

Original Article

Sweroside ameliorates α -naphthylisothiocyanate-induced cholestatic liver injury in mice by regulating bile acids and suppressing pro-inflammatory responses

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Aim: Sweroside is an iridoid glycoside with diverse biological activities. In the present study we investigated the effects of sweroside on α -naphthylisothiocyanate (ANIT)-induced cholestatic liver injury in mice.

Methods: Mice received sweroside (120 mg/kg⁻¹·d⁻¹, ig) or a positive control INT-747 (12 mg/kg⁻¹·d⁻¹, ig) for 5 d, and ANIT (75 mg/kg, ig) was administered on d 3. The mice were euthanized on d 5, and serum biochemical markers, hepatic bile acids and histological changes were analyzed. Hepatic expression of genes related to pro-inflammatory mediators and bile acid metabolism was also assessed. Primary mouse hepatocytes were exposed to a reconstituted mixture of hepatic bile acids, which were markedly elevated in the ANIT-treated mice, and the cell viability and expression of genes related to pro-inflammatory mediators were examined.

Results: Administration of sweroside or INT-747 effectively ameliorated ANIT-induced cholestatic liver injury in mice, as evidenced by significantly reduced serum biochemical markers and attenuated pathological changes in liver tissues. Furthermore, administration of sweroside or INT-747 significantly decreased ANIT-induced elevation of individual hepatic bile acids, such as β -MCA, CA, and TCA, which were related to its effects on the expression of genes responsible for bile acid synthesis and transport as well as pro-inflammatory responses. Treatment of mouse hepatocytes with the reconstituted bile acid mixture induced significant pro-inflammatory responses without affecting the cell viability.

Conclusion: Sweroside attenuates ANIT-induced cholestatic liver injury in mice by restoring bile acid synthesis and transport to their normal levels, as well as suppressing pro-inflammatory responses.

Keywords: sweroside; INT-747; α -naphthylisothiocyanate; bile acids; cholestasis; hepatotoxicity; hepatocytes; inflammation

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Introduction

Bile acids are the primary constituents of bile and facilitate the absorption of triglycerides, cholesterol and lipid-soluble vitamins. Bile acids play an important role in the enterohepatic system, but the corresponding cytotoxicity to hepatocytes should also be addressed^[1, 2]. Numerous studies have suggested that the intrahepatic accumulation of bile acids may directly or indirectly cause further damage to the liver^[3]. One

of the hypotheses regarding the pathophysiology of cholestasis is that accumulated hepatic bile acids directly induce cell apoptosis, as supported by studies on rat hepatocytes and human hepatoma lines^[4, 5]. Increasing attention has been paid the physiological role of bile acids acting as pro-inflammatory signals, which may trigger hepatic CXC chemokine formation during the development of cholestatic liver injury^[6, 7]. The abnormal circulation of bile acids partially reflects liver function^[8]. Therefore, bile acid homeostasis should be well controlled to avoid further liver injury due to their potential hepatotoxicity at high levels.

Cholestasis is an important symptom for liver injury. This

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condition represents an impairment of the hepatobiliary transport systems, which leads to disruption of circulating bile acid and results in more severe liver damage. Several liver diseases are accompanied by cholestasis, such as gallstones^[9], hepatitis^[10], and primary biliary cirrhosis (PBC)^[11]. Therefore, understanding the role of accumulated hepatic bile acids in cholestatic conditions is crucial to elucidate the underlying mechanism of cholestasis. Alpha-naphthylisothiocyanate (ANIT) is a well-characterized cholestatic agent that is widely used to mimic human intrahepatic cholestasis in rodents^[12-14]. The biochemical and pathological changes induced by ANIT administration in animal models are similar to the symptoms of clinical intrahepatic cholestasis^[15]. Early studies on the causes of ANIT-induced cholestatic liver injury have emphasized the response of cytotoxic and pro-inflammatory mediators^[16]. Recently, ANIT has been reported to directly induce bile acid overload in hepatocytes, which may partially account for cholestasis^[17]. Therefore, our study investigated the relationship between bile acid homeostasis and inflammatory mediators, which substantially contribute to ANIT-induced cholestatic liver injury.

To date, available drug treatments have proven to be non-specific and exhibited limited efficacy in cholestasis therapy^[18, 19]. Ursodeoxycholic acid is the only drug approved by the Food and Drug Administration for cholestasis treatment^[20, 21]. INT-747 has been investigated in a phase II trial for the treatment of PBC, liver fibrosis, and diet-induced atherosclerosis^[22]. However, side effects of INT-747 on lipid metabolism have been observed in pre-clinical studies, and these side effects may limit drug development. Therefore, the development of novel therapies for cholestasis is urgently needed.

Numerous studies have investigated the use of natural phytochemicals for the prevention and treatment of cholestasis. Sweroside is an iridoid glycoside that exhibits diverse biological activities, such as anti-fungal, anti-diabetic, anti-inflammatory, and anti-tumor effects^[23-26]. Many studies have indicated that sweroside ameliorates liver injury by decreasing oxidative damage and inhibiting the production of pro-inflammatory kinases^[27, 28]. However, the effects of sweroside on mice with intrahepatic cholestasis is not clear. The current study performed bile acid profile analyses to evaluate the hepatoprotective effects of sweroside. The expression levels of bile acid metabolism-related genes and pro-inflammatory kinases were measured to identify the underlying mechanism. Subsequently, we determined whether sweroside ameliorated ANIT-induced cholestatic liver injury by regulating bile acids and further mediating pro-inflammatory responses.

Materials and methods

Materials

Sweroside (>98.5% purity) was purchased from NanJing Goren Biotechnology Co Ltd (Nanjing, China). INT-747 was purchased from Adipogen (San Diego, CA, USA). ANIT and olive oil were purchased from Sigma-Aldrich (St Louis, MO, USA). All bile acid standards were purchased from Steraloids (Rhode Island, USA) and Sigma-Aldrich. Ammo-

nium acetate, formic acid, methanol (MeOH), and acetonitrile (HPLC-grade) were purchased from Fisher Scientific (Nepean, Ontario, Canada). Ultrapure water was purified with a Milli-Q system (Millipore, Bedfordshire, MA, USA). Other solvents of analytical grade were purchased from Shanghai Chemical Factory (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), L-glutamine, and penicillin-streptomycin were purchased from Fisher Scientific. Non-essential amino acids, insulin, and hydrocortisone were purchased from Sigma-Aldrich.

Ethics statement

The Guide for the Care and Use of Laboratory Animals was strictly followed, and the Institutional Animal Committee of Shanghai University of Traditional Chinese Medicine approved the animal experiment protocols (Permit number: SCXK (Hu) 2012-0002).

