

TND1128, a Redox-Active 5-Deazaflavin Derivative, Mitigates α -Naphthyl Isothiocyanate-Induced Intrahepatic Cholestasis in Rats via Antioxidant, Anti-Inflammatory, and Anti-Apoptotic Pathways

Athraa Lateef Khaleel^{1*}, Alaa Radhi Khudhair²

¹ Ministry of Health And Environment, Kirkuk Health Directorate, Kirkuk, Iraq

Email: azraa.reda2400m@copharm.uobaghdad.edu.iq, ORCID: 0009-0001-4375-6735

² Department of Pharmacology and Toxicology, College of Pharmacy, University of Baghdad, Baghdad, Iraq

Abstract

Background: Intrahepatic cholestasis is characterized by impaired bile formation or bile flow, leading to the accumulation of bile acid, oxidative stress, inflammation, and hepatocellular injury. Despite its clinical significance, effective therapeutic options remain limited. This study investigated the hepatoprotective potential of TND1128, a redox-active 5-deazaflavin derivative with mitochondrial-modulating capabilities, in an α -naphthyl isothiocyanate (ANIT)-induced model of intrahepatic cholestasis in rats.

Methods: Thirty-six male Sprague–Dawley rats were randomly assigned to six groups: control, ANIT, TND1128, ursodeoxycholic acid (UDCA), TND1128 + ANIT, and UDCA + ANIT. Cholestasis was induced by a single oral administration of ANIT (100 mg/kg). Biochemical, oxidative stress, inflammatory, apoptotic, and molecular parameters were subsequently evaluated, including serum liver enzymes [alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP)], bilirubin, bile acids, malondialdehyde (MDA), glutathione (GSH), interleukin-6 (IL-6), nuclear factor kappa B (NF- κ B), (nuclear factor erythroid 2-related factor 2) Nrf2, caspase-3, and farnesoid X receptor (FXR)/ bile salt export pump (BSEP) gene expression, alongside histopathological evaluation.

Results: ANIT administration induced significant hepatocellular injury, as evidenced by elevated levels of liver enzymes, bile acids, bilirubin, MDA, IL-6, NF- κ B, and caspase-3. Concurrently, it caused reduced GSH levels, downregulated expression of Nrf2, FXR, and BSEP, along with marked histological damage. Pretreatment with TND1128 significantly ameliorated these changes by restoring biochemical parameters, reducing oxidative stress and inflammatory mediators, enhancing antioxidant defense via Nrf2 upregulation, suppressing apoptotic signaling, and

substantially improving liver histoarchitecture. However, TND1128 did not significantly restore FXR or BSEP expression, suggesting that its hepatoprotective effects may involve modulation of redox, inflammatory, and apoptotic pathways without significant changes in FXR or BSEP Expression under the present experimental conditions.

Conclusion: These findings indicated that TND1128 protects against ANIT-induced cholestatic liver injury primarily through modulation of oxidative stress, inflammation, and apoptosis, highlighting its potential as a promising therapeutic candidate for further preclinical development and translational investigation.

Keywords

Cholestasis; ANIT model; TND1128; Oxidative stress; Inflammation; Nrf2; Caspase-3

1. Introduction

Cholestatic liver disease comprises a heterogeneous group of hepatobiliary disorders characterized by impaired bile production or flow, leading to bile acid accumulation and progressive liver injury. Persistent bile acid retention causes hepatocellular and cholangiocellular damage, inflammation, oxidative stress, and hepatic tissue remodeling, fibrosis and cirrhosis. Despite advances in understanding the pathophysiology of cholestasis, current therapeutic options remain limited and are ineffective in a substantial proportion of patients. Consequently, there is a growing interest in developing novel hepatoprotective agents that target the molecular mechanisms underlying cholestatic liver injury.^{1,2}

Oxidative stress is a known cause of cholestatic liver damage. The accumulation of toxic bile acids during cholestasis promotes excessive generation of reactive oxygen species (ROS), resulting in lipid peroxidation and oxidative damage to proteins, nucleic acids, and membrane lipids. Moreover, oxidative stress stimulates the release of pro-inflammatory cytokines, including interleukin-6 (IL-6), and activates transcription factors such as nuclear factor kappa-B (NF-κB), which regulates the expression of genes involved in inflammation, apoptosis, and immune responses.²⁻⁶

The interplay between oxidative stress and inflammatory mediators further activate apoptotic pathways. Mitochondrial dysfunction promotes the release of cytochrome c into the cytosol, initiating the intrinsic caspase cascade and subsequent activation of caspase-3, a key executioner

of apoptosis. Thus, oxidative stress, inflammatory responses, and apoptosis contribute to the progression of cholestatic liver injury.⁴⁻⁷

Hepatocytes possess intrinsic antioxidant defense mechanisms against oxidative stress, among which nuclear factor erythroid 2-related factor 2 (Nrf2) serves as a key cytoprotective transcription factor. Upon activation, Nrf2 induces the expression of antioxidant and detoxifying enzymes that protect hepatocytes from oxidative damage. Nrf2 signaling also regulates transporter expression and detoxification pathways involved in maintaining bile acid balance, thereby supporting antioxidant defense. Nrf2 activation thus protects the liver against oxidative stress-mediated injury.⁸⁻¹⁰

Maintenance of bile acid homeostasis is essential for normal hepatic function and is tightly regulated by nuclear receptors and membrane transporters, particularly the farnesoid X receptor (FXR). FXR activation reduces bile acid synthesis while increasing the expression of the bile salt export pump (BSEP), thereby facilitating bile acid excretion. Dysregulation of FXR signaling leads to bile acid accumulation and cholestatic liver injury. Therefore, targeting FXR-mediated signaling pathways represents a potential therapeutic strategy for the treatment of cholestatic liver disorders.¹¹⁻¹³

Among the animal models used to investigate the mechanisms of cholestatic liver injury and to evaluate candidate hepatoprotective drugs, α -naphthyl isothiocyanate (ANIT)-induced cholestasis is one of the most widely used models. ANIT selectively damages biliary epithelial cells, injuring the bile ducts and impairing bile flow. This disruption results in hepatocyte death, bile acid accumulation, oxidative stress, and inflammatory cell infiltration. Because ANIT-induced liver injury closely mimics clinical cholestatic liver disease, this model has been extensively employed in toxicological research evaluating hepatoprotective agents.¹⁴⁻¹⁶

Ursodeoxycholic acid (UDCA) is the standard treatment for several cholestatic liver disorders. Its hepatoprotective effects are primarily attributed to improved bile flow, stabilization of hepatocyte membranes, reduction of oxidative stress, and suppression of inflammatory signaling. However, many patients respond poorly to UDCA therapy. These limitations highlight the need for alternative therapies that target oxidative stress, inflammation, and apoptosis in cholestatic liver injury.^{17,18}

