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Papaverine attenuates the progression of alpha naphthylisothiocyanate induce cholestasis in rats

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ABSTRACT

Cholestasis is a hepatobiliary condition that manifests as acute or chronic and results from disruptions in the bile flow, formation, or secretion processes. The Farnesoid X receptor (FXR) is a vital target for the therapy of cholestasis since it regulates BA homeostasis. Despite the discovery of multiple active FXR agonists, there are still no effective treatments for cholestasis. Papaverine is identified as an FXR agonist.This study investigates papaverine's efficacy and probable mechanism in protecting against alpha naphthylisothiocyanate (ANIT) induced cholestasis. Thirty male albino rats were divided into three groups, each with ten rats. Group I (control) rats were administered 1 mL/kg corn oil 48 h before sacrifice; group II rats were orally administered 100 mg/kg ANIT. Group III received a 200 mg/kg dosage of papaverine over seven consecutive days. A single dose of ANIT at a concentration of 100 mg/kg was orally administered on the fifth day; group II and III animals were euthanized 48 h after inducing cholestasis, and serum concentrations of liver function tests and total bile acid (TBA) were measured. Besides measuring the inflammatory mediator's tumor necrosis factor-alpha (TNF-α) and interleukin 1 (IL-1β), antioxidant markers such as superoxide dismutase (SOD) and glutathione (GSH) were also assessed. The findings indicated the enhancement in the liver function test and total bile acids, as well as in liver histology; papaverine significantly lowered TNF-α and IL-1β while SOD and GSH significantly increased. Additionally, papaverine upregulates *Fxr* gene expression, bile salt export pump (*Besp*), small heterodimer partner (*shp*), hepatocyte nuclear factor 1α (*Hnfα*), nuclear factor erythroid 2-related factor (*Nrf2*), heme oxygenase (*Ho-1*), NAD (P)H quinone oxidoreductase 1 (*Nqo1*). Furthermore, papaverine increased protein expressions of Sirtuin1.

(SIRT 1), FXR, HO-1, and BSEP levels in the rats' livers. The protective effects of papaverine may be attributed to the activation of FXR signaling pathways. These findings revealed that papaverine protects against ANIT-induced Cholestasis.

1. Introduction

The liver is the primary site for the metabolism of drugs and toxicants, and it is the biggest organ in the body, accounting for about 2 % of adult body weight; the liver is the organ most vulnerable to the effects of toxic chemicals (Rusyn et al., 2022; Ahmad and Kathem, 2021). One of the most common hepatology consultations is cholestasis, caused by abnormal bile production, secretion, or flow through the biliary tract; elevated alkaline phosphatase and gamma-glutamyl transferase levels define Cholestasis (José et al., 2020). The Food and Drug Administration approved three medications: bezafibrate, ursodeoxycholic acid, and obeticholic acid For the treatment of cholestasis (Zou et al., 2021). Bile acids are formed in the liver from cholesterol and secreted as bile into the duodenum; bile acids aid in absorbing fat, cholesterol, and fat-soluble vitamins and act as ligands for bile acid receptors (Bertolini et al., 2022). Specific bile acid metabolites activate FXR; FXR directly activates transcription of genes encoding small heterodimer partner (*Shp*) in the liver and fibroblast growth factor 15 (*Fgf15* in mice; *Fgf19* in humans) in the intestine, both of which repress the expression of enzymes involved in hepatic bile acid synthesis, such as cytochrome P450 7A1 (*Cyp7a1*) (Sun, 2021; Al-Khfajy et al., 2018).

Alpha-naphthylisothiocyanate (ANIT) is a hepatotoxin known to cause intrahepatic cholestasis by selectively damaging bile duct epithelial cells; these cells attract neutrophils, which then damage the hepatocytes, resulting in intrahepatic cholestasis, Acute ANIT Hepatotoxicity is characterized by necrosis of bile duct epithelial cells, cessation of bile flow, hepatic parenchymal cell injury, and hyperbilirubinemia,

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Abbreviations

ALP	alkaline phosphatase
ALT	alanine aminotransferase
ANIT	Alpha-naphthylisothiocyanate
ARE	antioxidant response element
AST	aspartate aminotransferase
B. A	bile acids
BCA	Bicinchoninic acid
BSEP	bile salt export pump
CA	cholic acid
CMV	cytomegalovirus
Fgf	fibroblast growth factor
FXR	farnesoid X receptor
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase
GGT	gamma-glutamyltransferase
GSH	glutathione
H&E	Hematoxylin and Eosin
HIV	immunodeficiency virus
HMGB1	High Mobility Group box 1
Hnf1α	hepatocyte nuclear factor 1α
Ho-1	heme oxygenase
IL-1β	interleukin 1beta
Keap1	Kelch-like ECH-associated protein
MDA	Malondialdehyde
Mrp2	multidrug-resistant associated protein 2
Na3VO4	sodium orthovanadate
NFκβ	nuclear factor κβ
Nqo1	NAD(P)H quinone oxidoreductase 1
Nrf2	nuclear factor erythroid 2-related factor
Ntcp	Na^{+}-taurocholate cotransporting polypeptide
PBS	phosphate buffer saline
Oatp1b2	Organic anion transporting polypeptides
PMSF	phenylmethylsulfonyl fluoride
PPV	Papaverine
PVDF	polyvinylidene difluoride
RAGE	receptor of advance glycation end product
SDS	sodium dodecyl sulphate
Shp	small heterodimer partner
SRIT1	Sirtuin1
SOD	Superoxide dismutase
TBA	Total bile acid
T.BIL	total bilirubin
TBST	Tris-buffered saline with Tween 20
TNF-α	tumor necrosis factor-alpha
UDCA	ursodeoxycholic acid

the administration of ANIT to experimental animals, such as rats, mice, and guinea pigs, can generate an accurate model of intrahepatic cholestasis and hepatic damage in humans (Waters et al., 2001; Li et al., 2019).

