group showed early signs of cirrhosis along with localized fat accumulation (x100).

4 DISCUSSION:

QDH significantly reduced the incidence of hepatocellular carcinoma (HCC), macroscopic no dules, and preneoplastic liver lesions in the DEN-induced HCC model. DEN promotes liver cancer by inducing DNA strand breaks and carcinogenic adducts(24) .

The biological characteristics of QDH were assessed in rats with DEN-induced HCC. QDH, an alkaloid, increased the appropriate food intake in HCC rats and restored the healthy liver and body weight, according to the results of our current study. Histopathological analysis of the liver tissues of rats with DEN-induced HCC revealed severe hepatic dysplasia, loss of normal architecture, hepatocyte degradation, and a notable amount of tumor development. Significant hepatocyte production with restoration of normal architecture was observed in the liver tissues of HCC rats treated with QDH. With proper liver function, QDH has been shown to safeguard the architecture of the hepatocyte.

This study aimed to prevent liver cancer in Wistar rats using QDH. As reported by Deepika Singh et al., QDH is a liver-abundant biomolecule with strong efficacy against DEN-induced hepatocarcinogenesis, highlighting its potential as a protective agent in liver cancer prevention (25). The QDH has therapeutic

effectiveness and is non-toxic and cost-effective (26). The DEN-induced hepatocarcinogenesis model in rats is a widely recognized method for studying liver cancer, enabling the evaluation of anticancer drugs at various stages of neoplastic development and cellular transformation(27). Preneoplastic liver nodules arise from hepatocytes showing significant hyperplasia, increased cell proliferation, and elevated enzymatic markers, as documented in various studies, indicating early stages of liver cancer development (28). According to published research, the growth of hepatocyte nodes plays a critical role in the spread of hepatic cancer. QDH reduced hepatic nodules, normalized enzymes, lowered AFP levels dose-dependently, and showed chemoprotective effects in DEN-induced rats (29,30).Liver tissue enzyme leakage is closely linked to cell death, with serum AST, ALT, and ALP serving as key indicators of liver disease. In DEN-induced liver damage, elevated levels of these enzymes result from cytosolic enzyme leakage into the bloodstream. This reflects early hepatocellular destruction caused by hepatic hypofunction, impaired production of serum markers, and increased liver cell membrane permeability, marking the onset of liver dysfunction and injury (31). By altering the permeability and integrity of the hepatic membrane, QDH treatment restored the levels of these enzymes.Albumin and globulin indicate liver function. Elevated non-hepatic enzymes suggest mild hemolysis and impaired bile flow, while increased globulin levels indicate enhanced liver physiological activity and a potential compensatory response (32). Bilirubin, a hemoglobin byproduct conjugated with glucuronic acid in hepatocytes, is a key marker of liver function. Elevated blood bilirubin levels signal hepatobiliary disorders and liver inflammation, making its measurement essential for assessing hepatic capacity and health(33) . Serum bilirubin levels were lowered by dose-dependent QDH treatment, which also helped the liver's normal activity to regenerate.Evaluating hematological parameters helps assess the harmful impact of substances like DEN on blood profiles. Hemoglobin (Hb), essential for oxygen transport in red blood cells, is a key indicator. A reduction in Hb and red blood cell count leads to anemia, reflecting compromised blood health due to toxic exposure (34). Rats in the DEN group showed increased WBC counts, which are linked to liver cancer and other pathological diseases.The QDH improved the range of RBC and Hb in a dose-dependent manner while downregulating the amount of WBC. When superoxide anion is broken down by enzymatic antioxidant properties to produce hydrogen peroxide, the harmful effects of these radicals are subsequently reduced. Catalase and SOD are essential enzymes for the antioxidant's parameter (35). Reductions in these factors could have a number of negative effects. This study found that QDH elevated liver antioxidant enzymes SOD and catalase in DEN-induced liver cancer, enhancing enzymatic antioxidant activity. QDH also reduced reactive oxygen species and oxidative damage in hepatic tissues. Additionally, the liver contains high levels of glutathione (GSH), a key non-enzymatic antioxidant, which further contributes to its defense against oxidative stress and supports QDH's protective role in liver cancer prevention (36). DEN increased lipid peroxidation; QDH raised GPx and GSH dose-dependently (35). In our investigation, the DENA group showed a substantial rise in lipid peroxidation levels (as indicated by TBARS). One possible explanation for this is the overproduction of ROS during DENA metabolism, which can damage lipids in the endoplasmic reticulum membrane and induce lipid peroxidation. This leads to cell death when lipid peroxides build up and Ca^{2+} homeostasis is disrupted (37,38). TBARS levels were noticeably lower in the QDH or sorafenib-protected DEN group than in the DEN group, indicating that preventing the progression of HCC requires reversing this oxidative process. This aligned with a prior study that demonstrated QDH's effectiveness as a strong free radical scavenger (39).Monocytes/macrophages release TNF-α and IL-6 in inflammation. NF-κB regulates inflammation, cell survival, and promotes collagenase, cytokines, PGE2, and adhesion molecules production (40–42). When DEN causes hepatic cancer, it affects the liver's Kuffer cells by activating NF-κB and regulating the release of several inflammatory mediators (including TNF-α and IL-6) into the bloodstream. Synthesized QDH was predicted to decrease inflammation, which is linked to hepatic malignancy.