Mitochondrial dysfunction is an important cause of cholestatic liver damage. Accumulation of hydrophobic bile acids within hepatocytes affects mitochondrial respiration and electron transport

chain function, leading to increased ROS generation. Mitochondrial oxidative stress causes lipid peroxidation, DNA damage, and loss of membrane integrity. In severe cases, activation of the mitochondrial permeability transition pore depletes ATP and releases pro-apoptotic factors like cytochrome c, which activates downstream apoptotic pathways, including caspase-3. Thus, mitochondrial damage links bile acid toxicity to hepatocyte death and the progression of cholestatic liver disease.¹⁹⁻²¹

Recent advances in mitochondrial pharmacology have demonstrated that redox-active compounds can modulate mitochondrial function and oxidative stress. Synthetic 5-deazaflavin derivatives, which are structurally related to flavin cofactors, can participate in redox cycling reactions. TND1128, a synthetic 5-deazaflavin derivative, has been proposed to modulate mitochondrial electron transport and energy metabolism through its intrinsic redox activity. Under conditions of oxidative stress, it has been suggested to reduce ROS generation and restore mitochondrial function by modulating cellular redox equilibrium. Such modulation of oxidative stress may activate Nrf2 signaling and bile acid homeostasis pathways, suggesting a potential hepatoprotective role of TND1128 in cholestatic liver injury.^{22,23}

Despite extensive investigation of oxidative stress and bile acid dysregulation in cholestatic liver injury, few studies have examined the therapeutic potential of mitochondrial redox modulators in this context. The role of synthetic flavin analogues such as TND1128 in modulating oxidative stress, inflammatory signaling, apoptosis, and bile acid balance during cholestatic liver injury therefore remains unclear.^{7,22,24}

This study aimed to evaluate the hepatoprotective effects of TND1128 against ANIT-induced intrahepatic cholestasis and to investigate its underlying mechanisms, including oxidative stress, inflammatory signaling, apoptosis, and bile acid regulatory pathways.

2. Methods

2.1. Chemical and reagents

TND1128 (purity > 98 %) was obtained from Shandong Longilat Biotechnology Co., Ltd. (China). ANIT was purchased from Sigma-Aldrich (St. Louis, MO, USA). UDCA and corn oil were obtained from Macklin Biochemical Co., Ltd. (China). Xylazine HCl (XYL-M2, VMD® Livestock Pharma, Belgium) and ketamine (10 %, Dutch Farm International, Netherlands) were used for anesthesia. Tween 80 (Polysorbate 80) was sourced from ChapTec (Canada).

Commercial biochemical assay kits for alanine aminotransferase (ALT) (Cat# 1105000), aspartate aminotransferase (AST) (Cat# 1109000), alkaline phosphatase (ALP) (Cat# 1103005), total bile acids (TBA) (Cat# E-BC-K181), total serum bilirubin (TSB) (Cat#1110005) were procured from Linear Chemicals S.L.U. (Spain). Enzyme-linked immunosorbent assay (ELISA) kits for IL-6 (Cat# E-EL-R0016), MDA (Cat# E-EL-0060), GSH (Cat# E-EL-0026), and NF- κ B (Cat# ER1186) were obtained from ELK Biotechnology (China).

For molecular analyses, RNA extraction was conducted using TransZol (Cat# ER501-01, TransGen Biotech, Beijing, China), and cDNA synthesis was performed using TransScript First-Strand cDNA Synthesis SuperMix (TransGen Biotech, China). FXR and BSEP primers were synthesized by Macrogen Inc. (South Korea). Primary antibodies for Western blot analysis, including caspase-3 (E-AB-63510), Nrf2 (E-AB-68254), and β -tubulin (E-AB-20033) were purchased from Elabscience (USA).

2.1.1. Preparation of TND1128 solution

A fresh TND1128 solution was prepared daily by dissolving 40 mg of TND1128 powder in 16 mL of corn oil, and administered orally by gavage at a dose of 10 mg/kg body weight. The selected dose was based on previous studies demonstrating the mitochondrial-activating and cytoprotective effects of TND1128 in vivo and ex vivo.²⁵

2.1.2. Preparation of ANIT solution

A fresh ANIT solution was prepared on the day of administration by dissolving 100 mg of ANIT powder in 1 mL of corn oil with continuous mixing until complete dissolution. The resulting solution was administered orally by gavage at a volume individually adjusted according to each animal's body weight (1 mL/kg). This dose was selected in accordance with established ANIT-induced cholestasis models.²⁶

2.2 . Experimental Design and Animal Treatment

The study was approved by the Research Ethical Committee of the College of Pharmacy, University of Baghdad (Approval No. RECAUBCPR1020250615A; approved on 12 October 2025). All experimental procedures were conducted in accordance with the principles of the Office International des Epizooties (OIE) guidelines for animal ethics. All procedures involving animals

complied with relevant regional and international regulations governing the ethical treatment and use of laboratory animals. This study was reported in accordance with the ARRIVE 2.0 guidelines [36]. Thirty-six adult male Sprague–Dawley rats (8 weeks old; 150–200 g) were obtained from the Animal House, College of Pharmacy, University of Baghdad. Animals were housed under standard laboratory conditions ($25 \pm 2^{\circ}\text{C}$, 50–60% relative humidity, and a 12/12 h light/dark cycle) with free access to food and water. Animals were acclimatized for one week prior to the experiment to minimize stress-related physiological variations and allow adaptation to the laboratory environment.

Rats were randomly divided into six groups ($n = 6$ per group) as follows:

Group I (Control): Rats received corn oil (1 mL/kg/day) by oral gavage for 14 consecutive days.

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Group II (ANIT, cholestasis group): Rats received corn oil (1 mL/kg/day) by oral gavage for 14 consecutive days, and a single oral dose of ANIT (100 mg/kg, dissolved in corn oil) was administered on Day 12.

Group III (TND1128): Rats received TND1128 (10 mg/kg/day, dissolved in corn oil) by oral gavage for 14 consecutive days.

Group IV (UDCA): Rats received UDCA (100 mg/kg/day, prepared in corn oil) by oral gavage for 14 consecutive days.

Group V (TND1128+ANIT): Rats received TND1128 (10 mg/kg/day) for 14 consecutive days and followed by a single oral dose of ANIT (100 mg/kg) was administered on Day 12 following a 4-hour fasting period.

Group VI (UDCA + ANIT): Rats received UDCA (100 mg/kg/day) for 14 consecutive days, followed by a single oral dose of ANIT (100 mg/kg) on Day 12 after following a 4-hour fasting period (Figure 1).

To ensure consistency and stability, all treatments were administered according to a defined schedule, with doses freshly prepared as described in Sections 2.1.1 and 2.1.2. Rats were monitored daily for general health status and signs of toxicity, and body weights were recorded to assess animal well-being during the study. No unexpected mortality occurred before the scheduled euthanasia period. Any observed adverse effects were documented.