Papaverine is an alkaloid isolated from *Papaver somniferum* and *Rauwolfia serpentifera*; papaverine is a benzylisoquinoline-alkaloid used as an antispasmodic drug and to treat impotence (approved by the US Food and Drug Administration) (Gaber et al., 2020). Papaverine is identified as an FXR agonist (Hiebl et al., 2018). Papaverine enhances cerebral blood flow by immediate vasodilatory activity on cerebral blood vessels, which may be linked to its capacity to block phosphodiesterases and calcium channels (Sayama et al., 2006). Additionally, papaverine plays a role in pulmonary edema through its vasodilator effects on the major blood vessels, particularly the coronary and pulmonary arteries (Trejo et al., 2007). Papaverine is categorized as an antiarrhythmic drug because it decreases conduction and prolongs the refractory period (Karagueuzian et al., 2017). Furthermore, papaverine has demonstrated promising antiviral properties against the respiratory syncytial virus, cytomegalovirus CMV, measles, and human immunodeficiency virus HIV (Nokta et al., 1993). This study aims to investigate the potential protective effects of papaverine on ANIT-induced cholestasis in rats.

2. Material and methods

Ethics Approval

The University of Baghdad/College of Pharmacy Animal Research Local Ethics Committee accepted this protocol (protocol number 2006 on 15-2-2022).

2.1. Animals

Thirty male Wistar rats weighing 100-150gm at 4–6 weeks were supplied from the College of Pharmacy/Baghdad University. Animals were housed in an air-conditioned room at 25 ± 2 C^{o} with a 12/12 h light/dark cycle with free food and water. Animals were acclimatized for one week before treatment.

2.2. Chemicals and supplies

Papaverine (PPV) and ANIT were purchased from Sigma Aldrich (St Louis, USA). Biochemical kits for alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TBIL), and alkaline phosphatase (ALP) were obtained from Linear Chemicals SLU(Spain). A total bile acid (TBA) colorimetric assay kit was purchased from Elabscience (Houston, USA).

The Elisa kits for tumor necrosis factor-alpha (TNF-α) and interleukin 1 (IL-1β) were purchased from Elabscience (Houston, USA). Superoxide dismutase (SOD), glutathione (GSH), malondialdehyde (MDA), and gamma-glutamyl transferase(GGT) Elisa kits were obtained from MyBioSource (USA). Genezol reagents for RNA extraction were purchased from Geneaid (South Korea). Goscript® Reverse Transcription System RT-qPCR System and Go Taq® Master Mix were purchased from Promega (Madison, USA). PCR primers for farnesoid X receptor (*Fxr*), bile salt export pump (*Bsep*), small heterodimer partner (*Shp*), hepatocyte nuclear factor 1α (*Hnf1α*), nuclear factor erythroid 2-related factor (*Nrf-2*), heme oxygenase (*Ho-1*), NAD(P)H quinone oxidoreductase 1 (*Nqo1*), and Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) primer were synthesized and purchased from Integrated DNA Technologies (IDT, Iowa, USA). Antibodies against BSEP (MBS3009723), FXR (MBS2032490), and HO-1 (MBS2538072) were obtained from MyBioSource(USA). Antibodies against SIRT1 (E-AB-32901) and beta-actin (E-AB-40338) were provided by Elabscience (Houston, USA).

2.3. Experimental cholestasis induction and treatment

The animals were divided into three groups, and each group had ten rats: (I) control group rats received 1 mL/kg of corn oil orally via oral gavage 48 h before sacrifice; (II) ANIT (Cholestasis) group: oral administration of 100 mg/kg (Uchida et al., 2002) ANIT via gavage 48 h before sacrifice, 100 mg of ANIT dissolved in 1 ml of corn oil. (III) cholestasis + Papaverine group: Papaverine was administered (200 mg/kg) (Aneja et al., 2004) via oral gavage for seven consecutive days at day five ANIT (100 mg/kg) given orally, papaverine dissolved in water at a concentration of 25 mg/ml. Animals were euthanized 48 h after inducing cholestasis, and liver tissue and blood samples were taken after animal sacrifice.

2.4. Biochemical tests

Biochemical tests were evaluated on serum samples from each animal in all groups; the parameters of ALT, AST, TBA, TBIL, ALP, and GGT were assessed according to the manufacturer's protocol.

2.5. Determination of cytokine level and oxidative stress level

Following the sacrifice of the animals, the liver was extracted and washed in an ice-cold phosphate-buffered saline (PBS) with a pH of 7.4 to remove any remaining blood 1 g of liver tissue sample and 9 ml of PBS with a pH of 7.4 was subsequently prepared. Afterward, the tissue was homogenized with a homogenizer. Then, centrifuge for 10 min in a chilled centrifuge. According to the manufacturing protocol, GSH, SOD, MDA, TNF-α, and IL-1β concentrations were measured in the supernatant.

Briefly, the sample, standards, and blank were added to the pre-coated antibody plate and incubated at 37 °C for 1 h; then, biotinylated-specific antibody, enzyme conjugate, chromogenic substrate, and stop solutions were sequentially added. The optical density at 450 nm was measured by a microplate reader.

2.6. Quantitative real-time Polymerase Chain reaction

RNA was extracted from rat liver tissue using Trizol reagent, as directed by the manufacturer. On a spectrophotometer, the concentration and purity of total RNA were measured at 260 and 280 nm, and 2 μcg of total RNA was reverse transcribed with Go Script Reverse Transcriptase to generate cDNA. The synthesized cDNA was stored at −20 °C for future PCR reactions. The RNA amplification reaction was performed by CFX Opus 96 System Software (Version 2.1, Bio-Rad). The quantity of mRNA was normalized with the expression of *Gapdh,* and all comparison data were calculated using the $2-\Delta\Delta CT$; Table 1 lists the primers used in our study.

