

Silymarin protects the liver from α -naphthylisothiocyanate-induced cholestasis by modulating the expression of genes involved in bile acid homeostasis

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ABSTRACT

Cholestasis is characterized by impaired bile flow which results in inflammation, cirrhosis, and ultimately liver failure. The current study is aimed to evaluate the anti-cholestatic effect of silymarin against α -naphthylisothiocyanate (ANIT) induced cholestasis. Mice were gavaged with various doses of silymarin or ursodeoxycholic acid (UDCA) for 19 days. Then they were challenged with α -naphthylisothiocyanate (ANIT) and after 48 hours the animals were sacrificed to obtain blood and liver sections. Serum levels of bilirubin, aspartate transaminase (AST), alanine transaminase (ALP), and liver histology were analyzed. mRNA expression of selected transporters (Bile salt export pump (BSEP) and sodium taurocholate cotransporting polypeptide (NTCP)) and proteins (farnesoid x receptor (FXR) and Cytochrome P450 Family 7 Subfamily A Member 1 (Cyp7a1)) involved in bile acids biosynthesis, excretion and uptake were also evaluated by quantitative PCR. The results indicated that the serum levels of bilirubin, AST, and ALP were significantly higher in a cholestatic model group as compared to an untreated control group. However, in silymarin groups, the serum level of these parameters is significantly lower than in a cholestatic model group. Liver histology also showed that silymarin prevents ANIT-induced hepatic injury. mRNA expression of FXR, BSEP, and NTCP was downregulated and expression of Cyp7a1 was upregulated in a cholestatic model group as compared to an untreated control group. However, in silymarin treatment groups, the expression of FXR, BSEP and NTCP was upregulated and the expression of Cyp7a1 was downregulated as compared to the cholestatic model group. In conclusion, silymarin could alleviate hepatic injury by modulating the expression of genes involved in bile acid homeostasis.

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Introduction

Cholestasis is commonly characterized by impaired bile flow (1) and can be caused by genetic disorders or acquired diseases. Genetic disorders involve the mutation in the genes related to the bile formation, while in acquired disease it appears as a side-effect of systemically administered drugs (2-5). Regardless of the causative factors, in all types of cholestasis, the bile acids are piled up in the liver and systemic circulation. Elevated levels of bile acids in the liver result in oxidative stress, inflammation, fibrosis, cirrhosis, and ultimately liver failure (6, 7).

The treatment of cholestasis is limited to ursodeoxycholic acid (UDCA). Though amassing data have proved that UDCA improved cholestatic liver injury (8, 9), several studies have shown that the efficacy of UDCA is inconsistent (10-12). Therefore, alternative therapeutic agents for the treatment of cholestasis should be investigated.

Medicinal plants are rich in nutraceuticals and are used for the treatment of several ailments. *Silybum marianum* (Milk thistle), a well-known medicinal plant, has been traditionally used to cure many liver diseases for nearly 2000 years (13). Silymarin, the potent constituent of this plant, has been reported to possess therapeutic activities

against oxidative injury, fibrosis, inflammation, tumor formation, and atherosclerosis (14, 15). Furthermore, silymarin in combination with UDCA also showed an improvement in hepatic functions of patients with chronic ethylic hepatopathy (16). In this study, we have evaluated the anti-cholestatic potential of silymarin and its effect on the expression of genes involved in bile acids homeostasis. We focused on FXR, a transcription factor regulating the expression of genes involved in the synthesis and transport of bile salts. These include BSEP, NTCP, and Cyp7a1. BSEP transports bile salts from hepatocytes into bile canaliculi while NTCP imports the bile salts into hepatocytes from sinusoidal spaces. Cyp7a1 controls the rate-limiting step in the synthesis of bile salts.

Materials and Methods

Animal models

Swiss albino mice weighing 20-25g were used for the experiments. Mice were raised for 8 weeks in the animal house of the Department of Zoology, Government College University Lahore, Pakistan at standard laboratory conditions (24 ± 1 °C; 12/12 hours dark/light period). They were fed with commercially available feed *ad libitum*. Experi-

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mental approval was taken from the ethical committee of Government College University, Lahore (GCU-IIB-458), and experiments were performed according to the guidelines of the committee.

