

ORIGINAL ARTICLE

2-Chloro-3-Hydrazinylquinoxaline Mitigates Doxorubicin-Induced Hepatic Inflammation in Rats

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ABSTRACT

Background: In this research, 2-chloro-3-hydrazinylquinoxaline (CHQ) was evaluated for hepatoprotection and mechanism of action against the hepatotoxicity and liver inflammation in rats induced by the drug doxorubicin (DOX).

Methods: Twenty-four male Wistar rats were split into four groups (n = 6 per group): normal control, DOX-only (15 mg/kg, i.p. day 6), and two treatment groups (daily oral CHQ 30 mg/kg or 60 mg/kg for 28 days). Blood biochemical parameters and liver and tissue examinations for oxidative markers and inflammatory mediators were performed. Histopathology was done to check hepatic tissue changes (H&E).

Results: DOX treatment produced significant systemic toxicity, as evidenced by reduced body weight, hepatomegaly, and elevated serum liver enzyme levels compared with the control group (p < 0.05). It caused significant oxidative stress, leading to higher malondialdehyde (MDA) levels and lower glutathione, superoxide dismutase, and catalase levels (p < 0.05). Moreover, DOX triggered elevated levels of pro-inflammatory cytokines, activation of nuclear factor kappa- β , and decreased activity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (p < 0.001). On H&E sections, intense necrosis and leukocyte infiltration were present. Conversely, co-treatment with CHQ, in a dose-dependent manner, significantly reverses these changes (p < 0.05). It acts as a supplement to antioxidant mechanisms, reduces serum enzyme leakage, and regulates pro-inflammatory cytokines and signaling pathways, with a dose of 60 mg/kg producing near-mild liver histology.

Conclusions: CHQ was observed to be a significant protective agent against DOX-induced hepatotoxicity, with a dose-dependent effect, increasing antioxidant defense mechanisms, and decreasing DOX-mediated inflammatory responses, thereby making it a promising agent for the prevention of DOX-induced liver injury.

(Clin. Lab. 2026;72:xx-xx. DOI: 10.7754/Clin.Lab.2026.260619)

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KEYWORDS

inflammation, liver damage, doxorubicin, quinoxaline

LIST OF ABBREVIATIONS

ANOVA - Analysis of variance
 CAT - Catalase
 CHQ - 2-Chloro-3-hydrazinylquinoxaline
 COX-2 - Cyclooxygenase-2
 DOX - Doxorubicin
 ELISA - Enzyme-linked immunosorbent assay
 GAPDH - Glyceraldehyde 3-phosphate dehydrogenase
 GSH - Reduced glutathione
 H&E staining - Hematoxylin and eosin staining
 IFN- γ - Interferon-gamma
 IL-1 β - Interleukin-1 beta
 IL-6 - Interleukin-6
 iNOS - Inducible nitric oxide synthase
 MDA - Malondialdehyde
 NF- κ B - Nuclear factor kappa B
 PBS - Phosphate-buffered saline
 PGE₂ - Prostaglandin E₂
 ROS - Reactive oxygen species
 SOD - Superoxide dismutase
 TNF- α - Tumor necrosis factor-alpha

INTRODUCTION

Doxorubicin (DOX), an anthracycline chemotherapeutic antibiotic widely utilized for treating different malignancies such as breast cancer, leukemia, bronchogenic carcinoma, gastric cancer, sarcomas, thyroid cancers, and other hematological disorders [1]. Although DOX has potent antitumor properties, its use in clinical practice is limited by its toxicity to normal tissues [2]. The drug is metabolized in the liver via microsomal enzymes and cytoplasmic reductases, resulting in the formation and accumulation of reactive, potentially immunogenic intermediates that contribute to hepatic injury. Evidence from experimental studies indicates that DOX-induced hepatotoxicity is largely associated with excessive oxidative stress and inflammatory responses, which play a central role in the underlying mechanism of liver injury [3,4].

Natural and synthetic bioactive compounds have attracted considerable interest due to their potential protective effects against drug-induced hepatotoxicity, a major limitation of several therapeutic agents, including DOX. In recent years, several heterocyclic and pharmacologically active compounds have been extensively investigated due to their strong antioxidant and anti-inflammatory activities. These compounds have been shown to modulate multiple molecular pathways involved in liver injury, including oxidative stress, mitochondrial dysfunction, apoptosis, and inflammatory signaling [5,6]. Quinoxaline derivatives have emerged as promising "privileged structures" for their versatile pharmacological activities and are being investigated as more potent chemoprotective candidates. Among these, 2-chloro-3-hydrazinylquinoxaline (CHQ) has emerged as a promising dual-action agent. Recent evidence underscores its potent anti-inflammatory properties in gastric mucosal protection and in the regulation of important inflammatory mediators [7]. Furthermore, quinoxaline derivatives also exhibit anti-inflammatory activity by inhibiting inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). They also inhibit pro-inflammatory cytokines by modulating major signaling pathways such as NF- κ B [8,9]. Although quinoxaline compounds have been shown to have therapeutic benefits, their specific effects on in vivo DOX-induced hepatic inflammation remain unexplored. Thus, this study explored the hepatoprotective effects of CHQ in a rat model, focusing on its involvement in reducing oxidative stress and suppressing specific pro-inflammatory cytokines.

MATERIALS AND METHODS**Reagents**

In this study, DOX, xylazine, and ketamine were purchased from Sigma-Aldrich (CA, USA). 2-Chloro-3-hydrazinylquinoxaline (C₈H₇ClN₄; CHQ) was obtained from Sigma-Aldrich (CA, USA).

ELISA kits of rat IFN- γ (cat. no. MBS2019134); sensitivity typically ≤ 6.1 pg/mL; intra-assay CV $\leq 10\%$; inter-assay CV $\leq 12\%$, rat TNF- α (cat. no. MBS282960); detection range 6.25 - 400 pg/mL; sensitivity typically < 3.1 pg/mL; intra-assay CV $\leq 8\%$; inter-assay CV $\leq 12\%$, rat IL-1 β (cat. no. MBS265868); detection range 15.6 - 1,000 pg/mL; sensitivity usually < 5 pg/mL; intra-assay CV $\leq 8\%$; inter-assay CV $\leq 12\%$, rat IL-6 (cat. no. MBS269892); detection range 15.6 - 1,000 pg/mL; sensitivity up to 5 pg/mL; intra-assay CV $\leq 8\%$; inter-assay CV $\leq 12\%$, rat iNOS (inducible nitric oxide synthase; cat. no. MBS2505942); detection range 0.31 - 20 ng/mL; sensitivity typically ≤ 0.19 ng/mL; intra-assay CV $\leq 4.94\%$; inter-assay CV $\leq 5.19\%$, rat NF- κ B (cat. no. MBS287521); detection range 0.31 - 20 ng/mL; sensitivity typically ≤ 0.15 ng/mL; intra-assay CV $\leq 8\%$; inter-assay CV $\leq 12\%$, rat cyclooxygenase-2 (COX-2; cat. no. MBS266603); detection range 0.156 - 10 ng/mL; sensitivity typically up to 0.05 ng/mL; intra-

assay CV $\leq$ 8%; inter-assay CV $\leq$ 12%, GAPDH (glyceraldehyde 3-phosphate dehydrogenase, cat no. MBS7-27873), and rat PGE2 (prostaglandin E₂; cat. no. MBS2-62150); detection range 15.6 - 1,000 pg/mL; sensitivity typically up to 5 pg/mL; intra-assay CV $\leq$ 8%; inter-assay CV $\leq$ 12% were procured from MyBioSource Inc., CA, USA.