In vivo study

C57BL/6J mice (pathogen free, 25–30 g, 8–10 weeks of age) were obtained from the Laboratory Animal Center of Shanghai University of Traditional Chinese Medicine. The animals were housed at 20±2 °C with 60%–70% relative humidity under a 12-h light/dark cycle. Mice were provided food and tap water *ad libitum*. The mice were randomly assigned into four groups: Vehicle group ($n=8$), ANIT group ($n=8$), Sweroside+ANIT group ($n=8$), and INT-747+ANIT group ($n=8$). INT-747 has been used for cholestasis treatment because it activates the FXR. INT-747 was used as the positive control in the pharmacological experiments in the present study. Sweroside+ANIT and INT-747+ANIT groups were treated with sweroside (120 mg/kg, ig) or INT-747 (12 mg/kg, ig) for five continuous days (once daily). The Vehicle group and ANIT group received normal saline. ANIT was administered to the ANIT, Sweroside+ANIT and INT-747+ANIT groups on the 3rd day, and this was followed by normal saline, sweroside or INT-747 treatment 4 h later. Forty-eight hours after ANIT administration, all mice were euthanized by CO₂ inhalation after having fasted for 12 h. Retro-orbital blood samples were collected into tubes, and this was followed by centrifugation at 4 °C for 10 min (8000×g). Each mouse liver was dissected, and a portion was fixed in 10% buffered formaldehyde for further histological analyses. The remaining liver samples were immediately transferred to liquid nitrogen and stored at -80 °C for real-time PCR.

Serum biochemistry and hepatic myeloperoxidase assay

The serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total bilirubin (TBIL), direct bilirubin (DBIL), and total bile acids (TBA) were determined with commercially available kits in an automatic biochemical analyzer (HITACHI 7080, Japan). Hepatic myeloperoxidase (MPO) activity was determined with a commercial assay kit according to the manufacturer's protocol (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Histological analysis

Liver samples from all groups were fixed in 10% formaldehyde (neutral buffered formalin), dehydrated, embedded in paraffin, cut into 5- μ m sections, and stained with hematoxylin and eosin (H&E) according to standard protocols. Shanghai Showbio Biotech, Inc performed histological assessments.

Quantification of bile acids

Our bile acid quantification method was used and improved on the basis of our previous study. Briefly, approximately 50 mg of liver was homogenized in 2 volumes of 50% MeOH. Liver homogenate (300 μ L) was spiked with 2 mL of ice-cold MeOH, vortexed, was shaken continuously for 1 h, and was centrifuged at 12000 \times g for 10 min. The supernatant was aspirated, and the precipitate was extracted with another 1 mL of ice-cold MeOH. Supernatants from the two extraction steps were pooled and evaporated to dryness, and this was followed by reconstitution in a 100- μ L mixture of 0.1% formic acid containing 5 mmol/L ammonium acetate-methanol (45:55, *v/v*) prior to analysis. The sample solution was centrifuged at 12000 \times g for 10 min at 4°C, and a 5- μ L aliquot was injected for UPLC/MS analysis.

Bile acids were determined with a Waters Acquity ultra-performance lipid chromatograph system (Waters, MA, USA) equipped with an Acquity UPLC BEH C18 column (1.7 μ m, 2.1 mm \times 100 mm, Waters) at 45°C. The mobile phase consisted of 0.1% formic acid in an aqueous solution of 5 mmol/L ammonium acetate (A) and methanol (B) at a flow rate of 0.3 mL/min. The elution gradient was performed as follows. The eluent composition was set at 55% A and 45% B for the first minute, linearly changed to 62% A and 38% B at 2.6 min, and the proportion of B was increased to 80% in the next 8.8 min. The sample injection volume was 5 μ L.

MS analysis was performed with a ZQ 2000 quadrupole spectrometer equipped with an ESI probe and operated with selective ion monitoring in the negative-ion mode (Waters, MA, USA). The capillary and cone voltages were set at 3.0 and 55 V, respectively. The source temperature was 120°C, and the desolvation temperature was 300°C. The desolvation gas flow was set at 700 L/h, and the cone gas flow rate was set at 50 L/h. Data were acquired and processed with MassLynx 4.1 software.

In vitro study

Primary hepatocytes of male C57BL6 mice were purchased from the Research Institute for Liver Disease (Shanghai) Co, Ltd. Primary hepatocytes were cultured in DMEM supplemented with 10% fetal bovine serum, L-glutamine (2 mmol/L), non-essential amino acids (2 mmol/L), insulin (0.08 IU/mL), hydrocortisone (2.34 μ mol/L), and penicillin-streptomycin (1%). Cells were treated with the reconstituted bile acid mixture, which was composed of the hepatic bile acids that were most elevated after ANIT exposure *in vivo*. Cell viability was assessed with a CCK-8 assay kit (Shanghai Yeasen Biotechnology, Shanghai, China). The gene expression of pre-inflammatory mediators was also examined.

RNA isolation and quantitative real-time PCR

Total RNA was isolated from mouse liver samples and mouse primary hepatocytes with Trizol (Takara, Dalian, China) according to the manufacturer's protocol. RNA was reverse transcribed into cDNA (1 μ g/ μ L) with a PrimerScript RT Reagent Kit (Takara, Dalian, China). Gene-specific primers were designed online with Primers 3. Table 1 presents the primers for the detected genes. Quantification of gene expression was performed with real-time PCR (ABI, USA) with SYBR Green Master Mix (Takara, Dalian, China). All reactions were performed in triplicate with the following thermal cycling conditions: initial denaturation at 95°C for 30 s followed by 50 cycles of 95°C for 5 s and 60°C for 30 s. Beta-actin was used as an internal reference to calculate the relative expression of target genes.

Table 1. Primer sequence used for quantitative real-time PCR with β -actin as an internal control.