The animals were randomly divided into five groups (n=6). Group I (G-I): The mice in this group served as control and received saline (0.9% NaCl) for 19 days. On the nineteenth day, they were given olive oil (vehicle). Group II (G-II): The mice in this group served as a cholestatic model group and received saline (0.9% NaCl) for 19 days. On the nineteenth day, they were given orally a single dose of ANIT (75mg/kg dissolved in olive oil (17)). Group III (G-III): The mice in this group served as a low-dose treatment group and orally received silymarin (300mg/kg/day suspended in saline) for 19 days. On the nineteenth day, they were given orally a single dose of ANIT (75mg/kg dissolved in olive oil). Group IV (G-IV): The mice in this group served as a high-dose treatment group and orally received silymarin (600mg/kg/day suspended in saline (18)) for 19 days. On the nineteenth day, they were given orally a single dose of ANIT (75mg/kg dissolved in olive oil). Group V (G-V): The mice in this group served as the UDCA group and orally received UDCA (15 mg/kg/day suspended in saline (18)) for 19 days. On the nineteenth day, they were given orally a single dose of ANIT (75mg/kg dissolved in olive oil). After 48 hours of the ANIT (21st day), the mice were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine via intraperitoneal injection. Blood was collected by cardiac puncture and allowed to clot for 30 minutes at room temperature. Serum was isolated and kept at -20°C until further use. The livers were excised and a portion was placed in 10% formalin for histological studies, while the rest of the liver was snap frozen and kept at -80°C for RNA isolation and gene expression analysis.

Serum biochemical analysis

Serum was subjected to liver function tests (LFTs). Bilirubin, Aspartate aminotransferase (AST), and Alkaline phosphatase (ALP) were measured by using commercially available kits (Randox, UK) following the manufacturer's instructions.

Histological analysis

Liver samples (already suspended in 10% formalin) were washed, dehydrated, and embedded in paraffin. Then paraffin blocks were cut into thin sections (4–6 µm), stained with hematoxylin-eosin (HE), and examined under a

light microscope at 200x magnification.

Quantitative real-time PCR

Liver samples, stored at -80°C, were homogenized for total RNA isolation and cDNA synthesis as described earlier (Malkani, 2020). The purity and concentration of RNA were measured by NanoDrop 2000 (Thermo scientific). cDNA was synthesized by using RevertAid First Strand cDNA Synthesis Kit (Thermo scientific). qPCR was performed with gene specific primers enlisted in table 1 by using Maxima SYBR Green/ROX qPCR master mix (Thermo scientific). GAPDH primers were used for normalization. All reactions were carried out in triplicates.

Statistical analysis

Statistical analysis was performed using SPSS. One-way ANOVA was carried out, followed by Tukey's posthoc test. Data being given as mean ± SD and were considered statistically significant when P < 0.05.

Results

Silymarin and UDCA reduced levels of bilirubin, AST, and ALP in the cholestatic model

Serum bilirubin, AST, and ALP was significantly elevated in the cholestatic model group (G-II) as compared to the control group (G-I) which is an indication of liver injury (Figure 1). The treatment with silymarin (300mg/kg/day (G-III); 600mg/kg/day (G-IV)) significantly lowered the serum bilirubin, AST, and ALP as compared to the cholestatic model group (G-II). The effects of silymarin are comparable to UDCA, which was used as a reference drug in this study.

Silymarin improved liver architecture in the cholestatic model

In the control group (G-I), the liver histology was normal with intact hepatocytes, and normal lobular and sinusoidal spaces (Fig. 2A). In contrast, the cholestatic model group (G-II) showed infiltration of inflammatory cells and dilated sinusoids (figure 2B). The silymarin low as well as high dose groups (G-III and G-IV) exhibited improvement in the liver histology as evident by normal hepatocyte architecture and normal sinusoidal spaces (Fig. 2C-D). Similarly, in the UDCA group (G-V), the liver architecture is similar to that of the control (Fig. 2E).

Table 1. Primers used for qPCR.