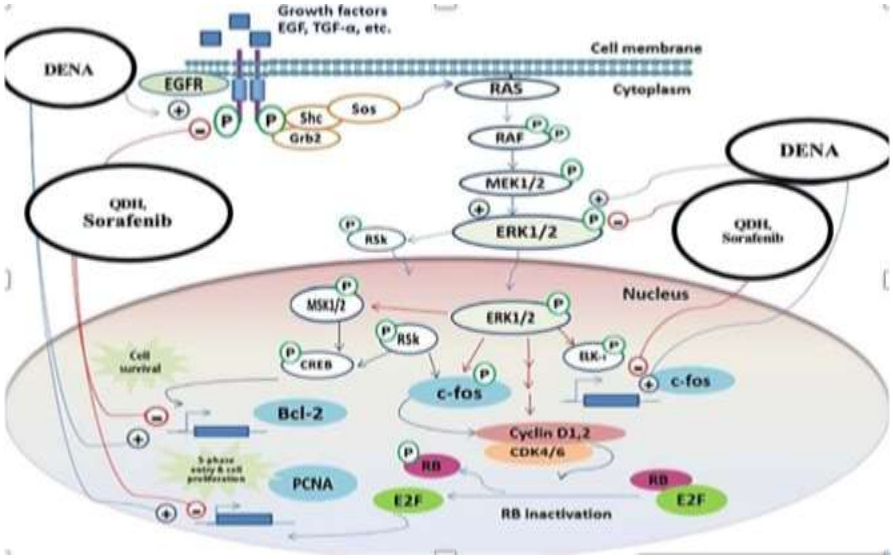

FIGURE 15: Schematic representation of the effect of QDH ,Sorafenib on EGFR/ERK1/2 signaling. QDH ,Sorafenib inhibited EGFR/ERK1/2 signaling and their transcription targets involved in cell proliferation and survival (PCNA, c-fos, and Bcl-2) as a suggested molecular mechanism for HCC prevention

Na^+/K^+ ATPase and Ca^{2+} ATPase enzymes, essential for ion transport using ATP, decreased in DEN-induced liver cancer rats but were restored in a dose-dependent manner after QDH treatment, indicating improved membrane function(43). We believe that the presence of bioactive chemicals in QDH gives it its efficacy and prevents the damage of liver hepatocytes. In HCC, dysregulated proliferation is one of the main features. The EGFR/MAPK/ERK1/2 signaling pathway plays a crucial role in HCC pathophysiology, especially in early stages, by regulating cell proliferation and survival, thereby significantly influencing cancer development and progression.(44,45). These results support earlier evidence that DENA activates the EGFR and ERK1/2 signaling pathways, highlighting their role in hepatocarcinogenesis and validating the model's effectiveness in studying liver cancer development (46). The DEN group's RAF/MAPK/ERK activation may be linked to a persistent c-raf activation, which might be brought on by either activation of their receptor EGFR or dysregulated overexpression of EGF family ligands (51). Additionally, it might be the result of DEN-induced oncogenic mutations in the RAS gene, which activate constitutive pathways via c-raf (47).