The animals were fasted for 24 hours after the final treatment to standardize biochemical measurements and subsequently anesthetized for sample collection. Anesthesia was induced by

intraperitoneal injection of ketamine/xylazine combination (50 mg/kg ketamine + 10 mg/kg xylazine) to achieve an adequate surgical plane.²⁸ Blood samples were collected by cardiac puncture prior to euthanasia. Cervical dislocation was subsequently performed under deep anesthesia as a humane method of euthanasia according to established animal welfare guidelines and the AVMA.²⁹ The liver was immediately excised following euthanasia and rinsed in ice-cold PBS. The harvested tissue was then processed for subsequent histological, molecular, and biochemical analyses.

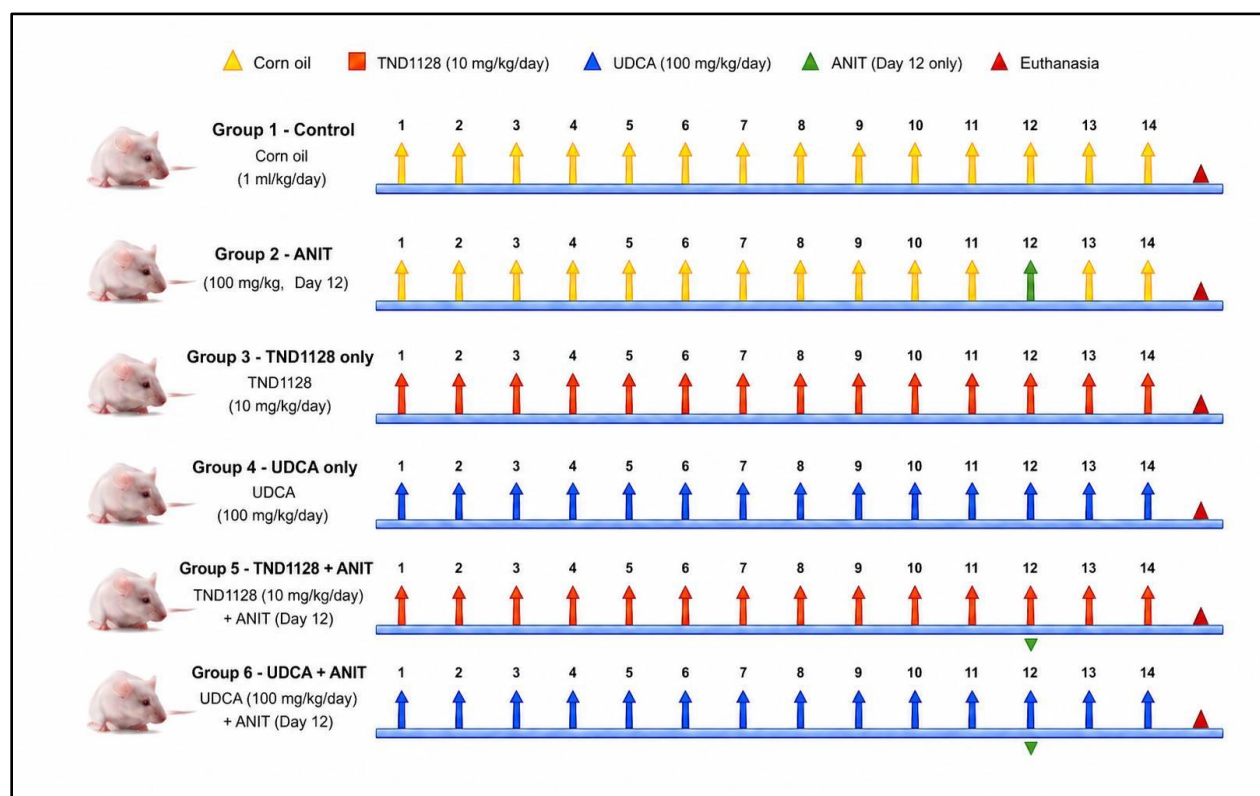


Figure 1. Study design flow chart.

2.3. Biochemical Analysis of Serum

Serum levels of ALT, AST, ALP, TSB, and TBA were determined using commercially available kits (Linear Chemicals, Spain) according to the manufacturers' protocols. ALT and AST activities were measured using kinetic enzymatic methods, whereas ALP and bilirubin levels were assessed using colorimetric assays.

2.4. Determination of Oxidative Stress and Inflammatory Markers

Following euthanasia, liver tissues were carefully excised and rinsed with ice-cold phosphate-buffered saline (PBS, pH 7.4) to eliminate residual blood. Tissue homogenates were prepared by homogenizing 1 g of liver tissue with 9 mL of PBS using a tissue homogenizer and subsequently centrifuged at 4000 rpm for 10 min. The resulting supernatant was collected for determination of the oxidative stress markers including malondialdehyde (MDA) and reduced glutathione (GSH), as well as the inflammatory marker nuclear factor kappa B (NF- κ B), using ELISA kits (ELK Biotechnology, China) according to the manufacturer's instructions. Serum IL-6 levels were determined using the same ELISA kits. For all assays, absorbance was measured at 450 nm, and concentrations were calculated from the corresponding standard curves.

2.6. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from liver tissue using TransZol reagent according to the manufacturer's instructions. RNA concentration and purity were measured at 260 nm and 280 nm on a spectrophotometer. Two micrograms of total RNA were reverse-transcribed into cDNA using TransScript First-Strand cDNA Synthesis Supermix kit (TransGen Biotech, China). Quantitative real-time PCR (qRT-PCR) was subsequently performed using a CFX Opus 96 Real-Time PCR system (Cat# 12011319, Version 2.1, Bio-Rad, Hercules, CA, USA) and SYBR Green master mix to determine the relative expression of *FXR* and *BSEP*. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an endogenous internal control. Relative gene expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method.^{30,31} The primer sequences are listed in Table 1.

Table 1. Primer sequences used for quantitative real-time PCR analysis.

Gene	Forward primer (5'→3')	Reverse primer (5'→3')
<i>FXR</i>	AGGCCATGTTTCCTTCGTTTC	CAGCTCCCCGACACTTTTATAG
<i>BSEP</i>	CAACGCATTGCTATTGCTCG	GTTCTGGATGGTGGACAAACG
<i>GAPDH</i>	TTCCAGGAGCGAGATCCCGCTAAC	CATGAGCCCTTCCACGATGCCAAAG

2.7. Western Blot Analysis

Hepatic protein expression of Nrf2 and cleaved caspase-3 was determined by Western blotting. Protein samples were extracted from liver tissue using radioimmunoprecipitation assay (RIPA) lysis buffer supplemented with phenylmethylsulfonyl fluoride (PMSF) and sodium orthovanadate (Na_3VO_4) at a ratio of 0.3 g liver tissue to 1 mL RIPA lysis buffer containing 10 μL each of PMSF and Na_3VO_4 . Protein concentrations were quantified using a bicinchoninic acid (BCA) assay. Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes, which were blocked with 5% fat-free milk at room temperature for 1 h and then incubated overnight at 4°C with primary antibodies against Nrf2, cleaved caspase-3, and β -tubulin. Membranes were subsequently incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Elabscience, dilution 1:10,000) for 1 h at room temperature. Protein bands were visualized using enhanced chemiluminescence (ECL) detection reagents (Elabscience, USA) and imaged using a ChemiDoc MP Imaging System (Bio-Rad Laboratories, USA). Band intensities were quantified using ImageJ software.^{32,33}

2.8 Histopathological Examination

Liver tissues were collected immediately after euthanasia and fixed in 10% formalin to preserve tissue architecture. Fixed tissues were routinely processed, embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E) for microscopic histopathological examination.