Primer ID	Nucleotide Sequence	References
FXR_mouse-F	GCTTGATGTGCTACAAAAGCTG	(19)
FXR_mouse-R	CGTGGTGATGGTTGAATGTCC	
BSEP_mouse-F	TCTGACTCAGTGATTCTTCGCA	
BSEP_mouse-R	CCCATAAACATCAGCCAGTTGT	
NTCP_mouse-F	CAAACCTCAGAAGGACCAAACA	(20)
NTCP_mouse-R	GTAGGAGGATTATTCCTGTTGTG	
Cyp7a1_mouse-F	GAACCTCCTTTGGACAACGGG	
Cyp7a1_mouse-R	GGAGTTTGTGATGAAGTGGACAT	
GAPDH_mouse-F	AGGTCGGTGTGAACGGATTTTG	
GAPDH_mouse-R	GGGGTCGTTGATGGCAACA	

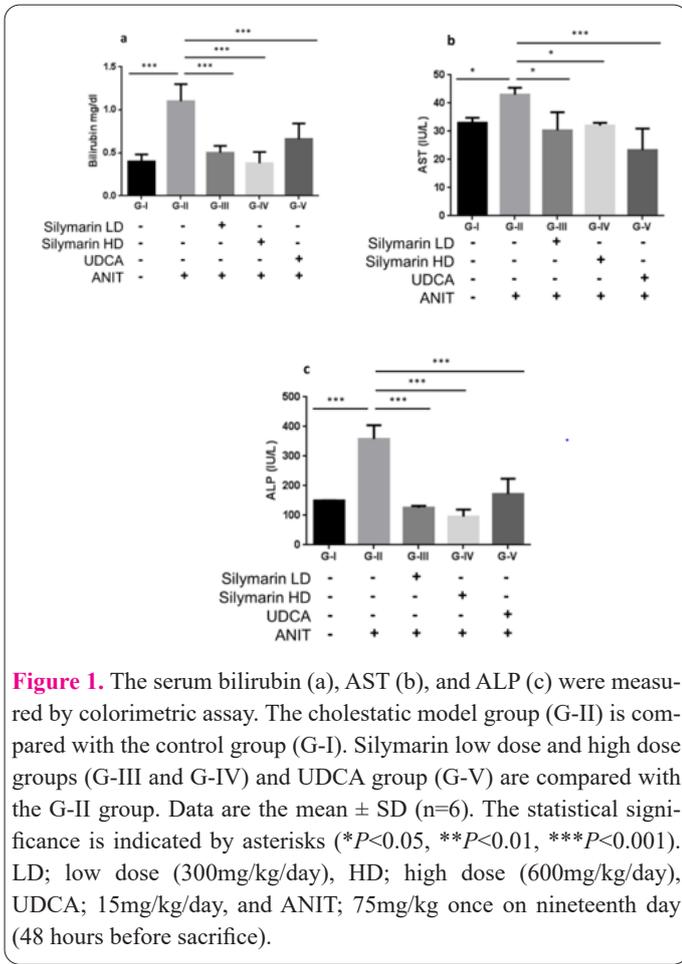


Figure 1. The serum bilirubin (a), AST (b), and ALP (c) were measured by colorimetric assay. The cholestatic model group (G-II) is compared with the control group (G-I). Silymarin low dose and high dose groups (G-III and G-IV) and UDCA group (G-V) are compared with the G-II group. Data are the mean ± SD (n=6). The statistical significance is indicated by asterisks (* P <0.05, ** P <0.01, *** P <0.001). LD; low dose (300mg/kg/day), HD; high dose (600mg/kg/day), UDCA; 15mg/kg/day, and ANIT; 75mg/kg once on nineteenth day (48 hours before sacrifice).