Experimental animals

The in vivo experimental study was carried out using 180 - 220 g, three-month-old male Wistar rats. Animals were housed under normal laboratory conditions of 12-hour light-dark cycles, $22 \pm 0.5^\circ\text{C}$, and 40 - 45% RH and were fed a pellet diet and water. Rats were acclimated for a period of 1 week before experimentation and were monitored daily for health status and behavioral changes. All experimental protocols adhered to the ARRIVE guidelines and the national regulations for laboratory animal care and were conducted under the approval of the Institutional Ethics Committee (approval no.: HAP0-02-K-012-2025-10-2956).

During the acclimatization period, a total of 24 animals ($n = 6/\text{group}$) were assigned unique identification numbers and were randomly allocated to the experimental groups using a simple randomization method [10,11]. All groups contained an equal number of animals. To minimize potential confounding factors, all animals were maintained under identical housing conditions throughout the study. Cage positions were rotated periodically, treatments were administered at the same time each day, and all behavioral evaluations, sample collection, and biochemical analyses were conducted using the same procedures and under consistent experimental conditions for every group.

Group I (Control): This group served as the normal control without any treatment;

Group II (DOX): Rats treated with a single intraperitoneal injection (i.p.) of DOX (15 mg/kg) on day 6 of the experimental protocol.

Group III (DOX + CHQ-30): received CHQ at 30 mg/kg orally once daily for 28 consecutive days and a single intraperitoneal injection of DOX (15 mg/kg) on Day 6.

Group IV (DOX + CHQ-60): received CHQ at 60 mg/kg orally once daily for 28 consecutive days and a single intraperitoneal injection of DOX (15 mg/kg) on Day 6.

The treatment protocol was developed to assess the hepatoprotective and anti-inflammatory potential of CHQ against DOX-induced liver injury through a prophylactic/co-treatment approach. No animals or samples were excluded from the final analysis.

Group allocation was carried out by the principal investigator, who was aware of the treatment assignments. The investigator responsible for administering the treatments was also aware of the group allocation. To minimize assessment bias, behavioral testing, biochemical measurements, histopathological evaluation, and statistical analysis were performed by investigators blinded

to the treatment groups. All samples and datasets were coded before analysis, and the group identities were revealed only after completion of the outcome assessment and statistical analyses.

Evaluation of body weight, liver weight, and histopathology

With xylazine (10 mg/kg) and ketamine (90 mg/kg), animals were anesthetized and euthanized by cervical dislocation. Liver tissues were carefully examined and photographed for gross morphological and histopathological evaluation.

The blood was drawn in plain vacutainer tubes (Becton Dickinson, NJ, USA) and centrifuged (Remi Elektrotechnik Limited, India) for 10 minutes at 4°C at $2,000 \times g$ to separate serum. Collected serum samples were stored at -80°C until biochemical analysis.

Effect of DOX on liver function and hepatic injury markers

The primary endpoint measured includes serum biochemical parameters to assess liver function and hepatic damage in experimental rats. Commercial assay kits were used for liver enzymes measurements, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP), following the manufacturer's instructions. Enzyme activities were expressed as U/L.

Effect of CHQ on oxidative stress markers in DOX-induced hepatic inflammation

The antioxidant efficacy of CHQ against DOX-induced hepatic inflammation was estimated by analyzing oxidative stress markers in liver tissue homogenates. Malondialdehyde (MDA) and reduced GSH levels were quantified using standard colorimetric methods. MDA was used as an indicator of lipid peroxidation, and GSH levels were used as an indicator of intracellular antioxidant status. In brief, the samples were treated with trichloroacetic acid, and the absorbance of the resulting chromogen was recorded at 532 nm [2]. The GSH assay was performed by reacting sulfhydryl (SH) groups with the assay reagent, 5,5'-dithiobis-(2-nitrobenzoic acid), and measuring absorbance at 412 nm. MDA was expressed in nmol/mg protein, and GSH in $\mu\text{mol}/\text{mg}$ protein.

The liver was also subjected to spectrophotometric determination of the antioxidant enzyme activities SOD and CAT. SOD activity was estimated by inhibition of nitro blue tetrazolium reduction, and absorbance was measured at 550 nm. Activity of catalase was determined in the diluted liver homogenate supernatant prepared in 1 M phosphate buffer (pH 7.4). Hydrogen peroxide (50 nM) was added only to initiate the reaction, and the reaction was incubated at 27°C . CAT-mediated activity was monitored spectrophotometrically (UV-1800, Shimadzu, Japan) by quantifying the absorbance at 240 nm every 15 seconds and was expressed as U/mg protein [12].

Effect of CHQ on inflammatory cytokines and mediators

The inflammatory response in liver tissue was assessed by measuring the concentrations of interleukin-6 (IL-6), interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF- α), and NF- κ B in liver homogenates using commercially available ELISA kits according to the manufacturers' protocols.

Effect of CHQ on other mediators related to inflammation

The effects of DOX and CHQ on other inflammatory markers were assessed by determining the levels of COX-2, prostaglandin E₂ (PGE₂), iNOS, GAPDH, and interferon- γ (IFN- γ) in liver tissue homogenates by using standard ELISA assay kits as per manufacturers' instructions.

Histopathological examination

Freshly collected liver tissues were fixed in 10% buffered formalin for histological studies. The fixed tissues were then embedded in paraffin and cut on a microtome (HM3358, Diamond Microscope Est., Saudi Arabia) into 5 μ m-thick sections. The prepared sections were mounted on glass slides and then stained with hematoxylin and eosin (H&E). Microscopic examination at 200 $\times$ magnification revealed histopathological changes in the hepatic architecture and cellular morphology.

Statistical analysis

Data are presented as mean $\pm$ standard deviation (SD). Statistical analyses were performed using GraphPad Prism version 8.0.2 (GraphPad Software, San Diego, CA, USA). Data were assessed for normality using the Shapiro-Wilk test and analyzed using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons test. For the primary outcome measures, effect estimates are presented with 95% confidence intervals (95% CIs). $p < 0.05$ was considered statistically significant.

RESULTS

Protective effect of CHQ against DOX-mediated hepatic damage

Increased serum ALT, AST, and ALP levels are well-established biochemical markers of hepatic cellular damage and altered hepatic function. The present study indicated that DOX administration resulted in marked hepatotoxicity in rats, with notable increases in serum AST (mean difference = -18.97 U/L, 95% CI: -25.38 to -12.56; $p < 0.001$), ALT (mean difference = -20.20 U/L, 95% CI: -28.99 to -11.41; $p < 0.001$), and ALP (mean difference = -35.50 U/L, 95% CI: -47.25 to -23.75; $p < 0.001$) compared with the control group. These results suggest that DOX-induced liver injury leads to membrane instability and leakage of hepatic enzymes into the circulation.

The elevations in enzyme levels were significantly attenuated by CHQ treatment, indicating its protective effect against DOX-mediated hepatic damage. Compared with the DOX-only group, CHQ at 30 mg/kg significantly reduced AST (mean difference = 8.367 U/L, 95% CI: 1.958 to 14.78; $p = 0.0079$), ALT (mean difference = 12.67 U/L, 95% CI: 3.875 to 21.46; $p = 0.0034$), and ALP (mean difference = 17.67 U/L, 95% CI: 5.912 to 29.42; $p = 0.0023$). Similarly, CHQ at 60 mg/kg produced greater reductions in AST (mean difference = 11.73 U/L, 95% CI: 5.324 to 18.14; $p = 0.0003$), ALT (mean difference = 15.07 U/L, 95% CI: 6.275 to 23.86; $p = 0.0006$), and ALP (mean difference = 22.67 U/L, 95% CI: 10.91 to 34.42; $p = 0.0002$), with enzyme levels approaching normal values (Figure 1A - C). The higher CHQ dose demonstrated greater hepatoprotective efficacy than the lower.