Histopathological examination was performed by a blinded pathologist to minimize observational bias. Hepatic structural alterations such as hepatocellular degeneration, necrosis, inflammatory cell infiltration, vascular and sinusoidal congestion, and fibrosis were assessed using a semi-quantitative scoring system ranging from 0 to 4, where 0 indicated the absence of lesions and 4 indicated severe pathological alterations. The histological grading was based on established criteria evaluating portal and lobular inflammation, necrosis, and the degree of fibrosis. Representative microscopic fields were selected for photomicrographic documentation. Histopathological findings were correlated with the morphological changes observed in liver tissue across the control, induction, and treatment groups.³⁴

2.9. Statistical analysis

Data were expressed as mean $\pm$ standard deviation (SD) and were analyzed using GraphPad Prism 10 (GraphPad Software, San Diego, CA, USA). Statistical comparisons were performed using one-way ANOVA followed by Tukey's post hoc test. A p-value < 0.05 was considered statistically significant.^{35,36}

3. Results

3.1. Effect of TND1128 on liver function tests

ANIT administration significantly increased serum ALT, AST, ALP, TBA, and TSB levels compared with the control group ($p < 0.0001$), indicating liver injury. Pretreatment with TND1128 significantly reduced all biochemical parameters relative to the ANIT group ($p < 0.0001$), with values approaching the control range for several markers, suggesting a hepatoprotective effect comparable to that of UDCA. UDCA treatment also markedly improved these biochemical indices compared with ANIT ($p < 0.0001$), with no statistically significant differences compared with TND1128 ($P > 0.05$), as seen in Figure 2.

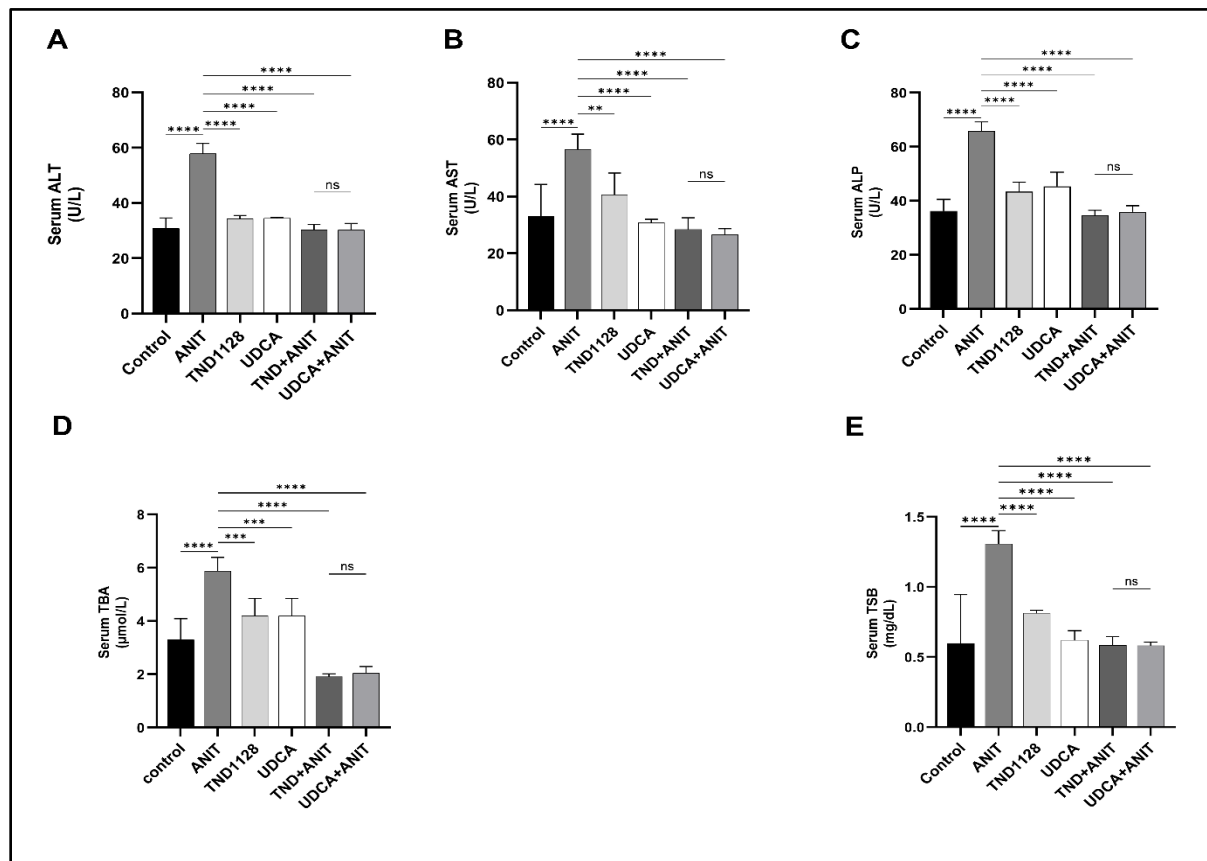


Figure 2. Effect of TND1128 and UDCA on serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total bile acid (TBA) and total serum bilirubin (TSB) levels in ANIT-induced cholestasis in rats. Data are presented as mean $\pm$ SD (n=6). Statistical significance: ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; ns = non-significant.

3.2. Effect of TND1128 on Inflammatory markers

ANIT exposure resulted in significant elevation of serum IL-6 and hepatic NF- κ B levels compared with control ($p < 0.0001$). TND1128 pretreatment significantly decreased both IL-6 and NF- κ B compared with the ANIT group ($p < 0.0001$), suggesting an anti-inflammatory effect. UDCA produced a comparable reduction in inflammatory markers to TND1128, with no statistically significant difference between the two treatment groups ($p > 0.05$), as seen in Figure 3.

