รายงานวิจัยฉบับสมบูรณ์

โครงการเรื่อง:

ผลทางการรักษาและกลไกการออกฤทธิ์ของฟ้าทะลายโจรต่อภาวะท่อน้ำดีอุดตัน

(Therapeutic Effects and Mechanisms of Andrographolide in Intrahepatic Cholestasis)

โดย

ผู้ช่วยศาสตราจารย์ ดร. จิตติมา วีระชยาภรณ์
ภาควิชาสรีรวิทยา คณะวิทยาศาสตร์
มหาวิทยาลัยมหิดล

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(Therapeutic Effects and Mechanisms of Andrographolide in Intrahepatic Cholestasis)

หัวหน้าโครงการวิจัยผู้รับทุน ผู้ช่วยศาสตราจารย์ ดร. จิตติมา วีระชยาภรณ์
ภาควิชาสรีรวิทยา คณะวิทยาศาสตร์
มหาวิทยาลัยมหิดล

นักวิจัยที่ปรึกษา ศาสตราจารย์ ดร. ภาวิณี ปิยะจตุรวัฒน์
ภาควิชาสรีรวิทยา คณะวิทยาศาสตร์
มหาวิทยาลัยมหิดล

Prof. Dr. Michael H. Nathanson
Department of Internal Medicine
Section of Digestive Diseases
Yale University, USA

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

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Investigator: Assist. Prof. Jittima Weerachayaphorn
Department of Physiology
Faculty of Science, Mahidol University
E-mail Address: jittima.wee@mahidol.ac.th
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Abstract:

Cholestasis is a cardinal manifestation of liver disease but effective therapeutic approaches are limited. Andrographolide is a natural diterpenoid lactone from an oriental medicinal plant *Andrographis paniculata* (Burm. f.) Nees (Acanthaceae) that has anti-tumorigenic effects and can protect against hepatocellular liver injury caused by carbon tetrachloride, acetaminophen, concanavalin-A, hexachlorocyclohexane, and ethanol. We investigated whether and how andrographolide could protect against cholestatic liver injury. Intrahepatic cholestasis was induced by intraperitoneal injection of alpha-naphthylisothiocyanate (ANIT). Rats orally received andrographolide or a control solvent for a total period of 4 days. On day 2 of the treatment, rats were injected with ANIT. Serum biochemistry and liver histology were evaluated at 48 hours after ANIT injection. Hepatic transporter expression, markers of inflammation, hepatic stellate cells activation, and bile duct proliferation were examined. Andrographolide treatment attenuated ANIT-induced liver injury. It resulted in marked reductions in serum alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, and gamma-glutamyltransferase plus bilirubin and bile acid levels. Andrographolide treatment also decreased the incidence and extent of inflammation, periductular fibrosis, and bile duct proliferation. Analysis of protein expression in livers from andrographolide-treated cholestatic rats revealed decreased expression of proliferating cell nuclear antigen, alpha-smooth muscle actin, and nuclear factor kappa-B (NF-κB p50 and p65 subunits). In conclusion, andrographolide ameliorates liver injury in ANIT-induced cholestasis. This involves (i) decreasing bile duct proliferation, (ii) suppressing activation of hepatic stellate cells, and (iii) diminishing NF-κB activation. These findings suggest that andrographolide could be a promising therapeutic option in the treatment of cholestatic liver disease.

Keywords: cholestasis, andrographolide, alpha-naphthylisothiocyanate, bile duct injury, NF-κB signaling

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ชื่อนักวิจัย: ผู้ช่วยศาสตราจารย์ ดร. จิตติมา วีระชยาภรณ์
ภาควิชาสรีรวิทยา คณะวิทยาศาสตร์
มหาวิทยาลัยมหิดล
E-mail Address: jittima.wee@mahidol.ac.th
ระยะเวลาโครงการ: 25 กันยายน 2556 – 24 กันยายน 2558