Protective effect of CHQ on oxidative stress markers in DOX-induced hepatic inflammation

Treatment with DOX profoundly altered the hepatic oxidant-antioxidant balance in rats. MDA levels were significantly increased (mean difference = -5.982 nmol/mg protein, 95% CI: -8.303 to -3.660; $p < 0.001$), whereas GSH (mean difference = 2.722 μ mol/mg protein, 95% CI: 1.068 to 4.376; $p < 0.001$), SOD (mean difference = 6.827 U/mg protein, 95% CI: 3.687 to 9.966; $p < 0.001$), and CAT (mean difference = 36.37 U/mg protein, 95% CI: 14.58 to 58.16; $p < 0.001$) activities were significantly reduced compared with the control group, indicating enhanced oxidative stress and impaired endogenous antioxidant defense (Figure 2A - D). Administration of CHQ significantly improved the oxidative stress profile. Compared with the DOX-only group, CHQ at 30 mg/kg significantly reduced MDA (mean difference = 3.305 nmol/mg protein, 95% CI: 0.9837 to 5.626; $p = 0.0037$) while increasing GSH (mean difference = -2.060 μ mol/mg protein, 95% CI: -3.714 to -0.4061; $p = 0.0115$), SOD (mean difference = -3.433 U/mg protein, 95% CI: -6.573 to -0.2937; $p = 0.0290$), and CAT (mean difference = -22.98 U/mg protein, 95% CI: -44.77 to -1.187; $p = 0.0365$). CHQ at 60 mg/kg produced greater improvements in all oxidative stress markers, significantly reduced MDA (mean difference = 4.395 nmol/mg protein, 95% CI: 2.074 to 6.716; $p = 0.0002$) while increasing GSH (mean difference = -2.658 μ mol/mg protein, 95% CI: -4.312 to -1.004; $p = 0.0012$), SOD (mean difference = -4.597 U/mg protein, 95% CI: -7.736 to -1.457; $p = 0.0029$), and CAT (mean difference = -30.53 U/mg protein, 95% CI: -52.32 to -8.742; $p = 0.0043$), demonstrating superior antioxidant activity compared with the lower-dose treatment.

Protective effect of CHQ on inflammatory cytokines and mediators

To evaluate the anti-inflammatory potential of CHQ, hepatic levels of major pro-inflammatory mediators were measured. DOX administration significantly in-

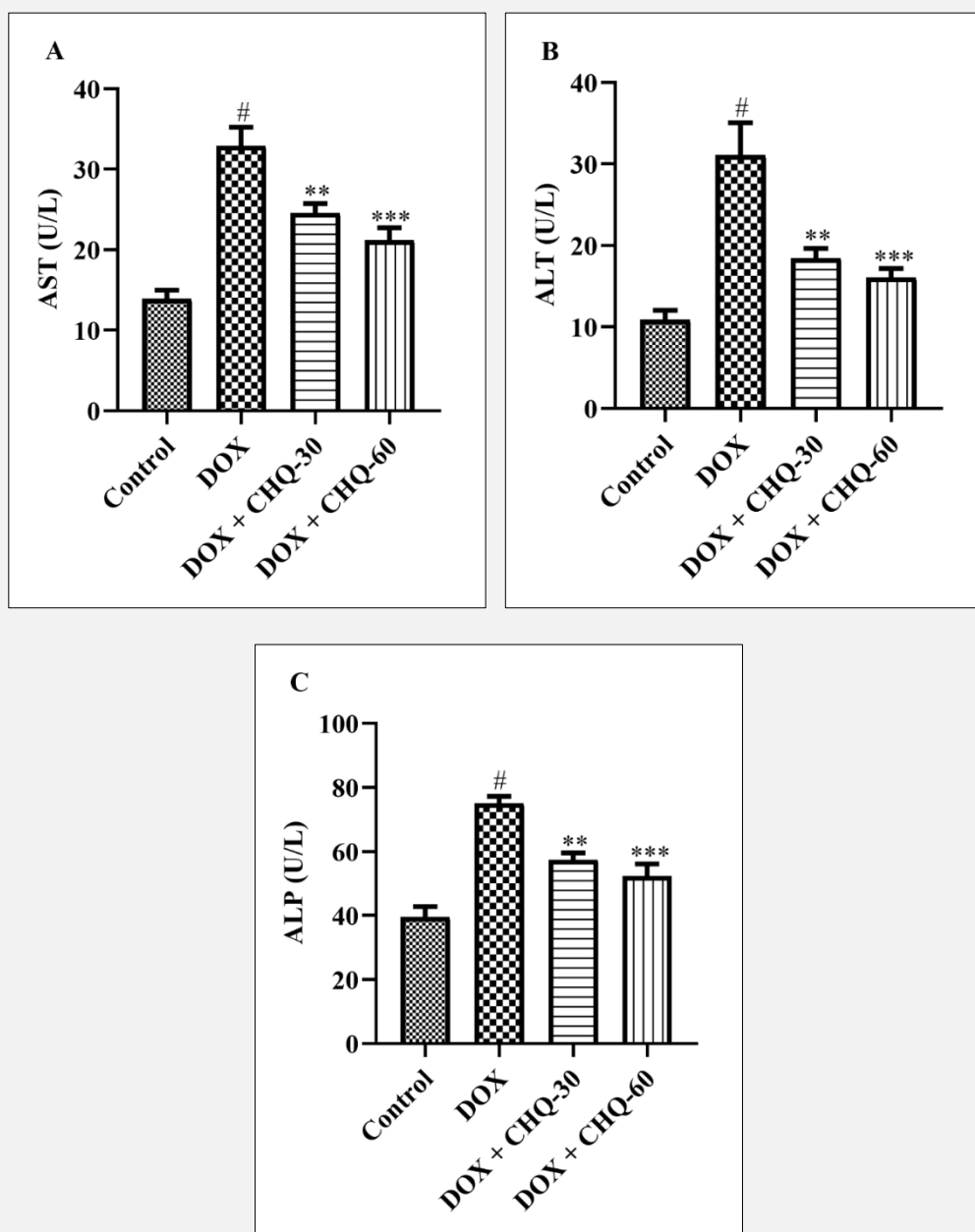


Figure 1. Effect of CHQ treatment on liver function markers in DOX-induced hepatotoxicity in rats.

A: AST, B: ALT, and C: ALP levels. Data are presented as mean $\pm$ SD (n = 6). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test.

[#] p < 0.001 vs. Control, ^{**} p < 0.01, ^{***} p < 0.001 vs. DOX.

creased hepatic IL-6 (mean difference = -4.800 pg/mL protein, 95% CI: -9.578 to -0.02182; p < 0.001), IL-1 β (mean difference = -13.70 pg/mL protein, 95% CI: -20.20 to -7.201; p < 0.001), TNF- α (mean difference = -13.50 pg/mL protein, 95% CI: -18.40 to -8.601; p < 0.001), and NF- κ B (mean difference = -11.59 pg/mL

protein, 95% CI: -15.68 to -7.494; p < 0.001) compared with the control group, indicating a pronounced inflammatory response and hepatic injury.