2.7. Western blot analysis

Protein samples from hepatic tissue were extracted, and RIPA lysis buffer was added with phenylmethylsulfonyl fluoride PMSF and sodium orthovanadate Na3VO4 in the following ratio: (0.3 g liver:1 ml RIPA lysis buffer: 10 μL of both PMSF and Na3VO4) then the protein content was measured using a BCA Protein Assay Kit (Elabscience, USA), then 10 μL of protein sample added per lane were separated by SDS PAGE gel and gels were transferred into polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5 % fat-free milk at room temperature for 1 h, then incubated overnight at 4 °C with antibodies directed against FXR, SIRT1, BSEP, HO-1, and beta-actin. After that, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Elabscience, dilution: 1:10,000) for 1 h at room temperature. Finally, ECL detection reagents (Elabscience, USA) were used to detect protein expression signals and images by ChemiDoc MP Imaging System (Bio-Rad Laboratories, USA). Densitometric scanning of band intensities was quantified by ImageJ (NIH). Beta-actin was used as an internal control in the analysis of the samples.

2.8. Histopathological Evaluations

The liver tissues were preserved in 4 % formaldehyde in 10 mM phosphate buffer (pH 7.4) for 48 h before histology. Hematoxylin and eosin (H&E) stained tissue slices on glass slides at a thickness of 5 μm were analyzed under a microscope. Slices were checked for anomalies under a microscope.

2.9. Statistical analysis

The data were analyzed with GraphPad Prism 8 (San Diego, CA, USA). Mathematical data was provided as the mean ± standard deviation. ANOVA and Tukey multiple comparison tests were performed. This study regarded the data as significant if the *p*-value was less than 0.05.

3. Results

3.1. Effect of papaverine on liver function tests

Serum AST and ALT levels were significantly higher in the ANIT group compared to the control group, indicating liver injury; in contrast, serum AST and ALT levels were significantly lower in the papaverine group compared to the ANIT group. Fig. 1(C and D) shows that in the cholestasis group, ALP and GGT levels, a biomarker for cholestasis, were significantly higher than in the control group, and the papaverine-treated group maintained much lower levels of ALP and GGT compared to the ANIT-treated group. Total bilirubin and bile acid remarkably increased in the ANIT-treated group and significantly decreased upon papaverine treatment, as shown in Figure (E, F).

3.2. Effect of papaverine on inflammatory marker and oxidative stress

To investigate the alterations in inflammatory biomarkers after papaverine treatment, the levels of serum IL1β and TNFα were examined, and it was observed that these biomarkers exhibited an increase in the ANIT group. However, the administration of papaverine resulted in a considerable reduction in these markers, as shown in Fig. 2 (A, B).

One of the features of cholestatic liver damage is oxidative stress. Antioxidant parameters such as SOD and GSH levels were measured, and the results showed that ANIT-treated groups had significantly lower GSH and SOD levels. In contrast, treatment with papaverine could successfully raise them, as shown in Fig. 2 (D, E). MDA is a biomarker of lipid peroxidation, and its level remarkably increased in the cholestasis group, while papaverine treatment reduced it.

3.3. Papaverine activated FXR signaling pathway in cholestasis rats

Because adaptive regulation has been postulated as a mechanism to limit hepatotoxicity, the expression of *Fxr* was determined. Compared to the control group, the mRNA expression of *Fxr* was significantly reduced in rats treated with ANIT, while it was restored in rats treated with papaverine as shown in Fig. 3 (A). These findings suggest that FXR activation may be responsible for the protective effect of papaverine against cholestasis. This study assessed the mRNA of *Hnf1α*, *Shp*, and

Table 1
Primer sequences for real-time PCR assay.

Gene	forward primer (5′–3′)	reverse primer (5′–3′)
Fxr	AGGCCATGTTCCTTCGTTC	CAGCTCCCCGACACTTTTATAG
Hnf-1α	AGAGTCCCTTCATGGCAAC	CTGAGGTTGGTGTCTGTGATC
Shp	GAGTCTTTCTGGAGCCTTGAG	AGGACTTCACACAATGCCC
Besp	CAACGCATTGCTATTGCTCG	GTTCTGGATGGTGGACAAACG
Nrf2	GCTATTTTCCATTCCCGAGTTAC	ATTGCTGTCCATCTCTGTCAG
Ho-1	CTTTCAGAAGGGTCAGGTGTC	TGCTTGTTTCGCTCTATCTCC
Nqo-1	CTGGCCAATTCAGAGTGGCAT	GAGTGGTGACTCCTCCCAGA
Gapdh	TTCCAGGAGCGAGATCCCGCTAAC	CATGAGCCCTTCCACGATGCCAAAG