Name	Forward primer (5'-3')	Reverse primer (5'-3')
<i>β-Actin</i>	GGCTGTATCCCTCCATCG	CCAGTTGGTAACAATGCCATGT
<i>Cyp7a1</i>	GGCTAGCATAGCCAACCTTGC	TGCCCTTCTGAAGCTCATCT
<i>Cyp8b1</i>	CCTCTGGACAAGGGTTTTGTG	GCACCGTGAAGACATCCCC
<i>Cyp27a1</i>	CCAGGCACAGGAGAGTACG	GGGCAAGTGCAGCACATAG
<i>Cyp7b1</i>	GGACCTGGAATTTCTGTCCA	ACGGATGATGCTGGAGTAGG
<i>Akr1d1</i>	TGGGCTTGGAACTACTCAG	GCCGGTACCCTCATCAATA
<i>Hsd3b7</i>	TCCACAAAGTCAACGTGCAG	TTCCATGCTGCTCGTGAGA
<i>Ost-β</i>	AGATGCGGCTCCTTGGAATTA	TGGCTGCTTCTTTCCGATTCTG
<i>Ntcp</i>	CAAACCTCAGAAGACCAACA	GTAGGAGGATTATCCCGTTGTG
<i>Oatp1a1</i>	AAGAAAGGCCCTGAGTGTGA	ATGGCTCGAGTGAAGAAGT
<i>Oatp1a4</i>	GCAAGACCCAACAGAGTGTG	AGGGCATGATGGGAGTTTCA
<i>Oatp1b2</i>	GGGAACATGCTTCGTGGGATA	GGAGTTATGCGGACACTTCTC
<i>Mdr1</i>	CTGTTGGCGTATTTGGGATGT	CAGCATCAAGAGGGGAAGTAATG
<i>Mrp2</i>	GTGTGGATCCCTTGGGCTTT	CACAACGAACCTGCTTGG
<i>Bsep</i>	GGACAATGATGTGCTTGTGG	CACACAAAGCCCTACCAGT
<i>mKC</i>	GAAGCTTGCCCTCAACTCTG	CTCCCTTCTGGTCAGTTGGA
<i>Mip-2</i>	TCCAGAACTTGAGTGTGACG	TTCAGGGTCAAGGCAAACCTT
<i>ICAM-1</i>	CTTCCAGCRACCATCCCAA	CTTCAGAGGCAGGAAACAGG
<i>TNF-α</i>	GGAACACGTCGTGGGATAATG	GGCAGACTTTGGATGCTTCTT
<i>IL-6</i>	GGCGGATCGGATGTTGTGAT	GGACCCAGACAATCGGTTG
<i>IL-17α</i>	TTCAGGGTGCAGAAGATGCT	AAACGTGGGGGTTCTTAGG

ELISA assay

Protein levels of TNF- α , IL-6, MIP-2, and ICAM-1 were determined in all treated groups by using commercial kits (Shanghai Xinyu Biotechnology Company, Shanghai, China).

Statistical analysis

Data were statistically analyzed by one-way ANOVA and the least significant differences test (SPSS 18.0 software; SPSS, Inc, Chicago, USA). Differences were considered to be statistically significant when $P < 0.05$. OPLS-DA analysis was performed with Simca-P software 13.0 (Umetrics, Umea, Sweden). All graphs were generated with Graphpad Prism 5 (GraphPad Software, CA, USA).

Results

Sweroside exhibited hepatoprotective effects against ANIT-induced cholestasis

Biochemical indicators were detected in all treatment groups to evaluate the hepatoprotective effects of sweroside. ANIT administration significantly increased serum ALT and AST in mice. However, sweroside treatment dramatically reduced both of these indicators. Sweroside treatment also reversed the ANIT-induced increases in serum ALP, TBIL, DBIL, and TBA, all of which indicated biliary toxicity. Oral INT-747 administration markedly decreased serum AST, ALP, TBIL, DBIL, and TBA levels but only slightly decreased ALT (Figure 1A). Liver sections were stained with H&E to further examine histological changes in the liver after ANIT exposure. Multifocal hepatic parenchymal necrosis with inflammatory cell infiltration was observed in the ANIT-treated group. Sweroside and INT-747 treatment significantly ameliorated the degree of hepatic parenchymal necrosis and attenuated inflammatory cell infiltration (Figure 1B).

Hepatoprotective effects of sweroside against ANIT-induced cholestasis were related to the regulation of bile acid homeostasis

Hepatic bile acid overload is a primary characteristic of ANIT-induced cholestasis. Figure 2A shows that ANIT administration markedly increased hepatic TBA levels. Individual hepatic bile acid levels were measured by UPLC-MS and followed by OPLS-DA analysis. Figure 2B shows that the vehicle, ANIT, Sweroside+ANIT, and INT-747+ANIT groups were markedly separated from each other in OPLS-DA score plots. The ANIT group was scattered far from the vehicle group, thereby indicating a severe disruption of bile acid homeostasis after ANIT administration. The sweroside pretreatment group was closer to the vehicle group than to the ANIT group, thereby indicating that sweroside normalized the ANIT-induced abnormality of hepatic bile acid levels. The INT-747 treatment group exhibited an identical trend to that of the sweroside group. Figure 2C shows that ANIT exposure markedly increased hepatic β -MCA, ω -MCA, CA, T- ω -MCA,