Co-treatment with CHQ significantly attenuated these inflammatory changes. Compared with the DOX-only group, CHQ at 30 mg/kg significantly reduced IL-6

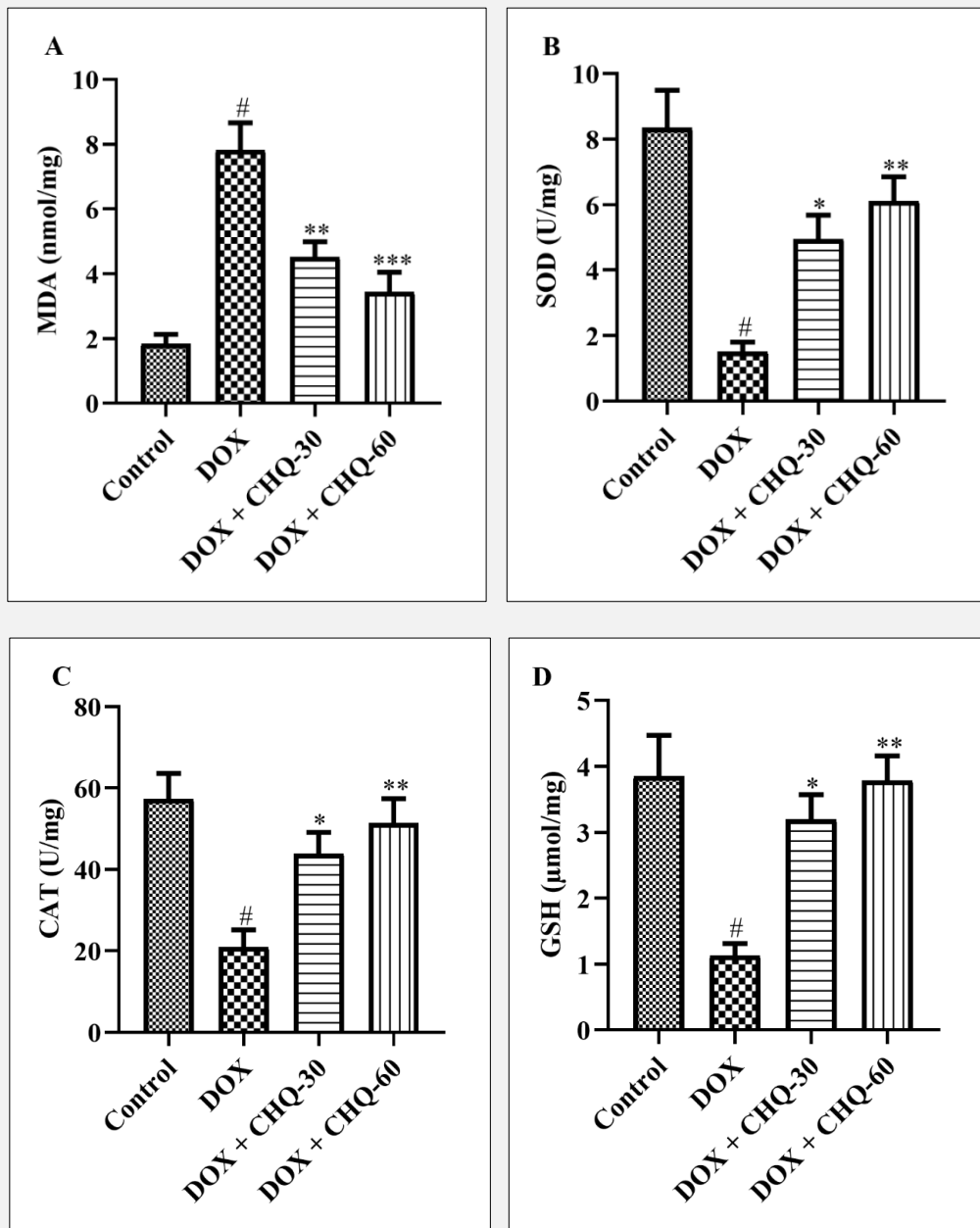


Figure 2. Effect of CHQ treatment on oxidative stress markers in DOX-induced liver injury in rats.

A: MDA, B: GSH, C: SOD, and D: CAT levels. Data are presented as mean $\pm$ SD (n = 6). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test.

p < 0.001 vs. Control, * p < 0.05, ** p < 0.01, *** p < 0.001 vs. DOX.

(mean difference = 7.233 pg/mL protein, 95% CI: 2.455 to 12.01; p = 0.0021), IL-1 β (mean difference = 10.13 pg/mL protein, 95% CI: 3.635 to 16.63; p = 0.0016), TNF- α (mean difference = 7.867 pg/mL protein, 95% CI: 2.968 to 12.77; p = 0.0012), and NF- κ B (mean dif-

ference = 4.380 pg/mL protein, 95% CI: 0.2878 to 8.472; p = 0.0332). CHQ at 60 mg/kg produced greater reductions in these inflammatory mediators than the lower-dose group, reduced IL-6 (mean difference = 9.067 pg/mL protein, 95% CI: 4.288 to 13.84; p =

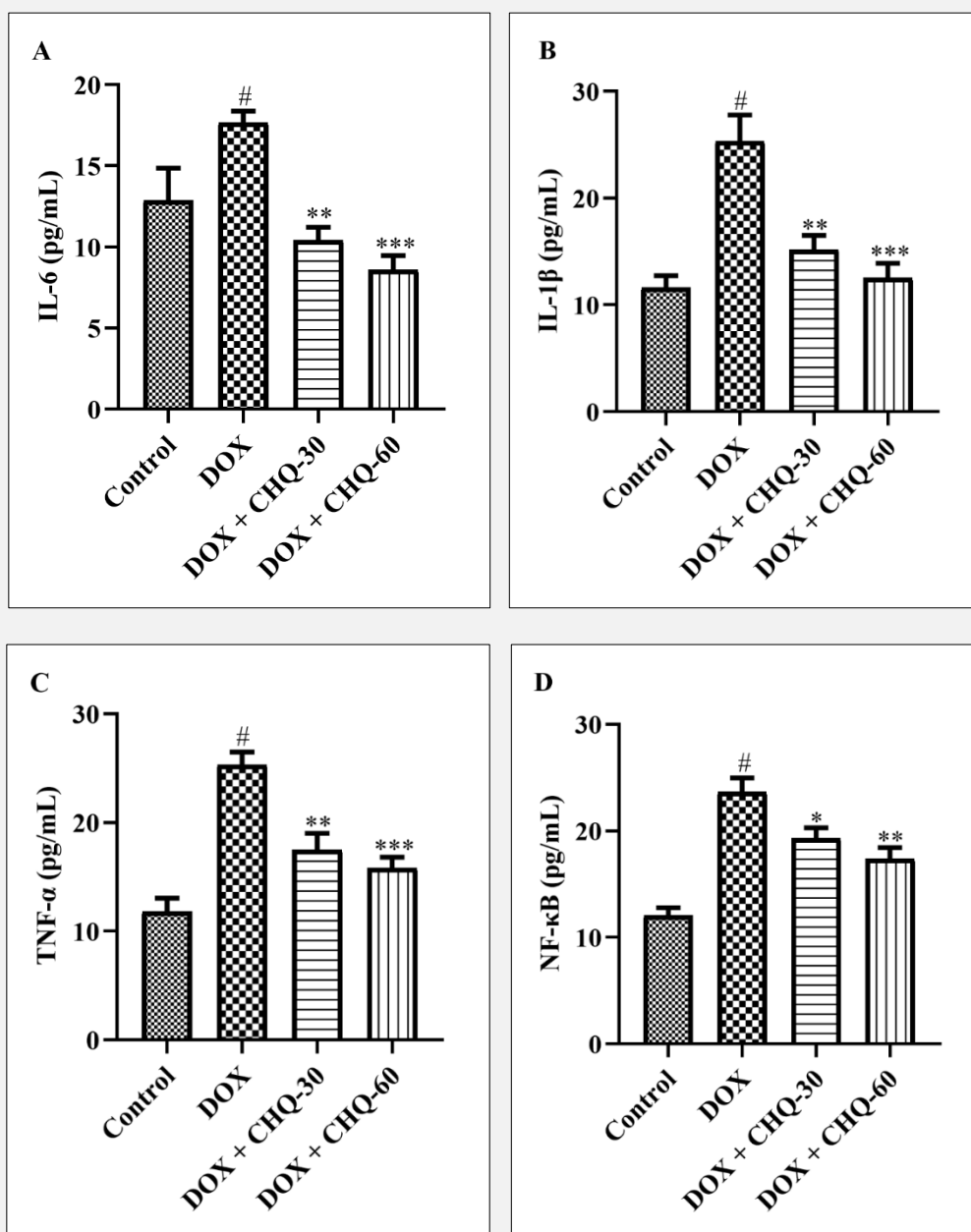


Figure 3. Effect of CHQ treatment on inflammatory cytokines in DOX-induced liver inflammation in rats.