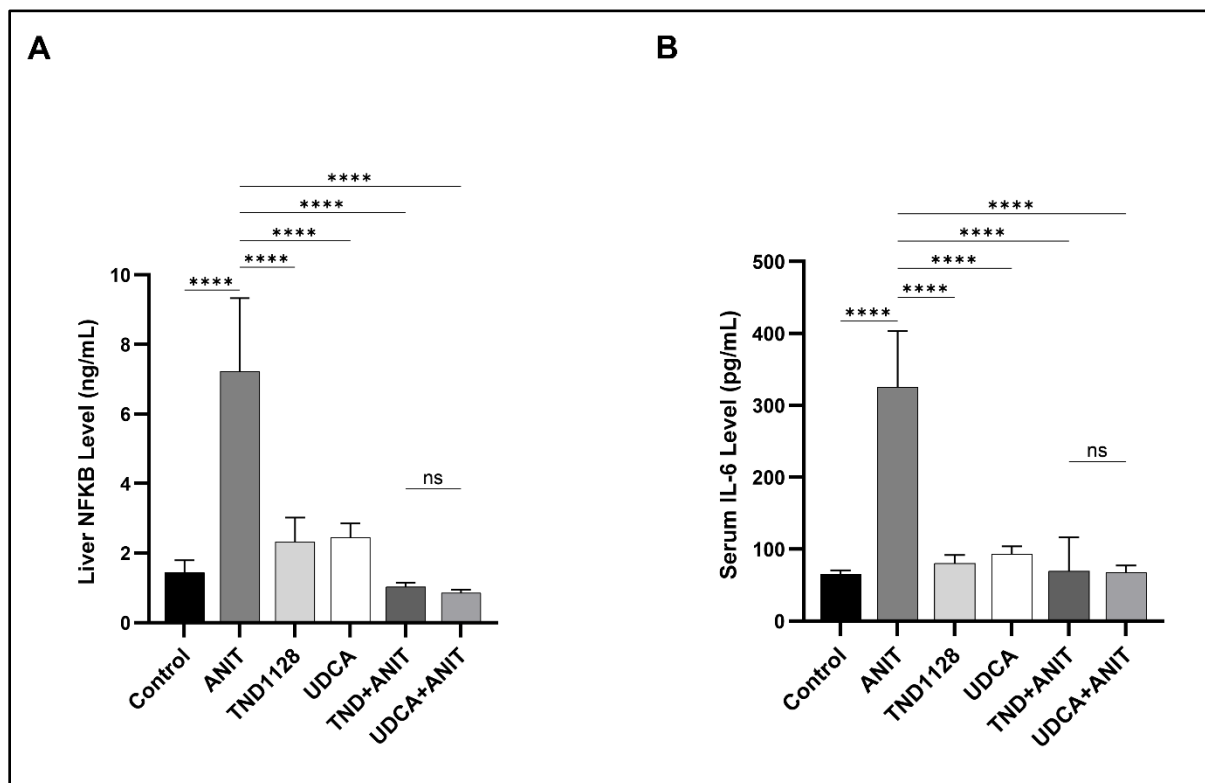


Figure 3. Effect of TND1128 and UDCA on hepatic NF- κ B and serum IL-6 levels in ANIT-induced cholestasis in rats. Data are presented as mean $\pm$ SD (n = 6). Statistical significance: **** $p < 0.0001$; ns = non-significant.

3.3. Effect of TND1128 on oxidative stress markers

The administration of ANIT significantly increased hepatic MDA levels and decreased GSH levels compared with the control group, indicating significant induction of oxidative stress ($p < 0.0001$). In comparison to the ANIT group, pretreatment with TND1128 significantly reduced MDA levels and restored hepatic GSH content ($p < 0.0001$), suggesting reduction of oxidative damage and enhancement of antioxidant defense. UDCA treatment also elicited comparable antioxidant effects to TND1128, with no statistically significant difference between the two treatment groups ($p > 0.05$), as seen in Figure 4.

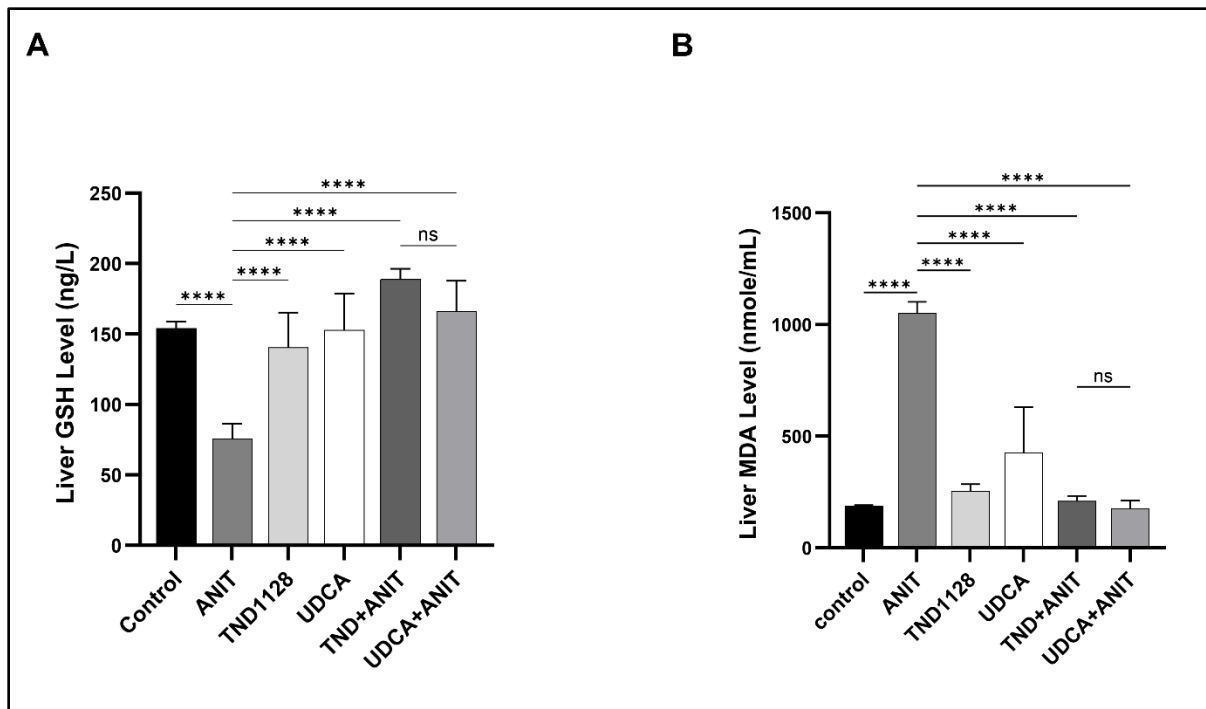


Figure 4. Effect of TND1128 and UDCA on hepatic MDA and reduced GSH levels in ANIT-induced cholestasis in rats. Data are presented as mean $\pm$ SD (n = 6). Statistical significance: **** $P < 0.0001$; ns = non-significant.