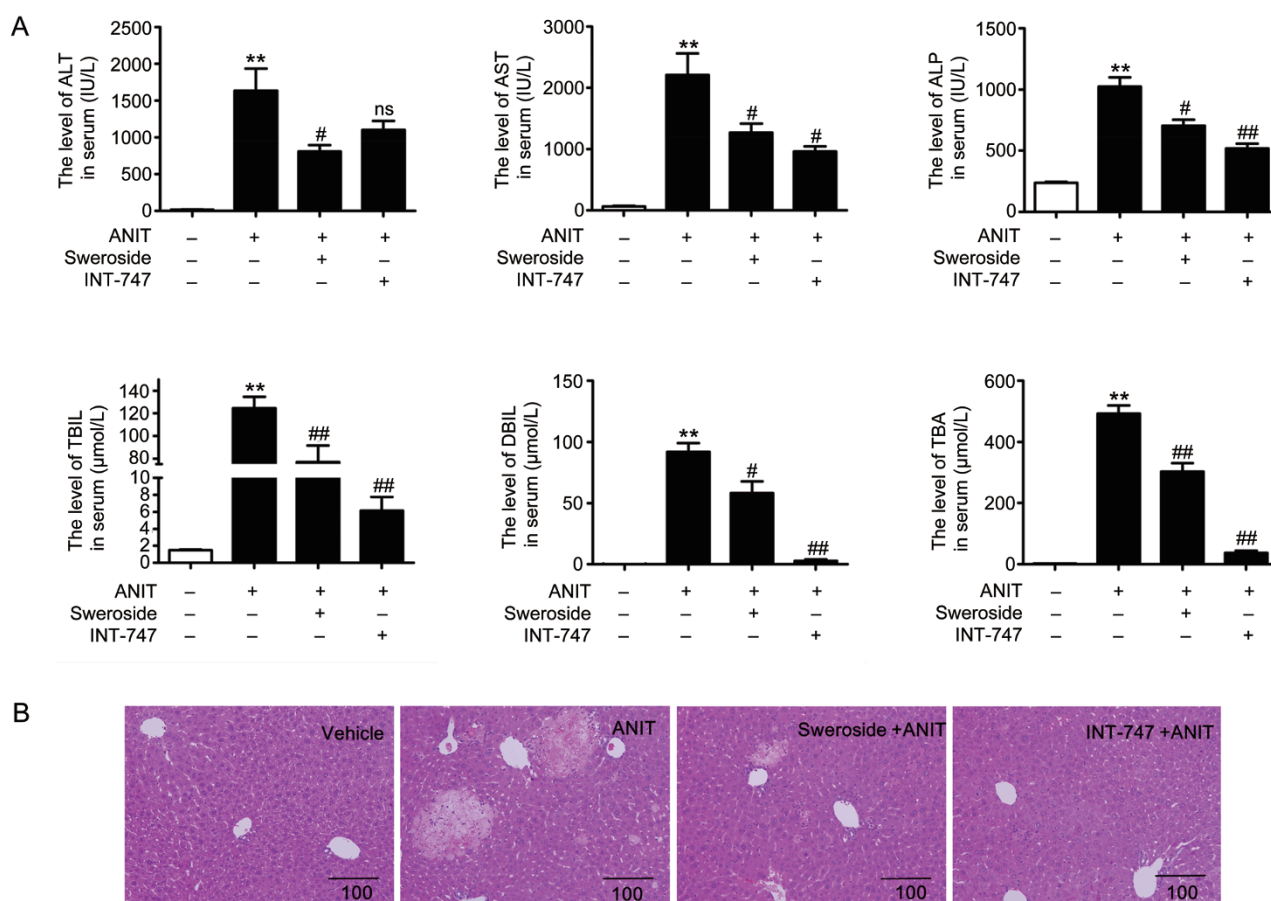


Figure 1. Sweroside alleviates ANIT-induced hepatotoxicity and cholestasis. (A) Biochemical indicators in mice orally administered sweroside (120 mg/kg, ig) or INT-747 (12 mg/kg, ig) for five consecutive days (once daily) were determined 48 h after vehicle or ANIT exposure. All data are shown as the mean \pm SEM ($n=8$). ^{ns} $P>0.05$, * $P<0.05$, ** $P<0.01$ versus vehicle group. ^{ns} $P>0.05$, # $P<0.05$, ## $P<0.01$ versus ANIT group. (B) The representative images of H&E-stained liver sections (100 \times magnification) were examined under a microscope.

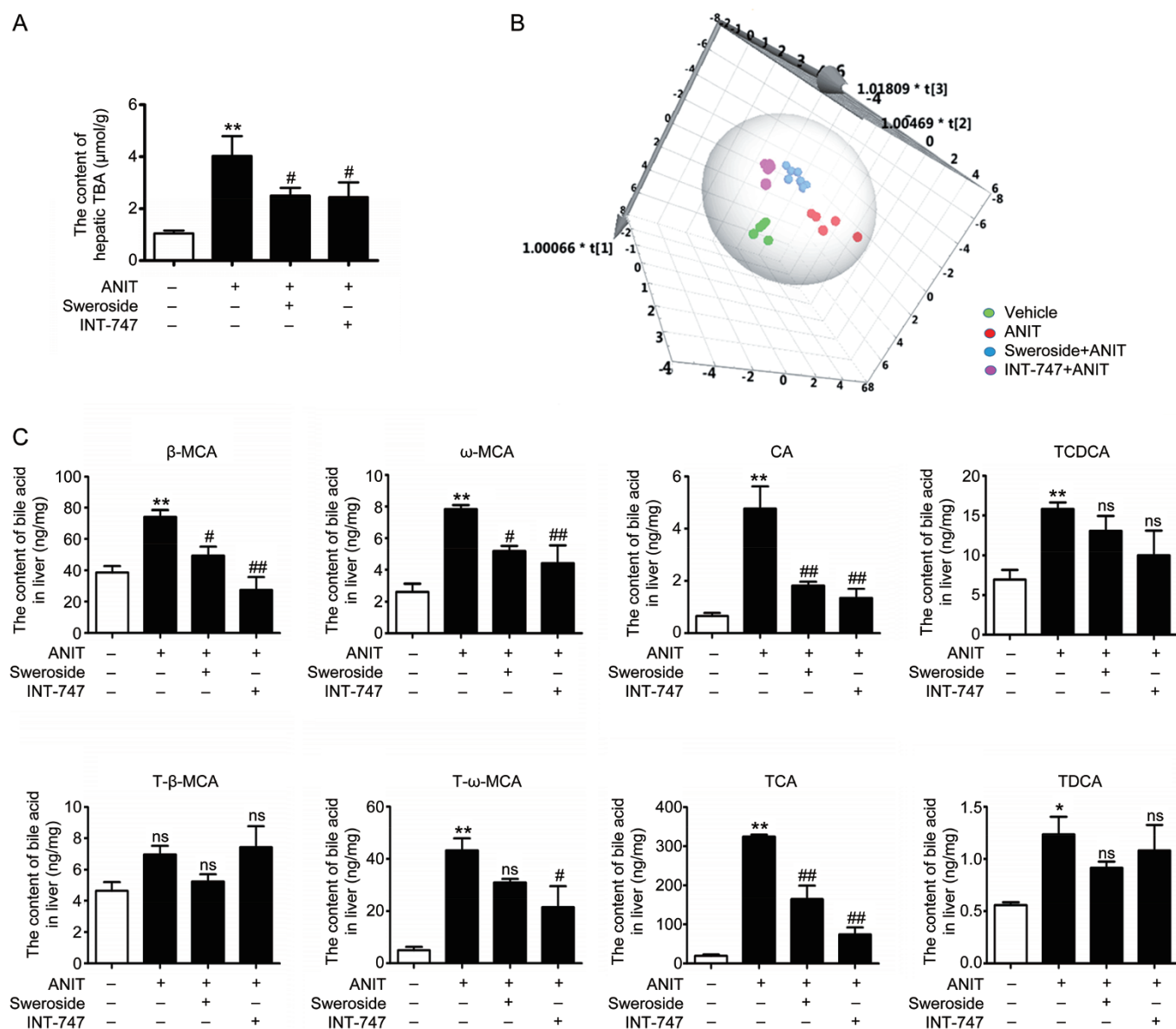


Figure 2. Sweroside alters hepatic bile acid profiles and protects livers against ANIT-induced cholestatic liver injury. (A) Total bile acid content was detected in the liver. (B) OPLS-DA score plots derived from the hepatic bile acid levels of the vehicle, ANIT, Sweroside+ANIT, and INT-747+ANIT groups. (C) Concentrations of individual bile acids were detected in the liver. All data are shown as the mean±SEM ($n=8$). ^{ns} $P>0.05$, ^{*} $P<0.05$, ^{**} $P<0.01$ versus vehicle group. ^{ns} $P>0.05$, [#] $P<0.05$, ^{##} $P<0.01$ versus ANIT group.