A: IL-6, B: IL-1β, C: TNF-α, and D: NF-κB levels. Data are presented as mean ± SD (n = 6). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test.

[#] p < 0.001 vs. Control, ^{**} p < 0.01, ^{***} p < 0.001 vs. DOX.

0.0002), IL-1β (mean difference = 12.80 pg/mL protein, 95% CI: 6.301 to 19.30; p = 0.0001), TNF-α (mean difference = 9.533 pg/mL protein, 95% CI: 4.634 to 14.43; p = 0.0001), and NF-κB (mean difference = 6.277 pg/mL protein, 95% CI: 2.184 to 10.37; p = 0.0019),

suggesting a dose-dependent anti-inflammatory effect (Figure 3A - D).

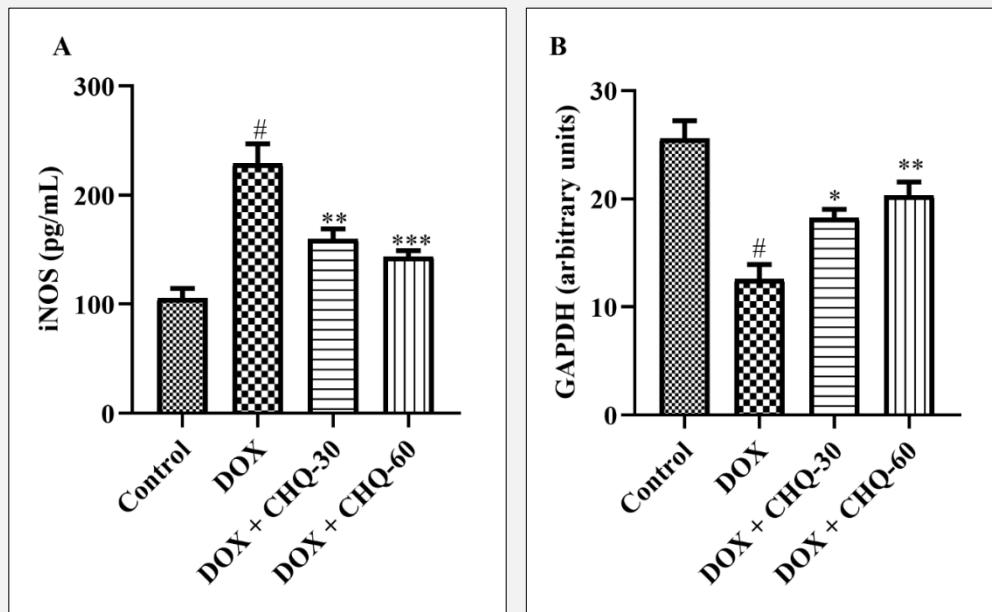


Figure 4. Effect of CHQ treatment on hepatic stress mediators in DOX-induced liver inflammation in rats.

A: iNOS and B: GAPDH levels. Data are presented as mean $\pm$ SD (n = 6). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test.

p < 0.001 vs. Control, * p < 0.05, ** p < 0.01, *** p < 0.001 vs. DOX.

Protective effect of CHQ on other inflammation-related mediators

DOX treatment significantly altered hepatic inflammatory and metabolic stress markers. Liver tissues from DOX-treated animals showed significantly increased iNOS levels (mean difference = -123.3 pg/mL protein, 95% CI: -168.4 to -78.23; p < 0.001) and decreased GAPDH levels (mean difference = 13.00, 95% CI: 7.884 to 18.12; p < 0.001) compared with the control group, indicating inflammatory stress and metabolic dysregulation.

Compared with the DOX-only group, CHQ at 30 mg/kg significantly reduced iNOS (mean difference = 69.33 pg/mL protein, 95% CI: 24.23 to 114.4; p = 0.0018) and increased GAPDH levels (mean difference = -5.687, 95% CI: -10.81 to -0.5673; p = 0.0261). CHQ at 60 mg/kg produced greater restoration in both biomarkers than the lower-dose group, reduced iNOS (mean difference = 85.17 pg/mL protein, 95% CI: 40.06 to 130.3; p = 0.0002), and increased GAPDH levels (mean difference = -7.750, 95% CI: -12.87 to -2.631; p = 0.0021), suggesting improved regulation of inflammatory and metabolic stress associated with DOX-induced hepatic injury (Figure 4A and B).

Protective effect of CHQ on the levels of PGE₂, IFN- γ , and COX-2

DOX administration significantly increased hepatic IFN- γ (mean difference = -1.890 pg/mL protein, 95% CI: -2.861 to -0.9185; p < 0.001), COX-2 (mean difference = -12.47 pg/mL protein, 95% CI: -16.29 to -8.644; p < 0.001), and PGE₂ levels (mean difference = -15.27 pg/mL protein, 95% CI: -20.48 to -10.05; p < 0.001) compared with the control group, indicating activation of inflammatory signaling pathways and disruption of inflammatory mediator homeostasis.

Co-treatment with CHQ significantly reduced IFN- γ (mean difference = 1.167 pg/mL protein, 95% CI: 0.1952 to 2.138; p = 0.0151) and COX-2 (mean difference = 5.833 pg/mL protein, 95% CI: 2.011 to 9.656; p = 0.0019), while partially restoring PGE₂ levels (mean difference = 6.633 pg/mL protein, 95% CI: 1.421 to 11.85; p = 0.0097) in a dose-dependent manner. The 60 mg/kg dose produced greater improvement in reducing IFN- γ (mean difference = 1.553 pg/mL protein, 95% CI: 0.5818 to 2.525; p = 0.0012), COX-2 (mean difference = 6.700 pg/mL protein, 95% CI: 2.877 to 10.52; p = 0.0005), and PGE₂ levels (mean difference = 8.650 pg/mL protein, 95% CI: 3.438 to 13.86; p = 0.0008) than the 30 mg/kg dose, suggesting superior modulation