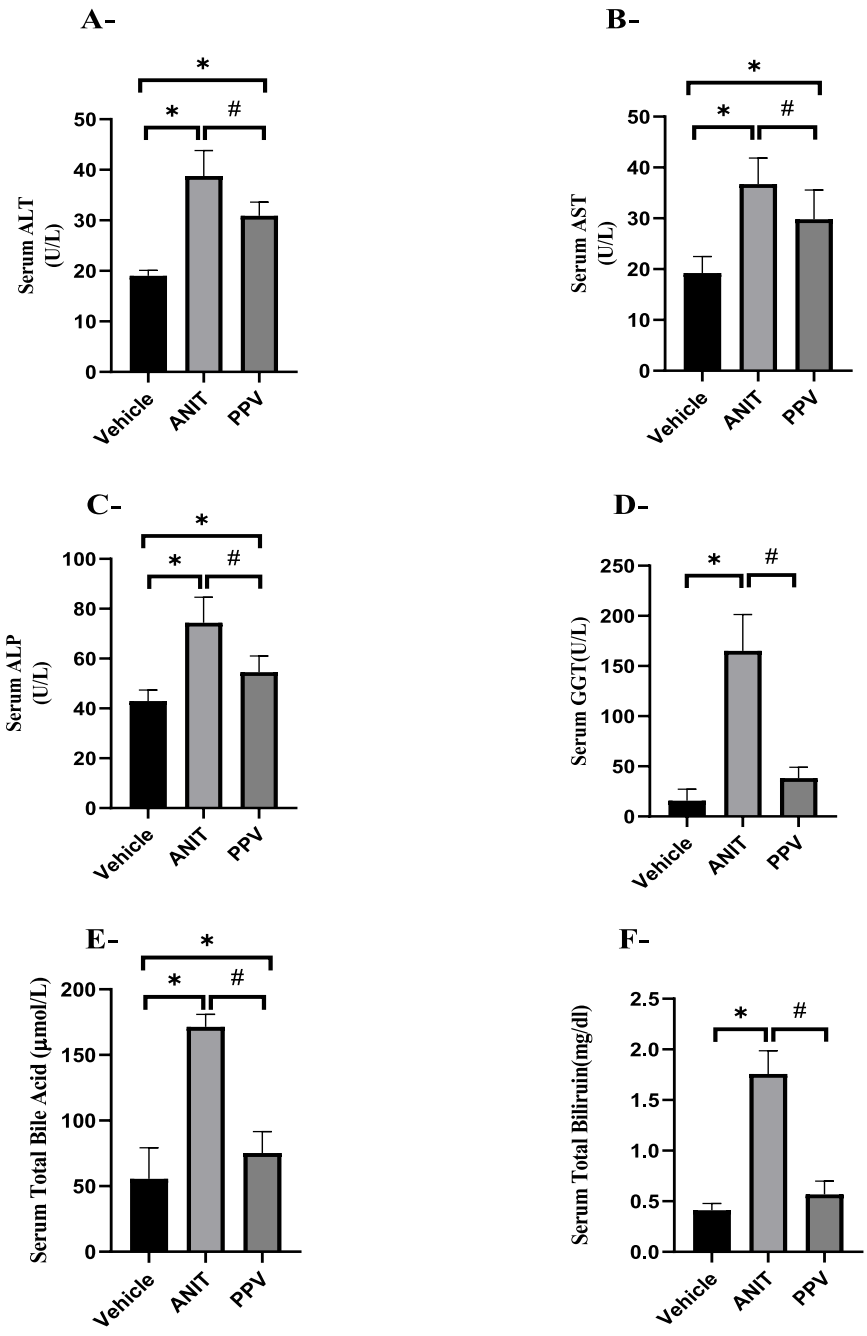

Fig. 1. Level of liver function test parameters (A)ALT,(B)AST, (C)ALP, (D)GGT, (E)Serum total bile acid, and (F)Serum total bilirubin. Data are the mean ± SD (N = 10)$P < 0.05$ #represent significant differences compared with ANIT -treated group $p < 0.05$ *represent significant differences compared with control $p < 0.05$.

Besp; the mRNA levels of *Hnf-1α*, *Shp*, and *Bsep* were decreased by 85.5 %, 82 %, and 64.8 %, respectively, in the ANIT-treated group, and papaverine significantly increased them as shown in Fig. 3 (B, C, D)

3.4. Papaverine activated Nrf2 signaling pathway in cholestasis rats

Fig. 3 (E, F, G) revealed that papaverine increased mRNA levels of *Nrf2* and its target genes *Ho-1* and *Nqo*. ANIT significantly decreased the mRNA levels of *Nrf2* and *H0-1* but not *Nqo-1*. These results demonstrated the antioxidant properties of papaverine.

3.5. Papaverine upregulated the protein levels of FXR, BSEP, Sirt-1, and HO-1 in rats

Western blotting was used to assess HO-1, SIRT-1, BSEP, and FXR protein levels to verify gene expression results. Fig. 4 (A) indicated that the administration of ANIT reduced the protein expressions of FXR, BSEP, SIRT-1, and HO-1. Conversely, using papaverine led to an elevation in the levels of these proteins, as shown in Fig. 4 (B, C, D, and E).

3.6. Effects of papaverine on liver tissue in experimental cholestasis

To determine structure change in the liver, the liver section in the ANIT group revealed glycogen depletion, neutrophile infiltration,