TCDCA, TDCA, and TCA levels. However, oral sweroside or INT-747 administration significantly reduced the hepatic accumulation of the above-mentioned bile acids, especially the reduction of TCA. Sweroside restored bile acid profiles to normal after ANIT exposure, thereby suggesting that bile acid profiles are good markers to evaluate the protective effects of sweroside.

Sweroside mediated bile acid homeostasis against ANIT-induced cholestasis by affecting bile acid synthesis and transport

The mRNA levels of major bile acid synthesis enzymes and transporters were measured to elucidate the protective mechanisms of sweroside on bile acid regulation. Figure 3 shows

that ANIT exposure significantly down-regulated most bile acid biosynthesis genes (*Cyp7a1*, *Cyp8b1*, *Cyp27a1*, *Cyp7b1*, *Hsd3b7*, and *Akr1d1*). However, sweroside treatment significantly reversed the ANIT-induced down-regulation of *Cyp7a1*, *Cyp8b1*, and *Hsd3b7* but further decreased the down-regulation of *Cyp7b1* (Figure 3). In contrast, INT-747 treatment, compared with ANIT treatment, further decreased the down-regulation of *Cyp7a1* and *Cyp8b1* and resulted in increased expression of *Cyp27a1* and *Cyp7b1*. However, INT-747 had no effect on *Hsd3b7* and *Akr1d1* expression levels. Efflux transporters were also studied, including *Ost-β*, *Mrp2*, *Bsep*, and *Mdr1*. ANIT exposure significantly increased the expression of *Ost-β* and *Mdr1*, but *Bsep* expression was only slightly

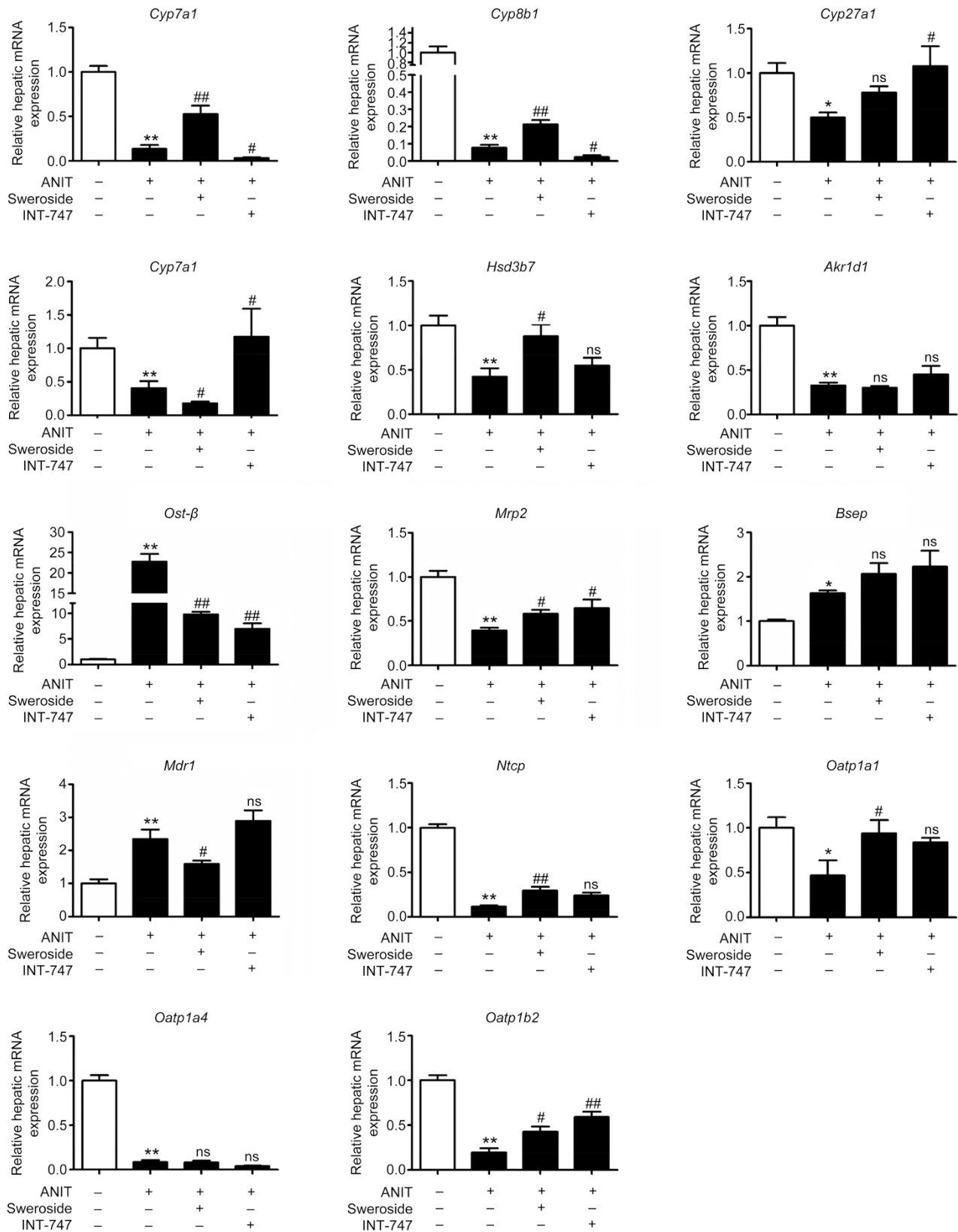


Figure 3. Sweroside alters the gene expression profiles involved in bile acid synthesis and transport. Quantitative real-time PCR analysis was performed to measure the gene expression of *Cyp7a1*, *Cyp8b1*, *Cyp27a1*, *Cyp7b1*, *Akr1d1*, *Hsd3b7*, *Oatp1a1*, *Oatp1a4*, *Oatp1b2*, *Ntcp*, *Ost-β*, *Mrp2*, *Bsep*, and *Mdr1* in the liver. All data are shown as the mean±SEM (n=8). ^{ns}P>0.05, *P<0.05, **P<0.01 versus vehicle group. ^{ns}P>0.05, #P<0.05, ##P<0.01 versus ANIT group.

increased compared with the vehicle group. ANIT exposure significantly decreased the expression of *Mrp2* mRNA. Sweroside treatment reversed the up-regulated *Ost-β* and *Mdr1* mRNA expression and slightly increased the up-regulation of *Bsep* mRNA, as compared with ANIT group. However, INT-747 treatment significantly reversed *Mrp2* and *Ost-β* expression and slightly enhanced the up-regulation of *Bsep* and *Mdr1* expression. *Ntcp*, *Oatp1a1*, *Oatp1a4*, and *Oatp1b2* play critical roles in the control of bile acid uptake, and ANIT exposure significantly decreased the expression of these mRNAs. Figure 4 shows that sweroside treatment enhanced the down-regulation of *Ntcp*, *Oatp1a1*, and *Oatp1b2*, but it did not affect the *Oatp1a4* mRNA level, as compared with the ANIT group. In contrast, INT-747 increased the *Oatp1b2* expression level, as compared with the ANIT group. The expression of *Ntcp*, *Oatp1a1*, and *Oatp1a4* remained unchanged after INT-747 treatment.