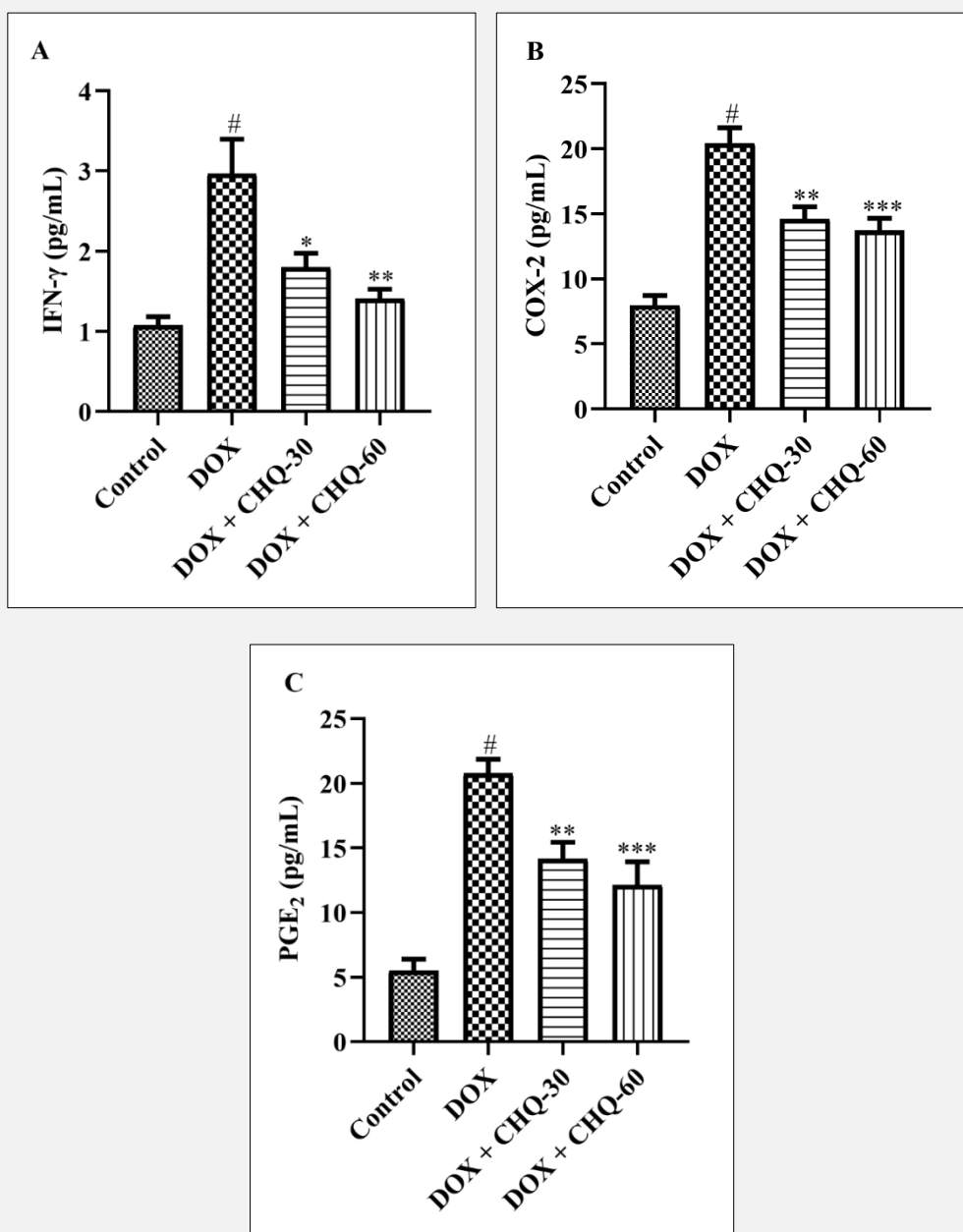


Figure 5. Effect of CHQ treatment on inflammatory mediators in DOX-induced liver inflammation in rats.

A: IFN- γ , B: COX-2, and C: PGE₂ levels. Data are presented as mean $\pm$ SD (n = 6). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test.

[#] p < 0.001 vs. Control, * p < 0.05, ** p < 0.01, *** p < 0.001 vs. DOX.

of inflammatory mediators and protection against DOX-induced hepatic inflammation (Figure 5A - C).

Improvement of DOX-induced histopathological changes by CHQ treatment

No abnormal hepatic structure was observed, with regular sinusoidal spaces, hepatocyte cell structure, and hepatocyte cords, and normal hepatic morphology was

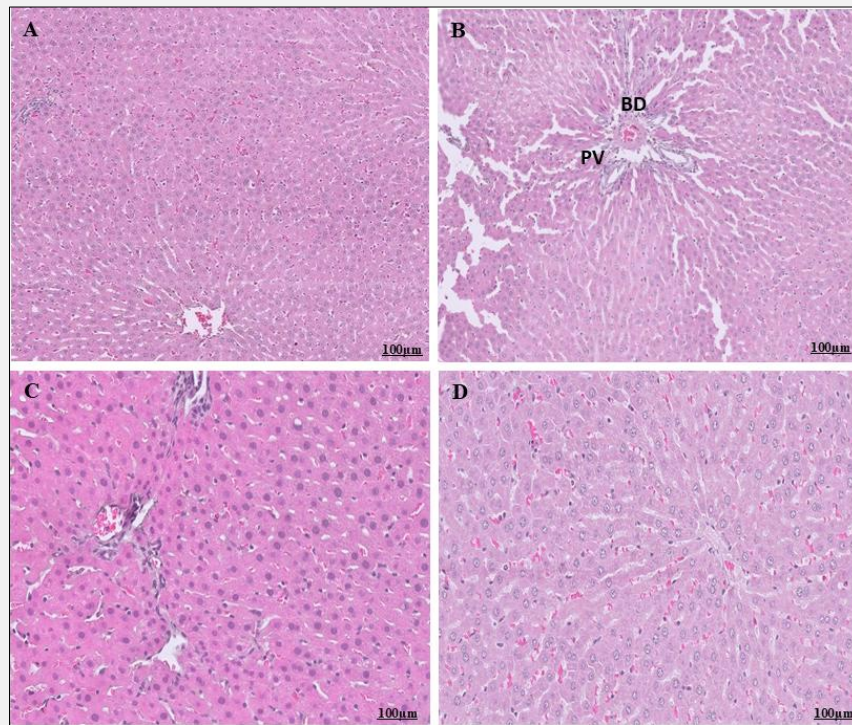


Figure 6. Histopathological evaluation of liver tissue following CHQ treatment in DOX-induced hepatotoxicity in rats.

Representative H&E-stained liver sections (200 ×). A: Normal hepatic architecture in the Control group. B: Severe hepatic damage in the DOX group. C: Moderate improvement in the DOX + CHQ-30 group [obvious presence of vein (PV) and bile duct (BD)]. D: Near-normal hepatic structure in the DOX + CHQ-60 group.

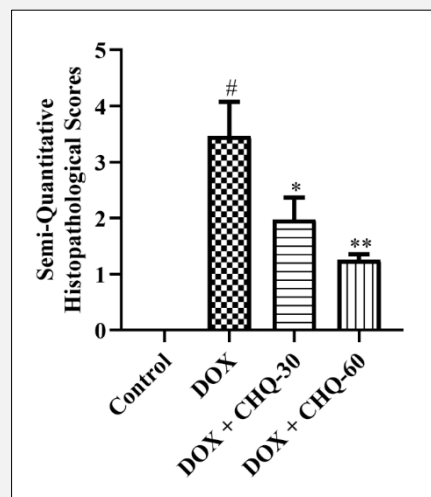


Figure 7. Semi-quantitative histopathological scoring of liver tissue in experimental groups.

Data are presented as mean ± SD (n = 6). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test. # p < 0.001 vs. Control, p < 0.05, * p < 0.01, *** p < 0.001 vs. DOX.

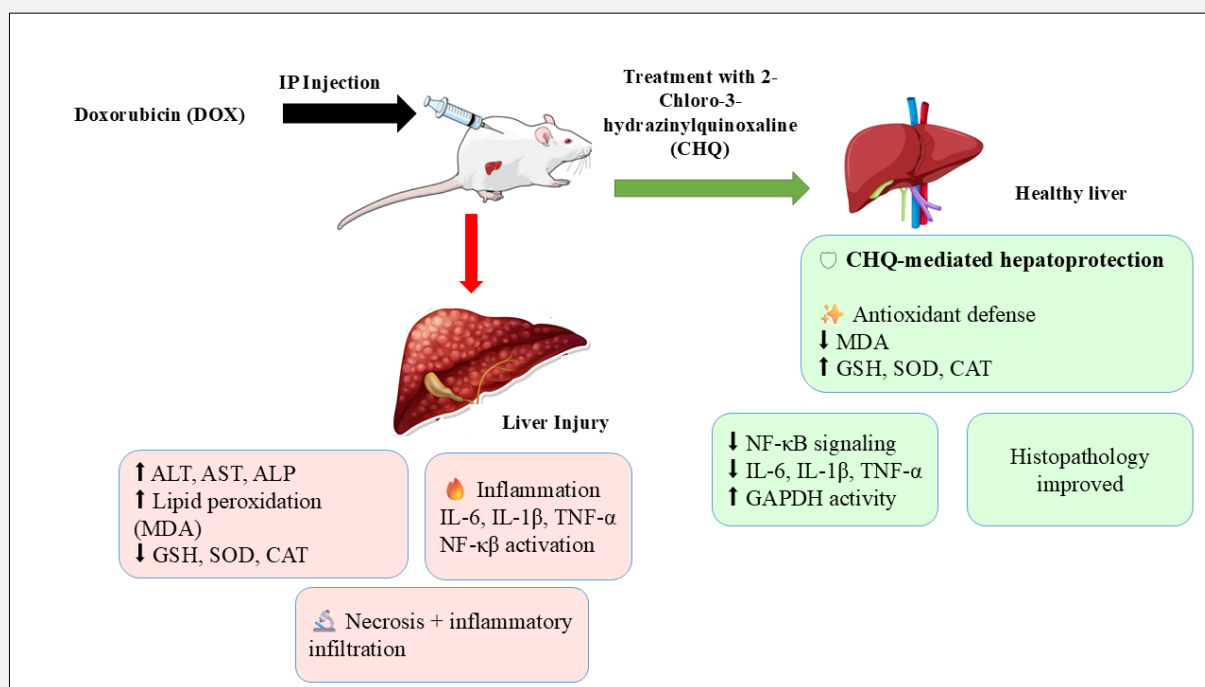


Figure 8. Graphical summary.