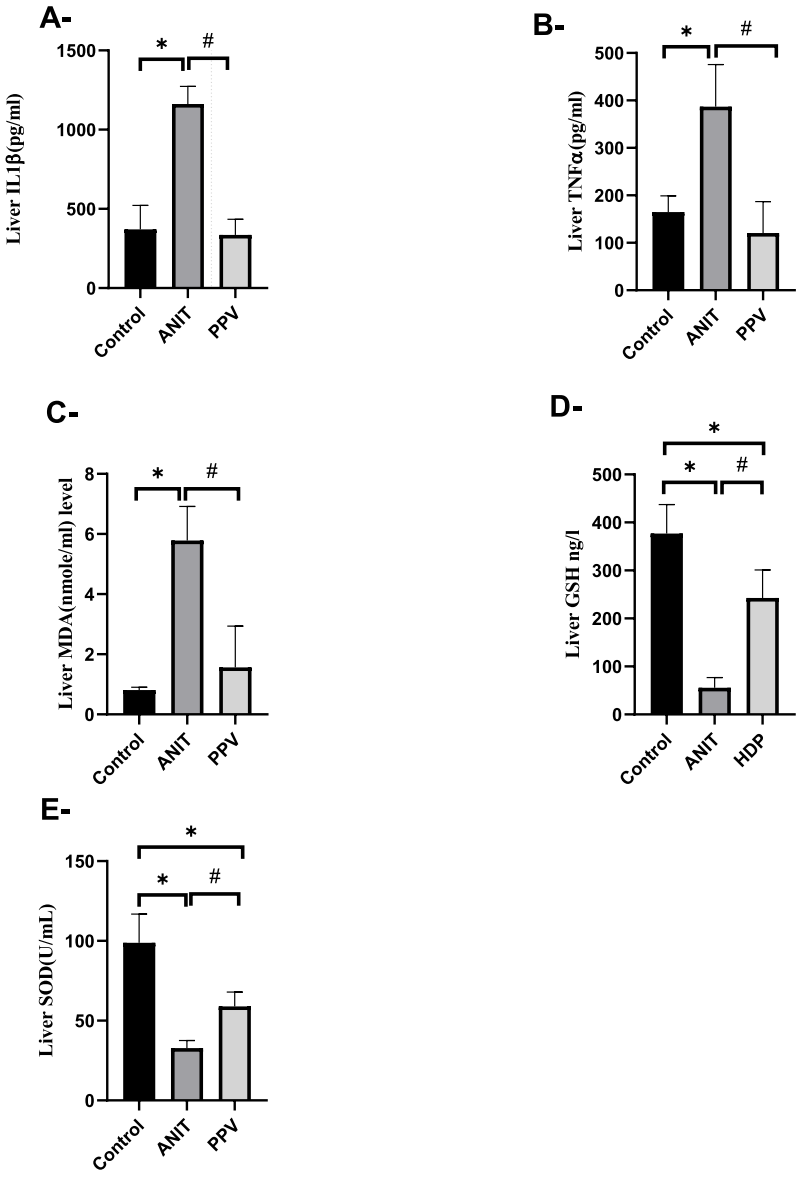

Fig. 2. Level of inflammatory cytokines and some of the oxidative stress parameters (A)IL-1β,(B)TNF-α, (C)MDA, (D)GSH and (E)SOD Data are the mean ± SD (N = 10)$P < 0.05$ #represent significant differences compared with ANIT -treated group $p < 0.05$ *represent significant differences compared with control $p < 0.05$.

bilirubin pigment deposit, and apoptosis, as shown in Fig. 5. However, liver injury was alleviated upon papaverine treatment (see Fig. 6).

4. Discussion

There is no effective medical treatment for intrahepatic cholestasis at this time; even though ursodeoxycholic acid (UDCA) is the standard treatment for cholestatic disorders, most patients exhibit only a partial response; therefore, it is necessary to develop an efficacious drug for intrahepatic cholestasis (Ou et al., 2016). In hepatocytes, ANIT is detoxified by conjugation with glutathione (GSH); the multidrug-resistant associated protein 2 (Mrp2) transports the ANIT-GSH conjugate into the bile, where The released ANIT specifically damages bile-duct epithelial cells, leading to cholangitis and intrahepatic cholestasis (Tanaka et al., 2009). ANIT can block bile flow, accumulating highly concentrated bile acids and other bile components in the liver, causing severe duct epithelial apoptosis or necrosis and hepatobiliary toxicity (Ding et al., 2012). AST and ALT are enzymes in hepatocytes and are released into the bloodstream in response to hepatocyte injury or death (Newsome et al., 2018).

Bile duct enzymes ALP and GGT are typical biochemical indicators of cholestasis; although blood ALP levels may rise in various disorders, GGT levels are nearly exclusively associated with the liver. Increased blood levels of these enzymes are most likely induced by bile acids, which function as detergents, causing the release of enzymes from the plasma membranes of hepatocytes as they accumulate within the liver on the intracellular and biliary membranes (Lu, 2022).

When ANIT causes cholestasis, the hepatocytes are damaged, bile acid cannot be reabsorbed efficiently from the gastrointestinal lumen, and it cannot be effectively drained, resulting in a higher TBA concentration in serum. (Cui et al., 2009). Unconjugated bilirubin is the product of hemoglobin catabolism conjugated by the liver and eliminated in the bile; cholestasis is frequently accompanied by conjugated hyperbilirubinemia (Pollock and Minuk, 2017).

This study showed pretreatment with papaverine significantly decreased the elevation of serum ALT, AST, ALP, GGT, TBIL, and TBA