QDH and sorafenib inhibited DEN-induced EGFR/ERK1/2 phosphorylation, reducing c-fos, PCNA, Bcl-2, and suppressing liver cancer cell proliferation (53). Furthermore, in a number of other cancer models, QDH downregulated Bcl-2 (54). Furthermore, it has been demonstrated that QDH suppresses the expression of Bcl-2 in neuroblastoma cell lines and PCNA in pulmonary arterial cells (48,49). QDH's anti-proliferative action because it has been demonstrated to decrease cholangiocyte proliferation by lowering PCNA levels and ERK1/2 activation (50) and human leukemia HL-60 cells' Bcl-2 level (58). QDH and sorafenib were safe, preserving liver structure; DEN activated EGFR/MAPK pathway, increasing PCNA, c-fos, Bcl-2, promoting cancer. However, treatment with QDH or sorafenib suppressed EGFR/ERK1/2 activation and down-regulated these gene targets, reducing cellular proliferation. These effects highlight the potential of QDH and sorafenib as chemopreventive agents against liver cancer. Moreover, their antioxidant properties help inhibit DEN-induced dysplastic liver changes, offering further protection. This study provides new evidence supporting QDH as a chemopreventive compound that may delay or reverse HCC development. Finally, the findings suggest that exposure to environmental N-nitrosamines could stimulate protective mechanisms against HCC, emphasizing the importance of exploring natural agents like QDH for liver cancer prevention. DENA exposure activates EGFR and its downstream MAPK/ERK1/2 signaling pathway during the development of liver cancer. This activation leads to increased expression of PCNA, c-fos, and Bcl-2, which are transcriptional targets of ERK1/2 and are associated with cell proliferation and survival. However, treatment with QDH and sorafenib mitigates the activation of the EGFR/ERK1/2 pathway and downregulates its associated gene targets (PCNA, c-

fos, and Bcl-2), thereby reducing cellular proliferation and supporting their role as chemopreventive agents in hepatocarcinogenesis (Figure 15). Additionally, QDH and sorafenib help prevent DENA-induced dysplastic liver changes through mechanisms involving their antioxidant properties. These findings suggest the potential of QDH as a chemopreventive agent that may delay or inhibit the onset of hepatocellular carcinoma.

5. CONCLUSION:

The present study comprehensively demonstrates that Quercetin Dihydrate (QDH) exerts a potent hepatoprotective and chemopreventive effect against diethylnitrosamine (DEN)-induced hepatocellular carcinoma (HCC) in Wistar rats. QDH administration significantly restored liver architecture, reduced hepatic nodules, and normalized both hepatic and non-hepatic biomarkers. It improved antioxidant enzyme levels (SOD, CAT, GPx, GSH) while reducing lipid peroxidation and proinflammatory cytokines (TNF-α, IL-6, IL-1β), indicating its strong antioxidative and anti-inflammatory potential.

Moreover, QDH inhibited DEN-induced activation of the EGFR/ERK1/2 signaling pathway and downregulated the expression of proliferative and anti-apoptotic genes such as PCNA, c-fos, and Bcl-2. These molecular changes were corroborated by histological findings that confirmed preserved liver tissue integrity and reduced cellular abnormalities. The study also confirmed the safety of QDH, with no significant toxicity observed even at higher doses.

In conclusion, QDH acts through multiple protective mechanisms—antioxidant defense, inflammation suppression, membrane enzyme restoration, and gene regulation—to mitigate hepatocarcinogenesis. These findings suggest that QDH is a promising natural compound with potential therapeutic applications in the prevention and management of liver cancer.

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