seen in liver histological sections from the control group, stained with H&E (Figure 6A). Rather, rats treated with DOX showed significant hepatic damage, as evidenced by hepatocellular degeneration, inflammatory cell infiltration, sinusoidal dilation, and focal necrosis (Figure 6B).

Administration of CHQ decreased the severity of these pathological changes in a dose-dependent manner. The DOX + CHQ 30 group showed moderate improvement, with reduced inflammatory infiltration and partial preservation of liver tissue (Figure 6C). The DOX + CHQ 60 group demonstrated greater protection, with histology showing near-normal findings and minimal degenerative and necrotic changes (Figure 6D).

DOX administration significantly increased the semi-quantitative histopathological score compared with the control group (mean difference = -3.467, 95% CI: -4.914 to -2.019; $p < 0.001$), indicating severe hepatic injury. CHQ treatment significantly reduced the histopathological score in both treatment groups compared with the DOX group, with the 60 mg/kg dose group (mean difference = 1.490, 95% CI: 0.04263 to 2.937; $p = 0.0422$) showing greater improvement than the 30 mg/kg dose group (mean difference = 2.215, 95% CI: 0.7676 to 3.662; $p = 0.0019$), suggesting attenuation of DOX-induced hepatic tissue damage (Figure 7).

DISCUSSION

Quinoxaline derivatives have attracted attention in medicinal chemistry because they display a broad range of biological effects. These benzopyrazine bicyclic scaffolds are reported to possess antimicrobial and antiviral activities, as well as anticancer, neuroprotective, and anti-inflammatory effects [8,13]. The quinoxaline nucleus is considered an important scaffold in drug design, because its structure can be modified to obtain compounds with different pharmacological properties. Its antioxidant activity is mainly due to its heterocyclic ring system's ability to donate an electron or hydrogen atom transfer, thus neutralizing free radicals. As in the case of the structure-activity relationship (SAR) observed in flavonoids, the biological potency of quinoxalines can be precision-engineered through strategic functional-group substitutions. Such modifications may improve target specificity and therapeutic performance [14].

DOX is a potent anticancer drug used to manage various malignancies. However, its therapeutic effectiveness is limited by toxicity to normal tissues, particularly the liver. DOX therapy-associated hepatic injury remains an important concern that can compromise patient safety and restrict long-term treatment outcomes [15].

Despite its broad therapeutic utility, prolonged administration of DOX has been associated with severe systemic toxicities, including hepatotoxicity, nephrotoxicity, and pulmonary toxicity. The liver is particularly susceptible to DOX-induced toxicity because of its central role in drug metabolism and its vulnerability to oxidative stress-mediated cellular injury. DOX-induced hepatotoxicity is mainly associated with increased generation of reactive oxygen species (ROS). This process occurs through redox cycling, leading to the formation of superoxide anions and hydrogen peroxide [2,5]. This oxidative disturbance leads to lipid peroxidation and hepatocyte membrane damage, resulting in increased MDA levels, a marker of oxidative stress and lipid peroxidation. It also elevates markers of hepatic injury, including ALT, AST, and ALP, and reduces endogenous antioxidant levels such as SOD and CAT [16]. Moreover, oxidative stress activates inflammatory signaling pathways, including NF- κ B, which induce the overproduction of pro-inflammatory cytokines, including interleukin-1 beta (IL-1 β) and interleukin-6 (IL-6), aggravating hepatic injury and dysfunction [2,4,17].

In the present study, administration of DOX (15 mg/kg) produced significant hepatic injury, consistent with previous experimental studies demonstrating that DOX therapy is associated with hepatotoxicity [18].

DOX administration caused marked alterations in hepatic function, as evidenced by elevated serum AST, ALT, and ALP levels. Increased activity of these enzymes reflects damage to hepatocellular cell membranes and leakage of intracellular contents into the circulation, indicating disruption of the integrity of normal liver cells. Similar results have been reported in the literature, showing a significant increase in hepatic enzyme markers after DOX exposure. These results support the role of DOX in the development of liver toxicity [19]. Treatment with CHQ was shown to have beneficial effects against DOX-induced hepatic injury. CHQ supplementation attenuated elevated AST, ALT, and ALP levels, indicating improved liver function and reduced tissue damage. These results imply that CHQ could protect against DOX-related liver toxicity.

Significant oxidative stress in hepatic tissues was observed after DOX exposure, as evidenced by increased lipid peroxidation and dysfunction of the endogenous antioxidant defense system [5,20]. The present study showed that DOX treatment significantly increased MDA levels, indicating greater membrane lipid damage due to excessive ROS generation. Meanwhile, decreased GSH content and reduced antioxidant enzyme activities, particularly SOD and CAT, indicated depletion of cellular antioxidant capacity during hepatic inflammation. SOD is important for scavenging superoxide radicals by converting them to hydrogen peroxide, and CAT further detoxifies hydrogen peroxide into water, limiting oxidative injury [21]. Thus, decreased activity of these enzymes may contribute to excessive accumulation of ROS and oxidative damage in the liver tissues [2]. In contrast, treatment with CHQ significant-

ly enhanced antioxidant status by decreasing MDA levels and increasing the activities of GSH, SOD, and CAT, indicating its potent antioxidant and hepatoprotective properties against DOX-induced hepatic inflammation.

DOX-induced hepatotoxicity is closely linked to a severe inflammatory response in liver tissue [17]. In the current study, we assessed the effects of CHQ on this damage by monitoring key inflammatory markers in rat liver homogenates. Our data showed that DOX exposure significantly elevated NF- κ B levels, which resulted in a substantial increase in downstream inflammatory markers. NF- κ B is a master genetic switch, and its activation leads to the release of these early-phase alarmins, accelerating leukocyte recruitment and parenchymal damage [22]. Treatment with CHQ markedly reduced NF- κ B expression in rat liver homogenates, thereby disrupting this self-perpetuating cycle and effectively blocking the overproduction of TNF- α , IL-1 β , and IL-6. This multi-cytokine suppression aligns with earlier findings [7], which pointed to robust anti-inflammatory activity of CHQ in animal models of tissue injury. Moreover, our findings are in line with the hepatoprotective trends reported [4], who mentioned that targeting the NF- κ B signaling axis and the attenuation of the cascade of inflammatory molecules is a primary defense mechanism against DOX-induced liver dysfunction. Structurally, quinoxaline skeletons are known to be potent small-molecule inhibitors that block these specific NF- κ B signaling pathways and inhibit inflammatory macrophage overactivation under metabolic stress [22]. Taken together, our findings confirm that CHQ exerts effective protection against Dox-induced liver inflammation in rats, as evidenced by reductions in inflammatory mediator levels and improvements in biochemical and histopathological parameters.