Sweroside repressed pro-inflammatory responses against ANIT-induced cholestatic liver injury

Most studies have suggested that inflammatory responses are histological features of cholestasis-associated pathophysiological changes. Our results demonstrated that hepatic MPO activity and the gene expression levels of *TNF-α*, *IL-6*, *mKC*, *MIP-2*, and *ICAM-1* increased significantly in mice after ANIT exposure (Figure 4A and 4B). However, oral sweroside administration significantly ameliorated hepatic MPO activity and *TNF-α*, *IL-6*, *mKC*, *MIP-2*, and *ICAM-1* levels, thereby indicating that sweroside exerts potential anti-inflammatory effects. INT-747 slightly decreased hepatic MPO activity and significantly ameliorated ANIT-induced increased mRNA levels of *TNF-α*, *IL-6*, *mKC*, *MIP-2*, and *ICAM-1* in our study (Figure 4A and 4B). Sweroside and INT-747 also decreased the ANIT-induced elevated protein levels of TNF-α, IL-6, MIP-2, and ICAM-1 in sera (Figure 4C).

Hepatic accumulation of bile acids induced pro-inflammatory responses

Primary hepatocytes in male C57BL6 mice were treated with a reconstituted bile acid mixture to investigate whether increased hepatic concentrations of bile acids after ANIT exposure were required for cell death or the release of pro-inflammatory mediators. Figure 2 shows the exact composition of the bile acid mixture. Treatment with the reconstituted bile acid mixture produced no significant cell death induction, which was supported by the cell viability study (Figure 5A). However, the reconstituted bile acids markedly increased *TNF-α*, *IL-6*, *IL-17a*, *mKC*, *MIP-2*, and *ICAM-1* mRNA expression levels (Figure 5B). These results are consistent with the results after ANIT administration *in vivo*. Our data indicate that bile acids may induce the inflammatory mediators that contribute to cholestasis.

Discussion

This study assessed the hepatoprotective effect of sweroside by using an ANIT-induced cholestasis model and bile acid

profile analysis. Many reports have focused on the beneficial role of sweroside^[24–28], but its effects on cholestatic liver injury remain largely unknown.

Sweroside exhibited the most dramatically protective effect at a dose of 120 mg/kg in our preliminary dose-response study, as evidenced by the reduction of ALT, AST, ALP, TBA, TBIL, and DBIL (Supplementary Figure S1). Therefore, this dose of sweroside (120 mg/kg) was chosen for the current study of the underlying mechanism. Our results demonstrated that the potential hepatoprotective benefits of sweroside against ANIT-induced liver injury occurred via bile acid regulation. We also discovered that sweroside significantly decreased TBA and TG levels in normal mice (Supplementary Figure S2 and Supplementary Figure S3B), thus partially suggesting its protective effect in the ANIT-induced model. An abnormal accumulation of bile acids in hepatocytes has been observed in cholestatic diseases, including intrahepatic cholestasis of pregnancy and progressive familial intrahepatic cholestasis 1, 2, and 3 (PFIC1, PFIC2, and PFIC3)^[29–31]. Therefore, maintaining the homeostasis of bile acids plays an importance role in cholestasis prevention and therapy. ANIT exposure significantly increased hepatic TBA and individual bile acids in the present study, which is consistent with results from a previous report^[32]. Sweroside demonstrated potential hepatoprotective effects through alleviation of bile acid accumulation in the liver. Bile acid metabolism-related genes warrant further study to investigate the mechanism underlying the roles of sweroside in bile acid regulation in an animal model.

Previous reports have demonstrated that bile acid synthesis may be inhibited during liver damage^[33, 34]. The present study demonstrated that sweroside significantly attenuated the down-regulation of *Cyp7a1*, *Cyp8b1*, and *Hsd3b7*, which is consistent with results from previous reports^[14, 17]. INT-747 further decreased the mRNA expression of *Cyp7a1* and *Cyp8b1* but reversed the *Cyp7b1* expression, as compared with the ANIT group. We hypothesize that sweroside affects the *de novo* synthesis of bile acids in a unique manner, which differs from that of INT-747. A previous report has demonstrated that ANIT also affects the function of bile acid transporters, ranging from efflux transporters to uptake transporters^[35]. ANIT significantly decreased *Mrp2*, *Ntcp*, *Oatp1a1*, *Oatp1a4*, and *Oatp1b2* levels and increased *Ost-β* and *Mdr1* levels in our study. These data are consistent with a results from a previous report^[36]. However, sweroside attenuated ANIT-induced changes in *Mrp2*, *Ost-β*, *Mdr1*, *Ntcp*, *Oatp1a1*, and *Oatp1b2* expression, thereby restoring the disruption of bile acid profile. *Mrp2* is an efflux transporter that mediates the secretion of toxic endogenous and exogenous compounds in the form of amphiphilic anionic conjugates via an ATP-dependent mechanism^[37, 38]. Sweroside treatment enhanced *Mrp2* expression, which may have contributed to the accelerated secretion of hepatic bile acids. Serum bilirubin is a substrate of *Mrp2*, and this factor negatively correlated with *Mrp2* expression. GSH is another substrate of *Mrp2*, which contributed to the further attenuation of the ANIT-induced liver injury by competitively