บทคัดย่อ

ภาวะท่อน้ำดีอุดตันเป็นภาวะที่ตับไม่สามารถหลั่งน้ำดีได้ตามปกติ ทำให้มีการสะสมของกรดน้ำดีและก่อให้เกิดการบาดเจ็บในตับ แต่อย่างไรก็ตามในปัจจุบันยังไม่มียาที่มีประสิทธิภาพที่ใช้ในการรักษาภาวะท่อน้ำดีอุดตัน ดังนั้นการศึกษาค้นคว้าเพื่อพัฒนาตัวยาชนิดใหม่จึงมีความจำเป็น จากการศึกษาก่อนหน้านี้มีรายงานว่าฟ้าทะลายโจร (*Andrographis paniculata* (Burm. f.) Nees) เป็นพืชสมุนไพรที่ใช้ในตำรับยาทางอายุรเวชของกลุ่มประเทศแถบเอเชียและมีสารออกฤทธิ์สำคัญทางยาคือสารแอนโดรกราโฟไลด์ ซึ่งมีผลทางยาในการต้านการอักเสบ และป้องกันการบาดเจ็บของตับจากสารพิษต่างๆ อาทิเช่น คาร์บอนเตตระคลอไรด์ อะเซตามิโนเฟน คอนคานาวาลิน-เอ และแอลกอฮอล์ จึงเป็นที่สังเกตว่าแอนโดรกราโฟไลด์อาจจะเป็นยาอีกทางเลือกหนึ่งในการใช้รักษาภาวะท่อน้ำดีอุดตันในตับได้ แต่อย่างไรก็ตามปัจจุบันนี้ยังไม่มีรายงานผลทางการรักษาของสารแอนโดรกราโฟไลด์ต่อภาวะท่อน้ำดีอุดตันในตับ ดังนั้นโครงการวิจัยนี้จึงมีวัตถุประสงค์เพื่อศึกษาผลของสารแอนโดรกราโฟไลด์ต่อการป้องกันการบาดเจ็บของตับ และความสามารถในการลดภาวะท่อน้ำดีอุดตันภายในตับรวมถึงกลไกการออกฤทธิ์ของสารแอนโดรกราโฟไลด์ ผลการศึกษาพบว่าการป้อนสารแอนโดรกราโฟไลด์แก่หนูแรทเป็นเวลาสี่วันติดต่อกัน และระหว่างในวันที่สองของการให้สารแอนโดรกราโฟไลด์ หนูแรทถูกเหนี่ยวนำให้เกิดภาวะท่อน้ำดีอุดตันภายในตับโดยการฉีดสาร alpha-naphthylisothiocyanate (ANIT) ทางหน้าท้อง พบว่าเมื่อให้สาร ANIT ไปแล้วเป็นเวลา 48 ชั่วโมง ระดับของเอนไซม์ตับ ระดับของกรดน้ำดีและบิลิรูบินเพิ่มสูงขึ้นในเลือด และมีความผิดปกติของพยาธิสภาพในตับ แต่ผู้วิจัยพบว่าสารแอนโดรกราโฟไลด์สามารถลดระดับการเพิ่มขึ้นของเอนไซม์ตับ ลดระดับของกรดน้ำดีและบิลิรูบินในเลือด ลดการอักเสบของตับและท่อน้ำดี ลดการกระตุ้นการทำงานของ hepatic stellate cells และลดการเพิ่มจำนวนของเซลล์ท่อบุน้ำดีในหนูแรทที่ถูกเหนี่ยวนำด้วยสาร ANIT นอกจากนี้ผู้วิจัยยังพบว่าสารแอนโดรกราโฟไลด์สามารถลดการแสดงออกของโปรตีน proliferating cell nuclear antigen, cyclin D1, alpha-smooth muscle actin และ nuclear factor kappa-B (NF-κB) ส่วนกลไกในการออกฤทธิ์ของสารแอนโดรกราโฟไลด์ในการป้องกันการบาดเจ็บของตับจากภาวะท่อน้ำดีอุดตัน พบว่าสารแอนโดรกราโฟไลด์ลดการเกิดการอักเสบในตับและท่อน้ำดี โดยผ่านกระบวนการซึ่งไปลดการแสดงออกของโปรตีน NF-κB ลดการเพิ่มจำนวนของเซลล์ท่อบุน้ำดี และยับยั้งการกระตุ้น hepatic stellate cells ซึ่งเป็นเซลล์ที่ก่อให้เกิดการสร้างพังพืดในท่อน้ำดี ดังนั้นจึงเป็นการช่วยทำให้ท่อน้ำดีไม่เกิดการอุดตัน รวมทั้งลดปัญหาการคั่งของกรดน้ำดีในตับ ผลการวิจัยสรุปได้ว่าสารแอนโดรกราโฟไลด์สามารถป้องกันภาวะท่อน้ำดีอุดตันในตับ ผลการวิจัยนี้ชี้ให้เห็นว่าสารแอนโดรกราโฟไลด์น่าจะนำมาใช้ประโยชน์ทางยาและเป็นอีกทางเลือกหนึ่งในการรักษาภาวะท่อน้ำดีอุดตันในตับ

คำสำคัญ: ภาวะท่อน้ำดีอุดตัน ฟ้าทะลายโจร แอนโดรกราโฟไลด์ เซลล์ท่อบุน้ำดี การอักเสบในตับ