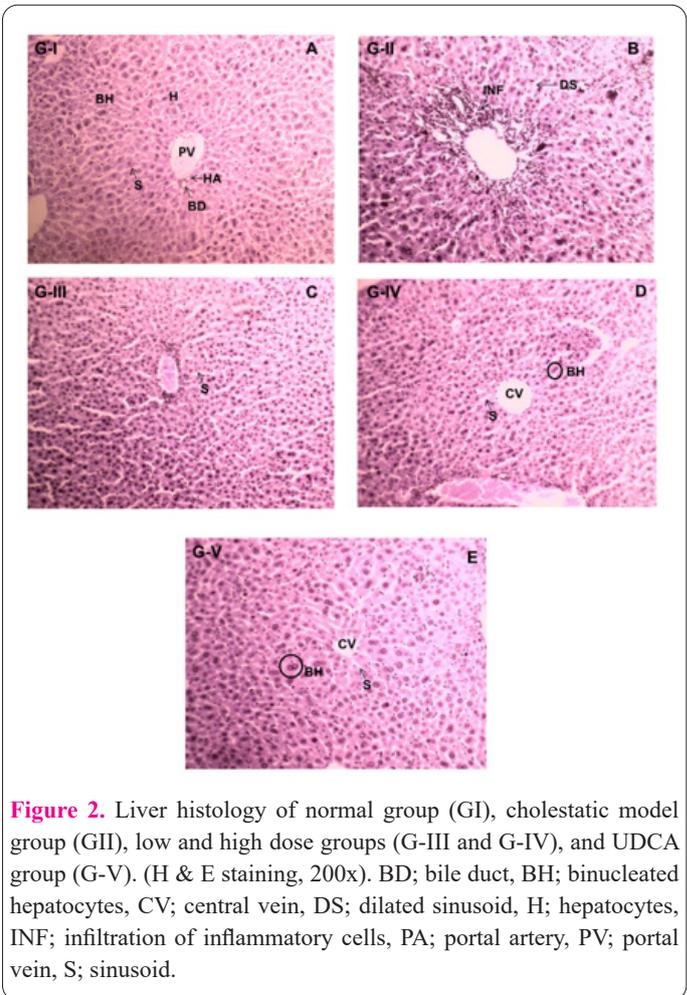


Figure 2. Liver histology of normal group (G-I), cholestatic model group (G-II), low and high dose groups (G-III and G-IV), and UDCA group (G-V). (H & E staining, 200x). BD; bile duct, BH; binucleated hepatocytes, CV; central vein, DS; dilated sinusoid, H; hepatocytes, INF; infiltration of inflammatory cells, PA; portal artery, PV; portal vein, S; sinusoid.

Silymarin modulated the gene expression in the cholestatic model

The mRNA expression of FXR, BSEP, NTCP, and Cyp7a1 was measured by quantitative PCR. In the cholestatic model group (GII), the expression of FXR, BSEP, and NTCP was significantly reduced, and the expression of Cyp7a1 was increased as compared to the control group (G-I) (Fig. 3). However, the expression of FXR, BSEP, and NTCP was upregulated in silymarin groups (GIII-G-IV) as compared to cholestatic model group (GII). Furthermore, the expression of Cyp7a1 is significantly reduced in silymarin groups (GIII-G-IV) as compared to the cholestatic model group (GII). Expression of FXR, BSEP, and NTCP is significantly elevated in the UDCA group (G-V) as compared to the model group. Furthermore, the expression of these genes in UDCA and silymarin groups is comparable. Expression of Cyp7a1 is significantly reduced in UDCA groups (G-V) as compared to the cholestatic model group (GII).

Discussion

ANIT is used to generate the cholestatic mice models (21). It is a classical biliary toxin that induces cholestasis in rodents by injuring bile duct epithelial cells and hepatocytes. Consequently, the hepatobiliary enzymes and components are leaked into the blood. These pathological and biochemical changes in the liver are similar to intrahepatic cholestasis in humans (22). In this study the serum level of liver markers (Bilirubin, AST, and ALP) was increased in the cholestatic model group as compared to the untreated control group, indicating liver damage. As ALP has been reported to accurately reflect the cholestasis