3.4. Regulation of apoptotic and antioxidant pathways by TND1128

Western blot analysis revealed that hepatic Nrf2 protein expression was significantly reduced in the ANIT group compared with the control group, whereas cleaved caspase-3 protein expression was markedly increased ($p < 0.001$), indicating enhanced oxidative stress and apoptotic activity. Pretreatment with TND1128 significantly restored Nrf2 expression and reduced cleaved caspase-3 levels relative to the ANIT group ($p < 0.01$), suggesting activation of antioxidant pathways and reduction of apoptosis. Similar regulatory effects were observed following UDCA treatment, with no statistically significant differences compared with TND1128 ($P > 0.05$), as demonstrated in Figure 5.

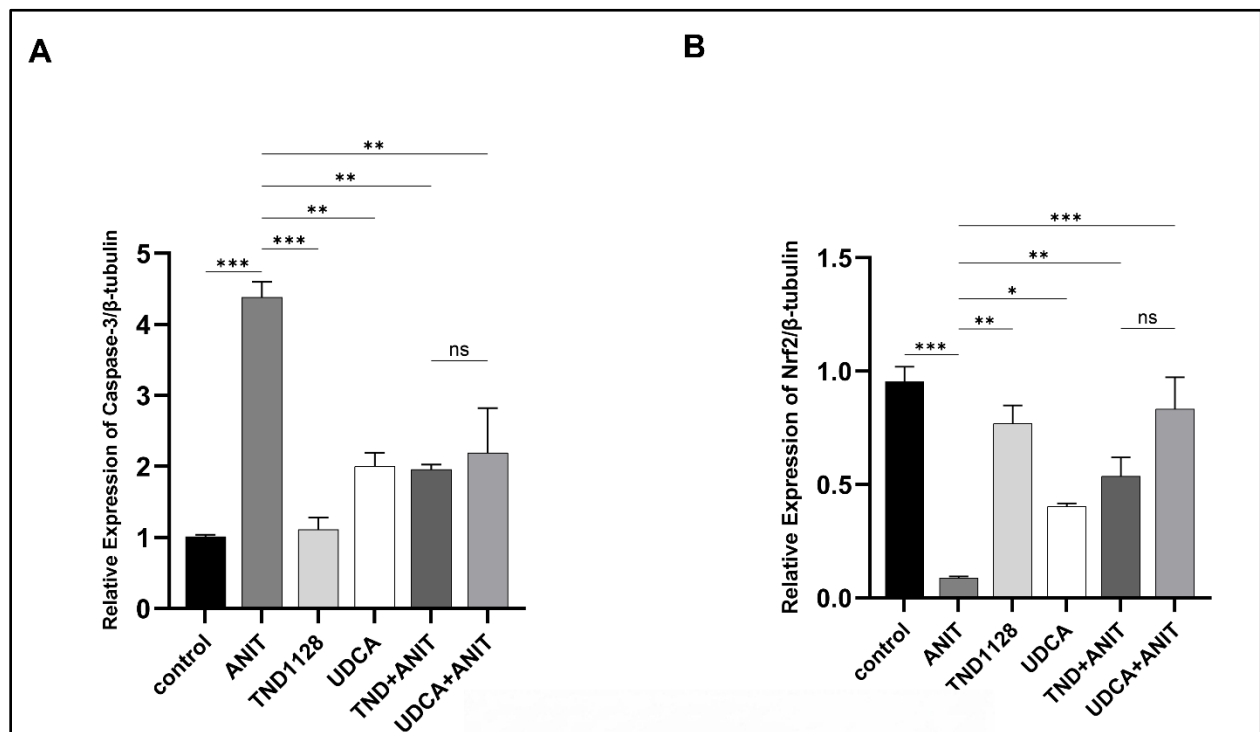


Figure 5. Effect of TND1128 and UDCA on hepatic cleaved caspase-3 and Nrf2 protein expression in rats with ANIT-induced cholestasis. (A) Cleaved caspase-3 protein expression normalized to β -tubulin. (B) Nrf2 expression normalized to β -tubulin. Data are presented as mean $\pm$ SD ($n = 6$). Statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; ns = non-significant.

3.5. Effect of TND1128 on hepatic FXR and BSEP gene expression

qRT-PCR analysis revealed that ANIT administration significantly downregulated hepatic FXR ($p = 0.0052$) and BSEP mRNA expression ($p = 0.0001$) compared with the control group. TND1128 pretreatment did not produce a statistically significant change in FXR and BSEP

expression relative to the ANIT group ($p > 0.05$). Similarly, UDCA did not significantly alter FXR expression compared with the ANIT group ($p > 0.05$). However, UDCA significantly restored BSEP mRNA expression ($p < 0.01$). These findings suggest that the hepatoprotective effects of TND1128 may not be mediated through the modulation of the FXR/BSEP signaling pathways (Figure 6).

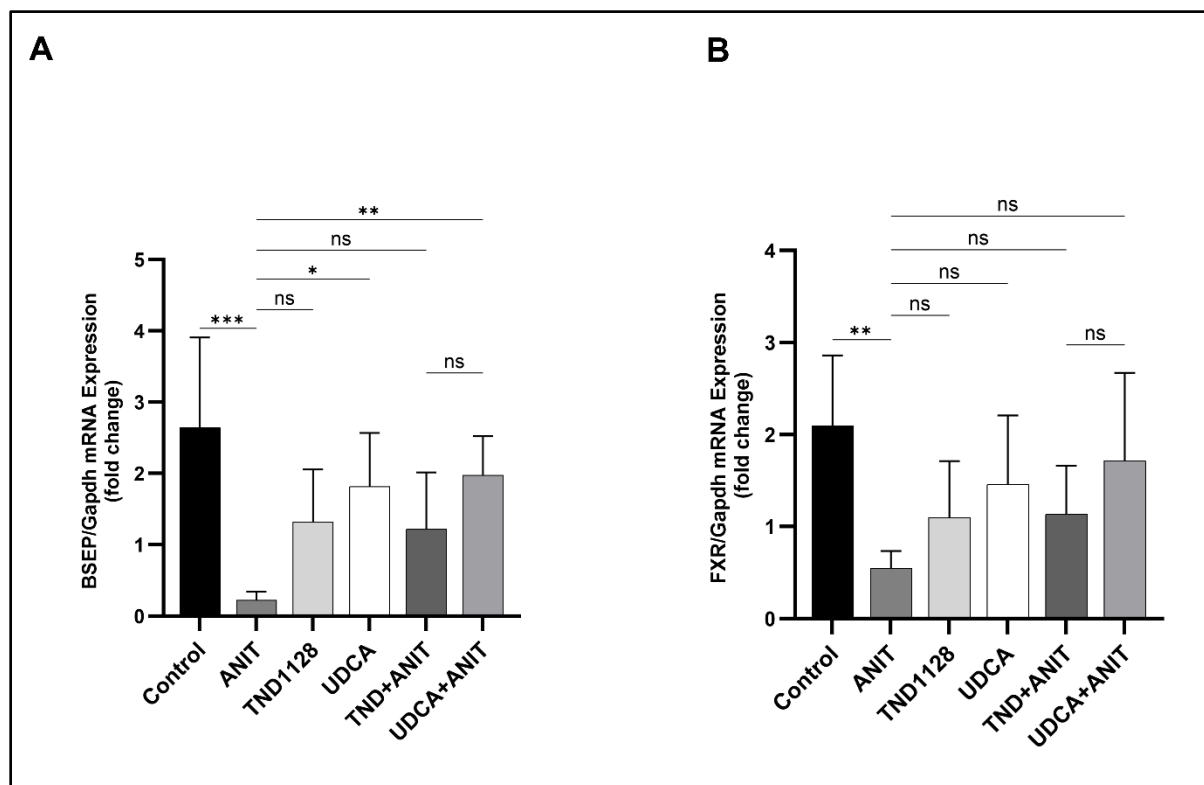


Figure 6. Effect of TND1128 and UDCA on hepatic BSEP and FXR mRNA expression in ANIT-induced cholestasis in rats. Data are presented as mean $\pm$ SD ($n = 6$). Statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; ns = non-significant.