Executive Summary

ความสำคัญและที่มาของปัญหา

Hepatobiliary transport systems are essential for the uptake and secretion of numerous compounds including bile acids. Disruption, disturbance and dysregulation of this excretory pathway that occurs from a variety of causes in humans result in cholestasis and that leads to the intrahepatic accumulation of bile acids and other toxic compounds with progression of liver injury and fibrosis. Prolonged, chronic cholestasis causes liver fibrosis, liver cirrhosis and eventually liver failure. Nearly one in five liver transplants in the United States is for chronic cholestatic conditions in patients with disorders such as primary biliary cirrhosis and primary sclerosing cholangitis, and cholestatic disorders are the most common indication for transplant among pediatric patients. Currently, ursodeoxycholic acid (UDCA) is the only drug approved by the Food and Drug Administration (FDA). UDCA has been the most successful drug to be used in cholestasis specifically for the treatment of primary biliary cirrhosis, nonetheless, not all cholestatic patients respond well to UDCA and the effectiveness of UDCA is limited to the early stages of primary biliary cirrhosis and the risk for disease progression still remains. Also UDCA has no proven benefit in primary sclerosing cholangitis (PSC) and even be harmful when administered in high doses to patients with PSC. Despite the molecular basis of cholestasis and the pathophysiology of hepatic fibrosis have been extensively studied, new therapeutic approaches have been still limited. Thus, agents need to be explored to find and develop alternative drugs and beneficial treatments for the cholestasis. Recently, we found that andrographolide, a Thai traditional herb plant, possess a variety of pharmacological activities including anti-inflammatory, anticancer, and hepatoprotective effects. Therefore, andrographolide appears to be an attractive, promising candidate for therapy of cholestatic liver diseases. However, to date, the effects of andrographolide on cholestasis have not been investigated. Therefore, a study on therapeutic potential of andrographolide and molecular mechanisms of action of andrographolide on cholestatic liver injury need to be elucidated. The objectives of this research project are to investigate the effect of andrographolide on the alpha-naphthylisothiocyanate (ANIT)-induced cholestatic liver injury and to determine the molecular mechanisms underlying the therapeutic potential of andrographolide on intrahepatic cholestasis. This present study indicates that andrographolide ameliorates liver injury in ANIT-induced cholestasis. This study is the first report providing *in vivo* evidence of hepatoprotection by andrographolide in an animal model of intrahepatic cholestatic liver disease, and suggests that andrographolide may be an effective alternative agent and supports the potential utility of andrographolide for the treatment of intrahepatic cholestasis.

เนื้อหางานวิจัย

Objectives:

The overall goal of this research project is to investigate whether andrographolide could be an alternative promising candidate for therapy of intrahepatic cholestasis. This study is aimed to investigate potential therapeutic effects and molecular mechanisms of action of andrographolide on the ANIT-induced cholestatic liver injury in rats.

The specific objectives of the research project:

Part I. To investigate the effects of andrographolide on ANIT-induced cholestatic liver injury

Part II. To determine the molecular mechanisms underlying the therapeutic potential of andrographolide on intrahepatic cholestasis

Methodology:

1. Reagents:

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). BSEP antibody was purchased from Kamiya Biomedical (Seattle, WA, USA). Monoclonal antibody to MRP2 was obtained from ALEXIS Biochemicals (San Diego, CA, USA). Anti-MRP3 and anti-MRP4 antibodies were purchased from Abcam (Cambridge, MA, USA) and Everest Biotech (Oxfordshire, UK), respectively. Monoclonal antibodies to β-actin and alpha-smooth muscle actin (α-SMA) were obtained from Sigma-Aldrich. Polyclonal antibodies to NF-κB p50, NF-κB p65 and proliferating cell nuclear antigen (PCNA) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2. Animals and experimental design:

The animal study (protocol no. MUSC54-027-273) was approved by the Institutional Animal Care and Use Committee, Mahidol University, in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health. Adult male Wistar rats weighing between 170 and 200 grams were obtained from the National Laboratory Animal Center, Bangkok, Thailand. Animals were housed in animal facility under a 12-hour light: 12-hour dark cycle at temperature 22°C, and allowed free to food and water. Animals were acclimatized for at least 5 days prior to the experiments. Rats were

randomized and pretreated orally with andrographolide at a dose of 50 and 100 mg/kg body weight, which are pharmacological doses and in the safe dose range, or the control solvent (1% carboxymethylcellulose, CMC) once daily for 4 days prior to receive the ANIT. On the second day of treatment, rats were intraperitoneally administered a single dose of ANIT (75 mg/kg body weight in olive oil) to induce cholestasis. A second set of vehicle-treated rats was given only olive oil to serve as the solvent control. At 48 hours after ANIT administration, animals were fasted overnight prior to sacrifice. Serum and liver samples were collected. Serum liver enzyme measurements for clinical chemistry parameters indicative of liver damage were made immediately after processing of blood samples. Liver tissues were snap frozen in liquid N_2 and stored in a −80°C freezer. A part of the liver from each rat was fixed in 10% neutral buffered formaldehyde for histological study.