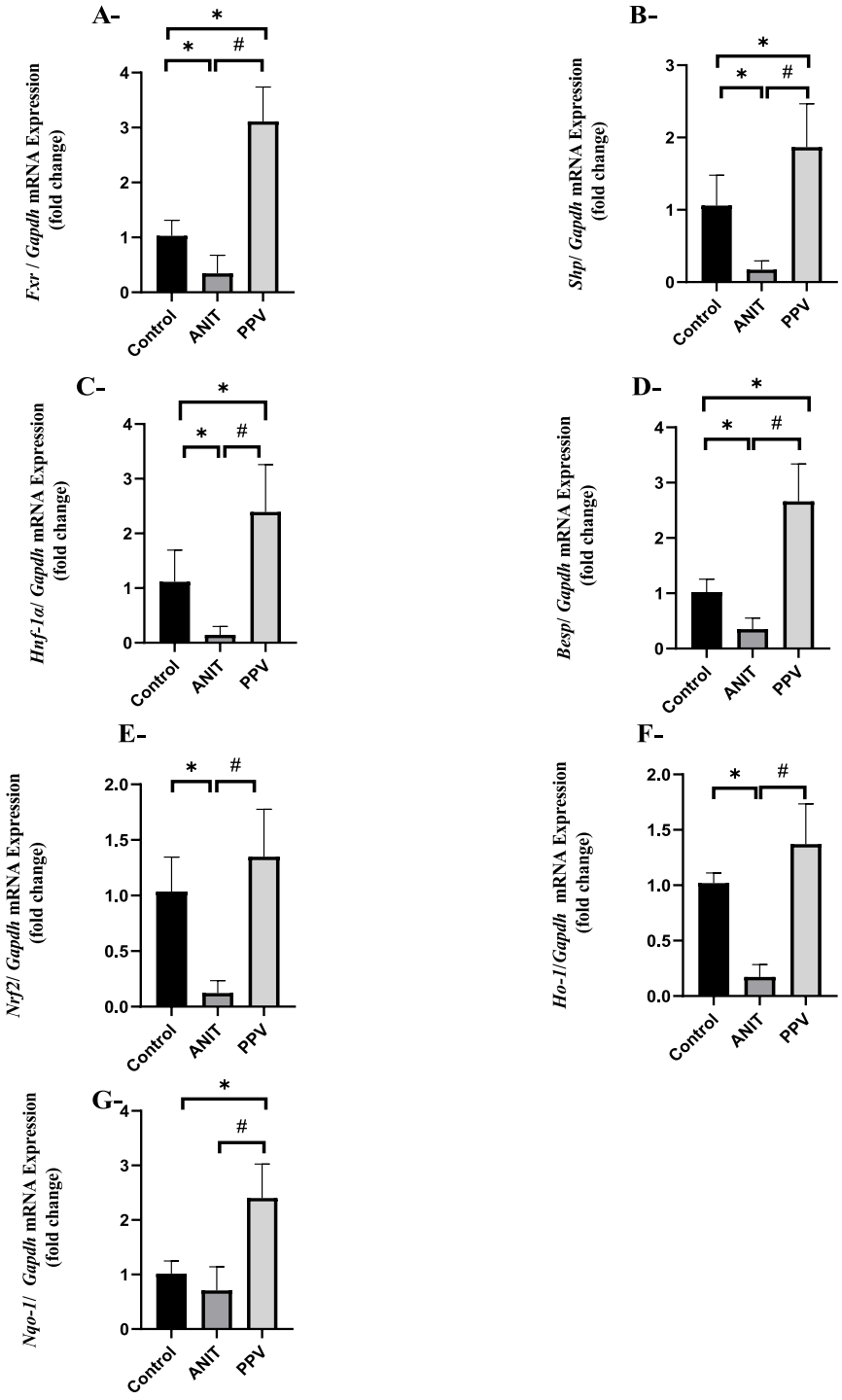

Fig. 3. Papaverine affected bile acid-related gene expression in rat livers. The levels of gene expression were determined by quantitative real-time PCR analysis. (A) *Fxr* (B) *Shp* (C) *Hnf1α* (D) *Besp* (E) *Nrf2* (F)*Ho-1*(G)*Nqo1*. Data are the mean ± SD (n = 6). *$P < 0.05$ versus vehicle; #$P < 0.05$ versus ANIT group.

levels induced by ANIT; furthermore, pathological injuries were reversed after papaverine treatment. These results indicate that papaverine is one of the medications that can be used to prevent ANIT-induced liver damage with cholestasis.

The FXR receptor primarily functions as a receptor for bile acids; bile acid metabolites trigger the FXR receptor. FXR directly initiates the transcription of genes encoding *shp* in the liver and fibroblast growth factor in the intestine. *shp*, and *Fgf,* in turn, suppress the expression of enzymes involved in synthesizing bile acids in the liver, such as cytochrome P450 7A1 (CYP 7A1). Furthermore, the activation of hepatic FXR directly impacts the upregulation of the *Abcb11* gene, which is responsible for encoding the *BSEP* (Sun, 2021).

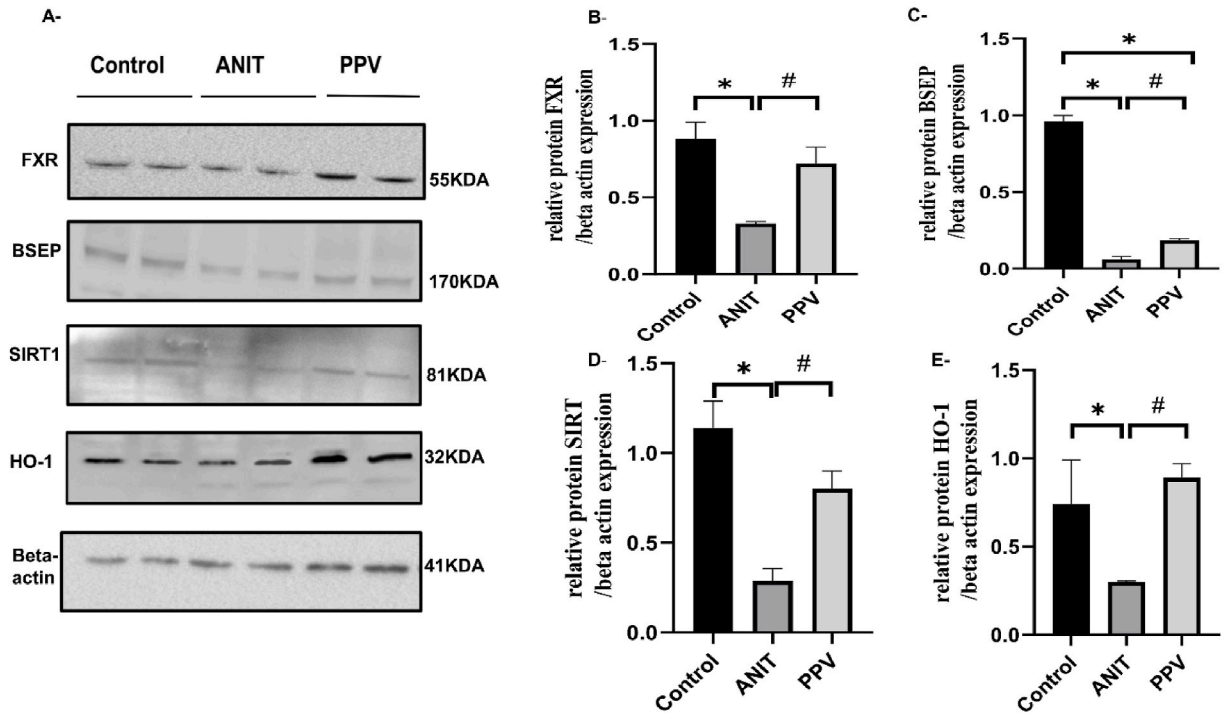

Fig. 4. Effect of Papaverine on FXR, BSEP, SIRT1, and HO-1 protein expressions in ANIT-induced cholestatic liver injury in rats. (A) Western blot images of FXR, BSEP, SIRT1, and H0-1 (B) relative protein expression of FXR; (C) relative protein expression of BSEP; (D) relative protein expression of SIRT1(E) relative protein expression of HO-1 Data were expressed as mean ± SD $^{\#}P < 0.05$ compared with the ANIT group; * $P < 0.05$ compared with the control group.