The marked elevation in hepatic iNOS levels and decreased GAPDH activity observed in the DOX-treated group suggested enhanced inflammatory and metabolic stress, consistent with previous studies in established models of anthracycline-induced hepatotoxicity [1,4]. Although GAPDH is commonly used as a housekeeping protein in molecular studies, it was evaluated in the present study as a parameter related to cellular metabolic status, because alterations in GAPDH activity may reflect changes in cellular energy metabolism associated with DOX-induced hepatic injury [23]. Excess iNOS-derived nitric oxide contributes to destructive nitrative stress, and the increased GAPDH activity may reflect altered cellular metabolism associated with DOX-induced hepatic injury. CHQ treatment reduced these pathological changes in a dose-dependent manner, with the higher dose of CHQ showing better efficacy in protecting hepatocytes from the destructive feedback loop of DOX-induced inflammation and metabolic exhaustion by inhibiting iNOS expression and restored GAPDH activity.

Elevated hepatic IFN- γ , COX-2, and PGE₂ levels following DOX exposure indicate the activation of a ro-

bust inflammatory response and disruption of inflammatory mediator homeostasis. These findings are consistent with toxicological models demonstrating that DOX-induced hepatotoxicity is primarily mediated through NF- κ B-dependent inflammatory pathways and severe oxidative stress [4,24-26]. Co-administration of CHQ significantly reduced IFN- γ , COX-2, and PGE₂ levels in a dose-dependent manner, with the 60 mg/kg dose showing greater efficacy than the 30 mg/kg dose. These findings suggest that CHQ attenuates DOX-induced hepatic inflammation by suppressing pro-inflammatory cytokine signaling and modulating inflammatory mediators, thereby protecting hepatic tissue against DOX-induced injury.

The histopathological findings closely support the biochemical protection conferred by CHQ against DOX-induced hepatotoxicity. In the DOX-alone group, pronounced structural damage was evident, encompassing hepatocellular degeneration, sinusoidal dilatation, focal necrosis, and inflammatory infiltration, indicating severe tissue disruption. These morphological alterations are in agreement with recent toxicological reports showing that DOX accumulation leads to extensive hepatocyte ballooning and localized tissue death [2]. Remarkably, CHQ administration preserved the liver architecture in a dose-dependent manner. While the 30 mg/kg dose provided moderate protection, the 60 mg/kg dose showed nearly normal histology with minimal degeneration. This structural recovery, validated by semi-quantitative scoring, demonstrates that CHQ effectively shields the hepatic structures from DOX-induced cellular injury.

This study demonstrates that quinoxaline offers significant protection against DOX-induced liver damage by targeting oxidative stress and inflammation. In the experimental model, DOX administration markedly altered liver function, causing a dramatic spike in hepatic enzymes such as ALT, AST, and ALP, which signal structural liver injury. It also triggers a harmful oxidative imbalance, depleting vital natural antioxidants such as SOD, CAT, and GSH, while elevating MDA levels, an indicator of lipid peroxidation and cell death. CHQ treatment successfully counteracts these effects, enhances antioxidant defenses, reduces lipid peroxidation, and brings liver enzyme levels to safeguard hepatic cellular integrity.

CHQ also attenuated the pronounced inflammatory response associated with DOX-induced hepatotoxicity. DOX administration was associated with significantly elevated hepatic levels of inflammatory mediators, including TNF- α , IL-1 β , IL-6, IFN- γ , NF- κ B, iNOS, COX-2, and PGE₂ levels, indicating marked inflammatory dysregulation. Treatment with CHQ significantly ameliorated these alterations in a dose-dependent manner, suggesting that its hepatoprotective effects are associated with modulation of oxidative stress and inflammatory responses (Figure 8).

Although the observed changes in inflammatory biomarkers are consistent with reduced inflammatory sig-

naling, the present study did not directly evaluate pathway activation using Western blotting, immunohistochemistry, or RT-qPCR. Therefore, the proposed involvement of specific molecular pathways should be considered preliminary and requires confirmation in future mechanistic studies. Nevertheless, the biochemical, inflammatory, and histopathological findings collectively support CHQ's potential as a promising hepatoprotective agent against DOX-induced liver injury.

A limitation of the present study is that only male rats were included, which may limit the generalizability of the findings. Future studies involving both male and female animals are warranted to determine whether sex-related differences influence the hepatoprotective effects of CHQ. This pilot study did not include a power calculation, as its purpose was to gather initial data and insights for future research. From a clinical perspective, the present findings suggest that CHQ may have potential as an adjuvant hepatoprotective agent for patients receiving DOX-based chemotherapy. Hepatic dysfunction is a common dose-limiting adverse effect of anthracycline treatment and may necessitate dose reduction or discontinuation, thereby compromising anticancer efficacy. By attenuating oxidative stress, suppressing NF- κ B-mediated inflammatory responses, and preserving hepatic architecture, CHQ could improve liver tolerance to chemotherapy without compromising DOX's antitumor activity. Such an approach may be particularly beneficial for patients requiring cumulative or prolonged anthracycline treatment and for those with pre-existing risk factors for hepatic injury. However, before clinical translation, several critical issues require investigation, including comprehensive pharmacokinetic and pharmacodynamic characterization, oral bioavailability, long-term toxicity, reproductive and genotoxic safety, potential interactions with chemotherapeutic agents, and confirmation that CHQ does not reduce the anticancer efficacy of DOX. Furthermore, mechanistic studies using molecular techniques, followed by studies in larger animal models and well-designed phase I and phase II clinical trials, will be essential to establish the safety, optimal dosing regimen, and therapeutic efficacy of CHQ in cancer patients. Therefore, although the current results are encouraging, CHQ should presently be regarded as a promising investigational hepatoprotective candidate rather than a clinically established therapeutic agent.

Nevertheless, the current findings are derived from an experimental animal model, and further investigations are required to evaluate the pharmacokinetic profile, long-term safety, potential drug-drug interactions, optimal dosing strategies, and efficacy of CHQ in clinically relevant models before its potential clinical application can be established.

CONCLUSION

DOX-induced hepatotoxicity is a significant limitation in cancer chemotherapy because of its association with oxidative stress, inflammatory activation, and progressive hepatic tissue injury. The findings of this study revealed the deleterious effects of DOX on endogenous antioxidant defenses, lipid peroxidation, inflammatory mediator levels, and significant histopathological alterations in rat liver tissue. Co-administration of CHQ produced a dose-dependent amelioration of these pathological changes, as evidenced by enhanced antioxidant enzyme activities, reduced oxidative stress, suppression of pro-inflammatory cytokines and mediators such as NF- κ B, iNOS, and COX-2, restoration of PGE₂ levels, and preservation of hepatic histological integrity. The results suggest that CHQ exerts hepatoprotective effects that are associated with antioxidant and anti-inflammatory activities. However, direct modulation of specific signaling pathways was not evaluated in the present study; therefore, further molecular investigations are required to elucidate the precise mechanisms underlying these protective effects. Collectively, CHQ may represent a promising therapeutic candidate to attenuate DOX-associated liver injury. Further molecular and clinical studies are required to confirm the long-term safety, pharmacological targets, and translational potential.

Ethical Approval Statement:

All research procedures were conducted in accordance with institutional guidelines and received approval from the Biomedical Research Ethics Committee of Umm Al-Qura University, with approval number HAPO-02-K-012-2025-10-2956.

Data Availability Statement:

All data supporting the conclusion of this study have been included in the manuscript.

Declaration of Generative AI in Scientific Writing:

During the summarization of this manuscript, the authors used grammarly to improve language and readability. After its use, the authors thoroughly reviewed, verified, and revised all AI-assisted content to ensure accuracy and originality. The authors take full responsibility for the integrity and final content of the published article.

Declaration of Interest:

The authors declare no conflicts of interest.

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