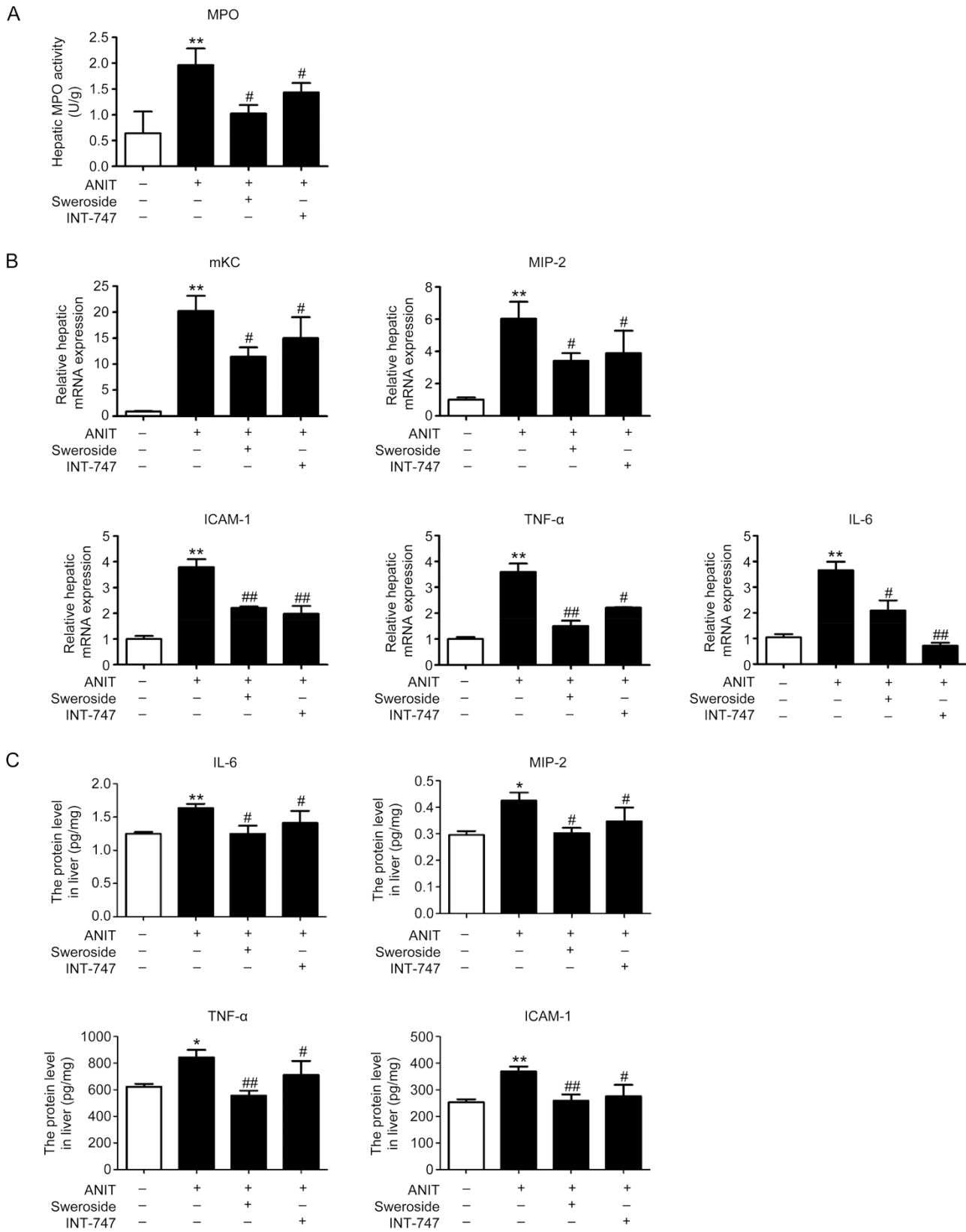


Figure 4. Sweroside decreases hepatic MPO activity and represses the pro-inflammatory response. (A) Hepatic MPO activity was assayed. (B) Quantitative real-time PCR analysis was performed to measure the gene expression of *TNF-α*, *IL-6*, *mKC*, *MIP-2*, and *ICAM-1* in the liver. (C) Commercially available ELISA test kits were used to determine the protein expression levels of *IL-6*, *TNF-α*, *ICAM-1*, and *MIP-2*. All data are shown as the mean±SEM (n=8). ^{ns}P>0.05, *P<0.05, **P<0.01 versus vehicle group. #P<0.05, ##P<0.01 versus ANIT group.

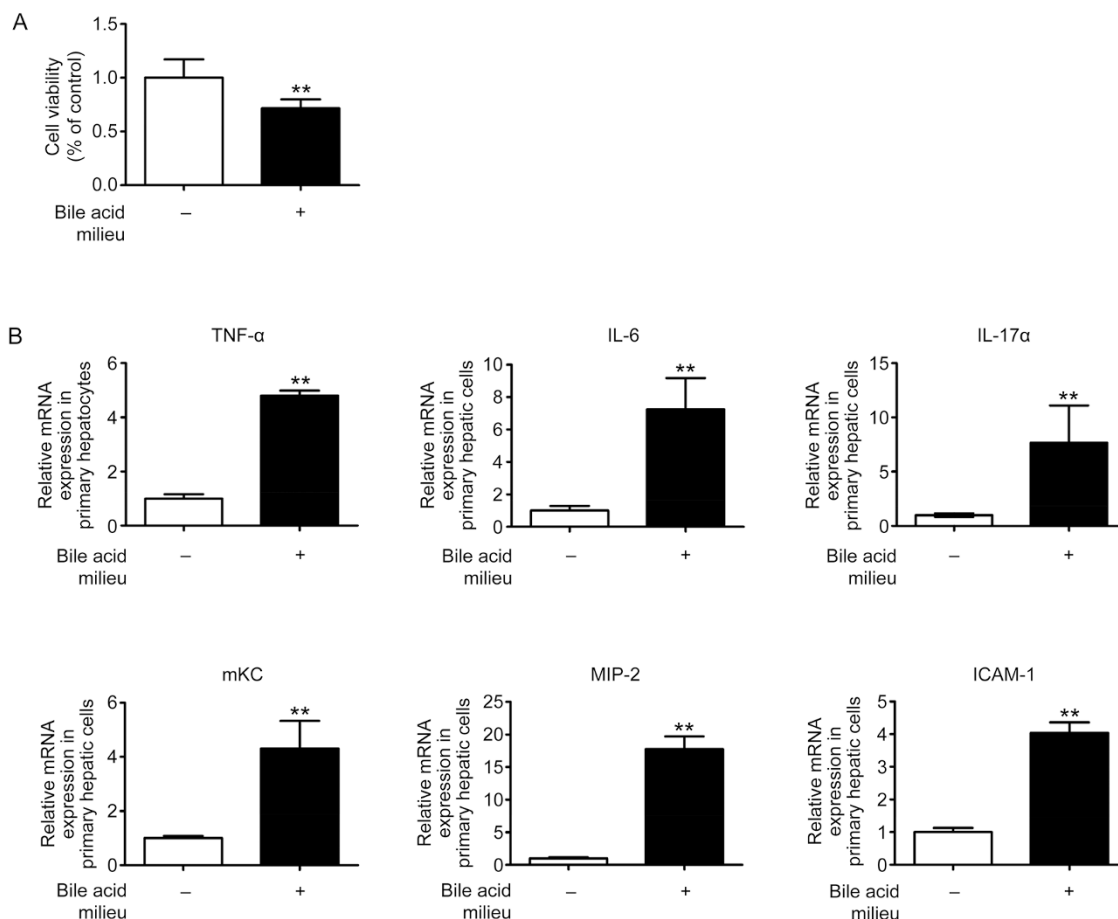


Figure 5. Cell viability study and expression of pro-inflammatory cytokines after exposure to the bile acid mixture. Primary mouse hepatocytes were treated with the indicated concentration of bile acid mixture. (A) Cell viability study using a CCK-8 kit. (B) Real-time PCR was used to assess the gene expression of *TNF-α*, *IL-6*, *IL-17α*, *mKC*, *MIP-2*, and *ICAM-1*. All data are shown as the mean±SEM ($n=8$). ** $P<0.01$ versus control group.