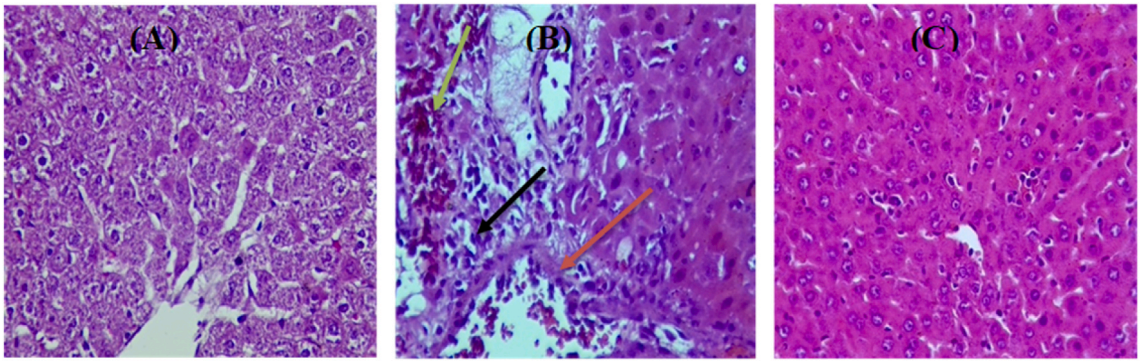

Fig. 5. The effect of papaverine on histological changes of liver tissue in the ANIT-induced cholestasis in rats. Image of H and E stained liver sections (40x magnification)at 48 h after ANIT administration was shown. (A) control group: no histological change was observed (B) ANIT group: sinusoidal congestion was marked by a red arrow, and the neutrophile infiltration was marked by a black arrow and bilirubin deposit marked by a yellow arrow (C) papaverine treated group: histological structure looked normal.

Sinal et al. demonstrated that fortifying the diet with 1 % cholic acid increased mortality in Fxr knock-out mice by approximately 30 % on day seven; Serum bile acid levels in *fxr* KO mice were 23-fold higher than in wild-type mice, which may be attributed to the impaired secretion of bile acids, resulting in the accumulation of bile acids within hepatocytes (Sinal et al., 2000). Another study demonstrated animals show *FXR−/−* mice exhibit rapid supersaturation of bile with cholesterol, cholesterol crystal precipitation in the gallbladder, increased hydrophobicity of bile salts, and gallbladder inflammation (Moschetta et al., 2004). Genetic studies revealed several defects in genes involved in FXR signaling pathways in pediatric and adult cholestasis (Petrescu and Demorrow, 2021).

The present study showed that papaverine increases the expression of FXR and FXR signaling pathways. It was demonstrated that papaverine markedly upregulates the mRNA level of *Fxr*, *Shp*, and protein expression of FXR.

Hepatocyte nuclear factors (HNFs) are a group of transcription factors that are primarily expressed in the liver. These factors are crucial in maintaining metabolic homeostasis by controlling the expression of genes involved in glucose, cholesterol, and fatty acid metabolism (Xu et al., 2015). HNF1α was identified as a transcriptional regulator of FXR, and inhibition of HNF1 α led to the downregulation of FXR and gallstone formation (Kinoo et al., 2023). HNF1 α has been referred to as the regulator of regulators. Shih et al. found that the plasma TBA level in HNF1 α knock-out mice was greater than that of normal mice. Since Na^{+}-taurocholate cotransporting polypeptide (Ntcp) and Organic anion transporting polypeptides (Oatp1*b*2), mice were direct targets of HNF's target genes (Shih et al., 2001). Bile acid reabsorption from the bloodstream to hepatocytes is facilitated by Ntcp and Oatp1b2 transporters (Yu et al., 2017). This study shows that ANIT down-regulates *Hnf1α* while papaverine treatment upregulates this gene.

Sirtuin1 (SIRT1) is a nicotinamide adenine dinucleotide-dependent

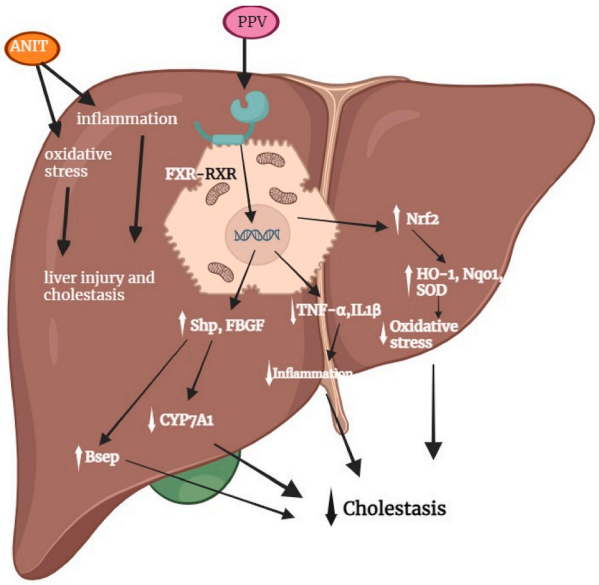

Fig. 6. The hypothetical mechanisms by which papaverine prevents ANIT-induced cholestasis through upregulating FXR, Papaverine upregulate SHP, BSEP, HNF-1α, Nrf2, HO-1and Nqo1. These effects may protect against cholestasis.

protein deacetylase that has been demonstrated to influence various activities, like growth, development, and metabolism (Kulkarni et al., 2016). SIRT1 modulates bile acid metabolism by modulating the expression of the farnesoid X receptor (FXR); SIRT1 impairment in the liver decreases HNF1α recruitment to the FXR promoter and FXR expression, impairing biliary bile acid and phospholipid transport and raising the risk of cholesterol gallstones; liver damage that is caused by 1 % C. A can be reversed by promoting SIRT1 expression with the small-molecule SIRT1 activator SRT1720 (Purushotham et al., 2012). Additionally, SIRT1 inhibits hepatic BA synthesis via the small heterodimer partner (SHP), and by deacetylating FXR, it promotes hepatic FXR/RXR heterodimerization at the FXR response element; it activates the expression of SHP and BSEP (Ma et al., 2021). Our work shows that treatment with papaverine significantly increases the protein expression of SIRT1.