3.6 Histopathological alterations in liver tissue

Histopathological examination of liver sections revealed well-preserved hepatic architecture in the control group, characterized by intact central veins, regularly arranged hepatic cords, normal hepatocyte morphology, and preserved sinusoidal spaces (Figure 7A). In contrast, the ANIT-treated group exhibited severe hepatic injury, including marked portal triad thickening, extensive vascular congestion, sinusoidal dilatation, and prominent inflammatory cell infiltration, periportal and pericentral hepatocellular necrosis, and disruption of the hepatic cords (Figure 7B).

Administration of TND1128 alone preserved near-normal hepatic architecture comparable to that of the control group, with no evident pathological alterations (Figure 7C), while the UDCA-treated group showed largely preserved liver structure with only minimal vascular changes (Figure 7D). Notably, pretreatment with TND1128 in ANIT-induced rats considerably reduced histological damage, as evidenced by reduced inflammatory infiltration, limited necrosis, and partial restoration of hepatic architecture, although mild sinusoidal congestion persisted (Figure 7E). Similarly, the UDCA + ANIT group demonstrated substantial histological improvement, with near-normal hepatic architecture and only minimal residual pathological alterations (Figure 7F). Semi-quantitative histopathological scoring further supported these observations, showing a significant increase in hepatic injury scores in the ANIT group compared with the control group ($p < 0.0001$). Both TND1128 and UDCA treatments significantly reduced the severity of hepatic lesions, with a significant difference observed between the two treatment groups (Figure 7G).

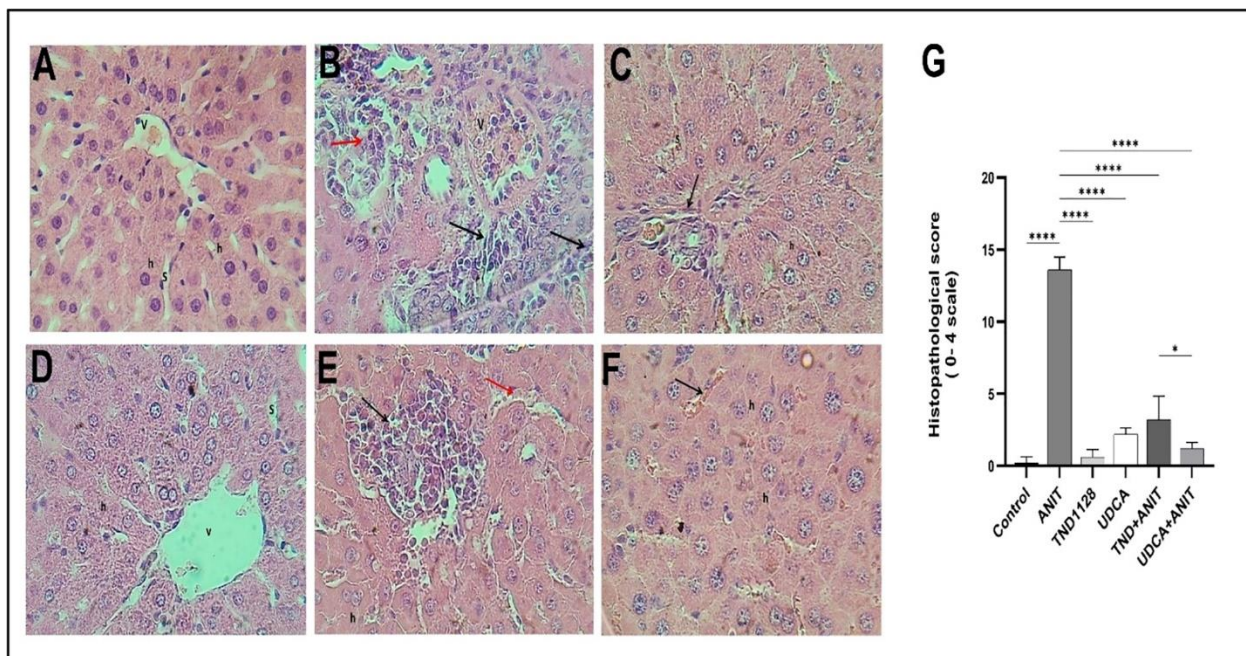


Figure 7. Histopathological evaluation of liver tissue sections stained with H&E (400×). (A) Control group: normal hepatic architecture with intact central vein, well-organized hepatocytes, and preserved sinusoids. (B) ANIT group: severe hepatic injury characterized by hepatocellular necrosis (black arrows), inflammatory cell infiltration and vascular congestion (red arrows). (C) TND1128-treated group: preserved hepatic architecture. (D) UDCA treated group: near-normal hepatic structure. (E) TND1128 + ANIT group: significant histological improvement with mild hepatic structure. (F) UDCA + ANIT group: near-normal hepatic structure.

sinusoidal congestion (red arrows). **(F)** UDCA + ANIT group: marked histological improvement. **(G)** Semi-quantitative histopathological scoring highlights the amelioration of hepatic lesions following TND1128 and UDCA treatment.

4. Discussion

Although UDCA remains the first-line therapy for cholestatic liver disease, some patients show only a partial therapeutic response.³⁷ Therefore, the development of more effective medications for intrahepatic cholestasis is needed. ANIT is a well-established experimental model of intrahepatic cholestasis and reproduces several biochemical and pathological features of cholestatic liver injury.

In the present study, TND1128 considerably decreased the ANIT-induced elevations in ALT, AST, ALP, TBA, and TSB, suggesting a hepatoprotective effect against liver injury.