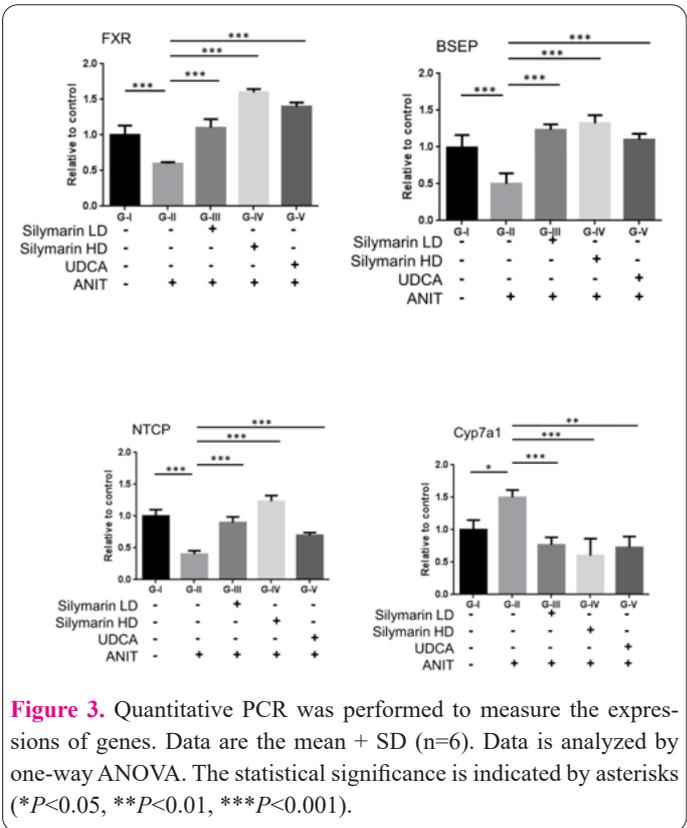


Figure 3. Quantitative PCR was performed to measure the expressions of genes. Data are the mean + SD (n=6). Data is analyzed by one-way ANOVA. The statistical significance is indicated by asterisks (* P <0.05, ** P <0.01, *** P <0.001).

in mice (23), it could be inferred that ANIT induced the cholestasis. However, in silymarin groups, the serum level of these markers was significantly lower than the cholestatic model group which indicates the protective effect of silymarin against ANIT-induced injury. This inference was

complemented by histological observations which showed severe cellular distortion in the cholestatic model group. This damage was alleviated in silymarin-treated groups. Altogether, the results provide evidence that silymarin protects the liver from cholestasis. Our results are also in accordance with Alaca and coworkers who showed that *Silybum marianum* alleviated the cholestatic liver injury in bile duct ligation murine models (18).

To explore the molecular mechanism behind the protective effect of silymarin, mRNA expression of the genes involved in biosynthesis, absorption, and excretion of bile acids was also measured. Among them, hepatocyte canalicular membrane-localized transporters, BSEP, MDR2, and MPR2 are involved in the export of bile acids and associated components into the bile canaliculi to form the bile (24). BSEP is the most critical of these transporters as it effluxes the unconjugated bile acids and provides the driving force for an enterohepatic circulation of bile acids (25). In the cholestatic model group, the expression of BSEP is reduced, consequently the efflux of bile acids from the hepatocytes into bile canaliculi is compromised. It results in piling up the bile acids in the liver and cholestasis-related cellular injuries. However, in silymarin treated groups the expression of BSEP is elevated. NTCP is located on the basolateral membrane of hepatocytes and involved in the uptake of bile acids from portal blood. In cholestatic model group the expression of NTCP is reduced thus disturbing the enterohepatic circulation of bile acids. This decrease might be because of the injury of hepatocytes (26). In silymarin treated groups the expression of NTCP is similar to that of the control group. FXR is a key component in bile acid homeostasis by its direct or indirect impact on bile acid formation, excretion, and reabsorption (27). Therefore it is recognized as a therapeutic target for cholestasis treatment (8, 9, 28, 29). In silymarin groups, FXR expression is elevated which in turn also triggers the expression of BSEP and other canalicular transporters of bile acids thus maintaining the enterohepatic circulation of the bile salts (30, 31). Furthermore, FXR also inhibits the expression of *Cyp7a1*, thus suppressing the load of bile acids in the liver (32). Our data also show that in the cholestatic model group the expression of *Cyp7a1* is increased (contrary to the decreased expression of FXR), thus worsening the cholestatic condition. However, in silymarin treated groups the expression of *Cyp7a1* is lower than in the cholestatic model group. Furthermore, the expression of *Cyp7a1* in silymarin groups is similar to the untreated control group. Silymarin probably interacts with FXR which ultimately alters the expression of other genes involved in homeostasis of bile acids. However a careful docking of silymarin with FXR is required to validate this hypothesis.

Overall, the data indicate that silymarin regulates the expression of genes involved in biosynthesis, excretion, and absorption of bile acids, thereby improving the ANIT-induced cholestasis. However, a clinical investigation is required before its prescription as a safe and alternative treatment of cholestasis.

Author contributions

Participated in research design: IS, NM

Conducted experiment: IS, NT, AK, SM

Performed data analysis: IS, NM, RA

Wrote/ contributed to writing manuscripts: IS, NM, AK, RA

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Conflict of interest

The authors declare that they have no competing interests

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