The BSEP protein is the primary transporter for monovalent bile acids, facilitating their transport into the bile canaliculi (Wagner et al., 2009). Mutations in the *Besp* gene have been linked to progressive familial intrahepatic cholestasis type II. Consequently, BSEP plays a crucial role in the biliary excretion of bile acid (Hirano et al., 2005). Our study showed that papaverine increases the protein and mRNA expression of *Bsep* while ANIT treatment downregulates *Bsep* expression.

ANIT causes direct injury to liver cells, facilitating the transmigration of neutrophils from the circulation into the liver via β2-integrins (Kodali et al., 2006). Also, in hepatocytes, the buildup of harmful BAs triggers the activation of nuclear factor κB NFκB, resulting in its translocation to the nucleus and subsequent upregulation of inflammatory mediators such as TNF-α and IL-6 (Kodali et al., 2006).

The High Mobility Group Box 1 (HMGB1) molecule is crucial in initiating inflammation following an injury; in the case of cholestasis, the release of HMGB1 occurs, creating inflammation and developing liver disease; this process is facilitated by the activation of TLR4, which subsequently triggers the synthesis of NFκB, TNFα, and interleukin 6 (IL6); consequently, there has been a growing interest in the development of medicinal approaches aimed at regulating the activity of HMGB1(Nabih & El-kharashi, 2019). The report shows that the opiate alkaloid papaverine, acting as a new receptor for Advanced Glycation Endproducts RAGE inhibitor, directly inhibited HMGB1/RAGE interaction in vitro, ex vivo, and in vivo in the setting of potentially fatal sepsis (Yoshizawa et al., 2021).

It has been reported that FXR downregulates NF-κB, and activating FXR signaling protects against liver injury (Stofan and Guo, 2020).

Our data showed that TNF-α and IL-1β were significantly elevated in the ANIT group. However, papaverine completely reversed this change; therefore, our data suggested that papaverine might suppress inflammation by activating the FXR receptor, which inhibits NFκB. also Papaverine is HMGB1/RAGE inhibitor.

The nuclear factor erythroid 2-related factor 2 (Nrf2) remains in an inactive state within the cytosolic compartment due to the presence of its repressor, known as Kelch-like ECH-associated protein 1 (Keap1). The dissociation of the Keap1-Nrf2 complex occurs due to an increase in reactive oxygen species (ROS) or electrophiles; this dissociation subsequently triggers the activation and translocation of Nrf2 into the nucleus and forms a complex with the antioxidant response element (ARE), leading to the upregulation of phase II detoxification enzymes and antioxidant enzymes such as NAD(P)H: quinone oxidoreductase 1 (Nqo1), glutathione-S-transferases, and heme oxygenase-1(He et al., 2021). A subsequent study demonstrated that mice treated with the Nrf2 activator oltipraz are protected from ANIT-induced histological changes (Tanaka et al., 2009). It has been shown that activation of the Nrf2 signaling pathway contributes to the treatment of cholestatic liver injury by regulating multiple pathways, such as the induction of hepatic phase II metabolic enzymes and BSEP and the reduction of the expression of BA synthesis regulators such as *Cyp7a1* (He et al., 2021).

Glutathione exhibits potent antioxidant characteristics by effectively reducing hydroperoxides, a chemical reaction facilitated by the enzyme glutathione peroxidase (I. Abd Al-Zahra et al., 2017). The decrease of glutathione (GSH) Also leads to lipid peroxidation and compromised activity of antioxidant enzymes (A. Aziz et al., 2017).

Superoxide dismutase (SOD) is an essential antioxidant enzyme that plays a critical role in catalyzing the dismutation reaction of two superoxide anions (O2 -) into hydrogen peroxide and molecular oxygen; this enzymatic process provides a certain degree of protection to the tissue against the harmful effects of superoxide radicals (Ms et al., 2020).

Malondialdehyde (MDA) is an oxidative stress marker that forms as a byproduct of lipid peroxidation(Abdul-Wahab and Al-Shawi, 2020)(R. Mahmood, 2017). MDA has the potential to induce an inflammatory response, resulting in the generation of proinflammatory cytokines, mitochondrial malfunction, and apoptosis (Petagine et al., 2022; Singh et al., 2014).

Papaverine significantly increased the antioxidant parameters GSH and SOD and decreased the lipid peroxidation marker MDA, which may be due to the activation of the Nrf2 signaling pathway. The results of our study suggest that papaverine can alleviate ANIT-induced cholestasis by negatively regulating inflammation, oxidative stress, and activation of the FXR signaling pathway.

5. Conclusion

In conclusion, the findings of this study indicate that papaverine exhibits a protective effect against ANIT-induced cholestatic liver injury in rats. This protective mechanism is likely attributed to the upregulation of the FXR signaling pathway and the inhibition of inflammatory mediators and oxidative stress. Consequently, papaverine shows promise as a potential therapeutic agent for managing cholestatic liver disease.

Credit authorship contribution

Doaa Adnan Atshan: Conducted animal experiment, Analysis and interpretation of data, and Drafted the article. **Munaf H. Zalzala**: Conception and design of the study, Revising the manuscript, and supervising the whole process.

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Declaration of Competing interest

None.

Data availability

The data that has been used is confidential.

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