inhibiting the conjugation between ANIT and GSH. *Ost-β* is also an important bile acid efflux gene that transports bile acids into the bloodstream. ANIT exposure robustly increased *Ost-β* expression, a result consistent with those from a previous report^[39]. The reduced expression of *Ost-β* by sweroside may contribute to the decreased counter-transport of bile acids into the blood, which further reduces bile acid levels in the systemic circulation. *Ntcp* and *Oatp* are located at the basolateral surface of hepatocytes and are responsible for the hepatic uptake of bile acids. A reduction of *Ntcp* has been observed in various cholestatic conditions, such as advanced PBC, cholestatic alcoholic liver injury, and biliary atresia^[40]. Therefore, the restoration of defective *Ntcp* in cholestasis may be an important target for pharmacotherapeutic interventions. In our previous study, the *Danning* tablet was used to treat cholestatic liver injury, and this treatment partially exerts its protective effect via the restoration of *Ntcp* expression^[13]. *Oatp1a1* that has a high affinity for TCA and TDCA is another gene involved in the hepatic uptake of bile acids^[41]. The up-regulation of *Oatp1a1* by sweroside treatment in the present study is consistent with the reduction of TCA and TDCA in

the liver.

The modulation of pro-inflammatory responses plays a critical role in cholestatic liver injury^[33]. The present study demonstrated that hepatic MPO activity was significantly increased after ANIT exposure, which is consistent with results from a previous study^[34]. However, oral sweroside administration alleviated the increase in hepatic MPO activity after ANIT treatment, which contributed to the inhibition of neutrophil infiltration into liver tissues. Sweroside also exhibits anti-inflammatory activity by inhibition of NO, IL-6, and TNF-α production induced by lipopolysaccharide (LPS) in RAW264 cells^[29]. Sweroside decreased hepatic mRNA and protein expression of pro-inflammatory cytokines in the present study, including TNF-α, IL-6, mKC, MIP-2, and ICAM-1 (Figure 4B and 4C). The relationship between pro-inflammatory cytokines and bile acid metabolism-related genes has recently received considerable attention. IL-1β has been identified as an inducer of *Mrp2*, *Cyp8b1*, and *Cyp7a1*^[42-44]. TNF-α mediates the down-regulation of the *Oatp* genes at the mRNA level^[45]. *Mrp2*, *Cyp8b1*, *Cyp7a1* and *Oatp* expression was negatively correlated with the pro-inflammatory cytokine expression in

our study, which indicates that the suppression of bile acid metabolism-related genes may be partially caused by an elevation of the pro-inflammatory response. However, further studies are necessary to verify this hypothesis.

ANIT induces pro-inflammatory mediators^[16]. However, the role of increased hepatic bile acids in the progress of ANIT-induced liver injury is not known. A bile acid mixture significantly increased the mRNA expression of pro-inflammatory cytokines *in vitro* in our study, which is consistent with results from a previous report^[5]. TCA exhibited the highest increase after ANIT treatment in our study, and this factor increases the expression of MIP-2 in rodents and IL-8 in humans^[6,7]. The reconstituted bile acid mixture markedly increased *mKC* and *MIP-2* expression *in vitro* in our study, which is consistent with the results after ANIT administration *in vivo*. Therefore, bile acids may act as a pro-inflammatory signal triggering CXC chemokine formation and result in liver injury.

Sweroside treatment of cells or mice exhibited no obvious toxicity (Supplementary Figure S3 and Supplementary Figure S4), thus supporting the safety of sweroside as a potentially reliable treatment option for further consideration. In conclusion, sweroside exerted a protective effect on ANIT-induced acute cholestatic liver injury in mice. Target profiling analysis of individual hepatic bile acids was used to evaluate the protective effect of sweroside against liver injury. The anti-inflammatory activity of sweroside also prevented ANIT-induced acute cholestatic liver injury. These combined results indicate that the hepatoprotective mechanism of sweroside is related to the regulation of bile acid homeostasis, and abnormal bile acid levels may result in pro-inflammatory responses that lead to liver injury.

Abbreviations

ANIT, a-naphthylisothiocyanate; INT-747, obeticholic acid; *mKC*, mouse keratinocyte derived factor; MIP-2, macrophage inflammatory protein 2; ICAM-1, intercellular adhesion molecule-1; TNF- α , tumor necrosis factor α ; IL-6, interleukin-6; IL-17 α , interleukin 17 α ; TBA, total bile acid; α -MCA, α -muricholic acid; β -MCA, β -muricholic acid; ω -MCA, ω -muricholic acid; T- α -MCA, tauro- α -muricholic acid; T- β -MCA, tauro- β -muricholic acid; T- ω -MCA, tauro- ω -muricholic acid; CA, cholic acid; DCA, deoxycholic acid; CDCA, chenocholic acid; LCA, lithocholic acid; UDCA, ursodeoxycholic acid; HDCA, hyodesoxycholic acid; GCA, glycocholic acid; TCA, taurocholic acid; GDCA, glycodeoxycholic; TDCA, taurodeoxycholic acid; GCDCA, glycochenodeoxycholic acid; TCDCa, taurochenodeoxycholic acid; GUDCA, glyoursodeoxycholic acid; TUDCA, tauroursodeoxycholic acid; THDCA, taurohyodesoxycholic acid; GLCA, glycolithocholic acid; TLCA, tauroolithocholic acid.

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Author contribution

Qiao-ling YANG, Li YANG, and Zheng-tao WANG conceived and designed the experiments; Qiao-ling YANG, Jun-ting GONG, Xiao-wen TANG, and Guang-yun WANG performed the experiments; Qiao-ling YANG, Jun-ting GONG, Xiao-wen TANG, and Guang-yun WANG acquired data; Qiao-ling YANG and Fan YANG analyzed data; Qiao-ling YANG, Fan YANG, and Li YANG wrote the manuscript; all authors revised the manuscript.

Supplementary information

Supplementary information is available on the website of *Acta Pharmacologica Sinica*.

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