Inflammation represents an important component of ANIT-induced hepatic injury. The reduction of NF- κ B and IL-6 following TND1128 treatment is consistent with previous studies showing that attenuation of oxidative stress leads to downregulation of NF- κ B activation and subsequent reduction in cytokine release, including IL-6 and other pro-inflammatory cytokines in cholestatic models.³⁸⁻⁴¹ Mechanistically, the anti-inflammatory effect of TND1128 may be attributed to its upstream effect on oxidative stress. ROS are well-established activators of NF- κ B signaling, thereby linking oxidative stress to inflammatory cascade activation in cholestatic liver injury.^{6,40,42} Oxidative stress is closely associated with the progression of cholestatic liver injury. The increase in Nrf2 expression following TND1128 treatment may reflect restoration of the endogenous antioxidant defence system and redox homeostasis.^{43,44} This aligns with the findings of Wang et al. (2026), who reported that amelioration of ANIT-induced cholestasis is associated with activation of Nrf2 signalling.⁴⁵ In agreement with previous studies⁴⁶⁻⁴⁸, the reduction in MDA levels and restoration of hepatic GSH content following TND1128 treatment suggest potent antioxidant activity that may be associated with enhanced Nrf2-mediated antioxidant defences.

The reduction in caspase-3 expression following TND1128 treatment may be explained by the established role of oxidative stress and mitochondrial dysfunction in triggering hepatocyte apoptosis during cholestatic liver injury.^{6,49} The anti-apoptotic effect of TND1128 is therefore

likely secondary to its upstream effects on redox balance and pathways associated with mitochondrial dysfunction, supporting a mechanism involving preservation of antioxidant defense and inhibition of apoptosis.^{22,50}

An important finding of the present study concerns the FXR/BSEP pathway. ANIT significantly reduced hepatic FXR and BSEP expression, indicating disruption of bile acid homeostasis. However, TND1128 did not produce a statistically significant restoration in the expression of either FXR or BSEP, suggesting that its hepatoprotective effects are unlikely to be mediated through direct modulation of FXR/BSEP signaling axis. Instead, the beneficial effects of TND1128 appear to result primarily from attenuation of oxidative stress, inflammation, and apoptosis. This is consistent with previous reports suggesting that recovery of bile acid regulatory pathways may occur secondary to reduced hepatic injury rather than direct activation of FXR signaling.⁵¹

The findings of the present study are consistent with those reported by Rasheed and Khudhair (2026), who demonstrated that TND1128 exerts significant cytoprotective effects through attenuation of oxidative stress, suppression of NF- κ B-mediated inflammation, and inhibition of caspase-3 activation. Their cardiotoxicity model also revealed preservation of tissue architecture and mitochondrial integrity, which closely parallel the hepatoprotective effects observed in the current study. This cross-organ consistency suggests that the protective mechanism of TND1128 may be mediated through conserved redox-dependent and mitochondrial-associated mechanisms rather than tissue-specific pathways.²⁵

The current findings also agree with previous studies investigating hepatoprotective agents in ANIT-induced cholestasis. For instance, papaverine has been reported to significantly attenuate cholestatic liver injury through modulation of oxidative stress, inflammatory signaling, and FXR signaling pathways. Similar to TND1128, papaverine restored antioxidant markers such as GSH and enhanced Nrf2 expression. However, while papaverine acts primarily as a direct FXR agonist, the effects observed in the present study suggest that TND1128 may exert its protective action indirectly through redox modulation and mitochondrial stabilization, which may contribute to improvement in cellular redox balance and hepatocellular protection.²⁶

Interestingly, the protective effects observed in the current study differ from earlier reports evaluating UDCA in drug-induced cholestasis models. Hillstrom et al. (1992) reported no significant improvement in biochemical or survival outcomes following UDCA administration in ANIT-induced and other drug-induced cholestasis.⁵² These discrepancies may be attributed to

differences in the experimental models and mechanisms of action. Although both UDCA and TND1128 demonstrated protective effects against oxidative, inflammatory, and apoptotic injury in the present study, their effects on bile acid regulatory pathways appeared to differ. UDCA significantly restored BSEP expression, whereas TND1128 did not significantly alter BSEP expression, suggesting that the two treatments may share some downstream protective mechanisms while differing in their modulation of bile acid-related pathways.

Conversely, more recent studies have demonstrated hepatoprotective effects of UDCA in oxidative stress-driven liver injury models, including carbon tetrachloride-induced hepatotoxicity. In these models, UDCA has been shown to improve antioxidant status, suppress inflammatory responses, and preserve liver architecture. These observations partially align with the current study, particularly regarding the reduction of oxidative stress and inflammation. Nevertheless, variability in UDCA efficacy among different models suggests that its protective effect may be context-dependent.

The structural improvements following TND1128 treatment are consistent with the observed biochemical and molecular findings, particularly the reduction in oxidative stress, inflammatory signaling, and apoptosis. Such findings are consistent with previous studies showing that restoration of liver histology in cholestatic models is closely associated with attenuation of oxidative stress, inflammation, and apoptosis.^{42,48}

Study limitations

This study has several limitations. First, the experimental design employed an acute prophylactic model, which may not fully reflect the pathophysiological features of chronic cholestatic conditions. Second, mitochondrial function was not directly evaluated despite its proposed role in the mechanism of action of TND1128. Third, only a single dose of TND1128 was investigated, limiting assessment of dose–response relationship. Therefore, future studies should include chronic disease models, detailed evaluation of mitochondrial function, and multiple dosing strategies to better define the pharmacological profile and therapeutic potential of TND1128.

Conclusion

TND1128 demonstrated significant hepatoprotective effects against ANIT-induced intrahepatic cholestasis, as evidenced by improvement in biochemical markers of liver injury, attenuation of

oxidative stress, suppression of inflammatory and apoptotic pathways, and enhancement of endogenous antioxidant defense. Notably, these protective effects were not accompanied by significant changes in FXR or BSEP expression under the present experimental conditions, suggesting that modulation of redox balance, inflammation, and apoptosis may contribute to the observed hepatoprotective effects.

Authorship contribution statement

Conceptualization: Athraa Lateef Khaleel, Alaa Radhi Khudhair

Methodology: Athraa Lateef Khaleel, Alaa Radhi Khudhair

Investigation: Athraa Lateef Khaleel

Data curation: Athraa Lateef Khaleel

Formal analysis: Athraa Lateef Khaleel

Visualization: Athraa Lateef Khaleel

Writing – original draft: Athraa Lateef Khaleel

Supervision: Alaa Radhi Khudhair

Validation: Alaa Radhi Khudhair

Writing – review & editing: Alaa Radhi Khudhair

Project administration: Alaa Radhi Khudhair

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Ethics statement

All animal experimental procedures were approved by the Local Ethics Committee of the College of Pharmacy, University of Baghdad (Approval No. RECAUBCPR1020250615A) and were performed in accordance with institutional guidelines for the care and use of laboratory animals and ARRIVE recommendations.

Declaration of Generative AI and AI-assisted Technologies in the Writing Process

During the preparation of this manuscript, the authors used ChatGPT (OpenAI) for language editing and improving the clarity and readability of the text. The authors subsequently reviewed and edited all AI-assisted content and take full responsibility for the accuracy, integrity, and originality of the final manuscript.

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