3. Biochemical analysis:

The levels of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), total bilirubin (TBIL), direct bilirubin (DBIL) and indirect bilirubin (IDBIL) levels were measured using a commercial kit (Human Gesellschaft für Biochemica und Diagnostica mbH, Wiesbaden, Germany). Bile acids were extracted from liver homogenates as previously described (Mennone et al., 2006). Bile acid concentrations in the serum and liver were determined using a commercial total bile acid kit (Diazyme Laboratories, Poway, CA, USA).

4. Liver histology and immunohistochemical analysis

Formalin-fixed liver was embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Immunohistochemical analysis of α-SMA and PCNA was performed on paraffin embedded rat liver sections. The liver tissues were deparaffinized in xylene and rehydrated through a graded alcohol series. Antigen retrieval using 10 mM sodium citrate (pH 6.0), in the microwave oven for 20 min (750 watts) was done prior to overnight incubation with anti-α-SMA or anti-PCNA antibodies. The tissue sections were followed by incubation with horseradish peroxidase-linked anti-mouse IgG for 1 hour at room temperature. Finally, tissue sections were developed for 60 second using the NovaRED staining kit (Vector Laboratories, Burlingame, CA, USA), and counterstained with hematoxylin. Tissue slides were examined and imaged using a Nikon Eclipse E600 microscope-fitted with Nikon digital camera DXM1200 (Nikon Inc., Melville,

NY). All images were captured using the same settings, and images were processed with Adobe Photoshop CS6 (Moutainview, CA, USA).

5. Western blot analysis

The rat liver was homogenized using a TissueLyser LT (QIAGEN, Valencia, CA, USA) in RIPA lysis buffer (Amresco, OH) containing Halt protease inhibitor cocktail (Thermo Scientific, IL, USA). The protein concentration of each sample was determined using a BCA protein assay kit (Pierce, IL, USA). Western blot analysis was performed. The proteins separated by SDS-PAGE were blotted onto nitrocellulose membranes. Blots were probed with the primary antibody at 4°C overnight and followed by incubation with secondary antibody conjugated with HRP for 1 hour at room temperature. The immunoreactive protein was detected with the SuperSignal West Pico Chemiluminescent Substrate kit (Pierce). Immunoblots were visualized and the relative quantities of protein expression were analyzed using the ImageJ software developed at the National Institutes of Health (NIH, Bethesda, USA). The β-actin was used as internal control and was used as a reference to normalize data.

6. TaqMan reverse transcription quantitative PCR

Total RNA was prepared from rat liver tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The RNA was then cleaned up and purified using the RNeasy MinElute Cleanup kit (Qiagen). RNA was converted to cDNA using RT-PCR kit-RNA to cDNA EcoDry Premix (Clontech Laboratories, Mountain View, CA, USA). Quantitative TaqMan real-time PCR was performed on an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster city, CA, USA). All primers were TaqMan Gene Expression Assays and were purchased from Applied Biosystems. The expression of target genes was normalized to *Gapdh* and quantification of relative expression was determined by the Pfaffl's method.

7. Cell culture

The HepG2 cell line was obtained from American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (Gibco-Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Gibco). Cells were maintained in 37°C, 5% CO2 incubator.

8. Transient transfection and luciferase reporter gene assay

HepG2 cells (2×10^5 cells per well) were grown in 24-well plates for 24 hours. Cells were cotransfected with the NF-κB luciferase reporter plasmid (luc2P/NF-κB-RE/Hygro, Promega, Madison, WI, USA) or empty vector pGL4.13 (Promega) with NF-κB p65 along with a pRL-CMV renilla luciferase (Promega) as a control for transfection efficiency using XtremeFuGENE HP DNA transfection reagent (Roche, Indianapolis, IN, USA). Fourty-eight hours following transfection, cells were treated with either DMSO control or andrographolide for an additional 48 hours. Cells were lysed in passive lysis buffer (Promega). Cell lysates were used to measure firefly and renilla luciferase activity using the dual-luciferase reporter gene assay system (Promega) according to the manufacturer's instructions. Luciferase activity was measured using the Synergy 2 plate reader (BioTek, Winooski, VT, USA). Results are presented as firefly luciferase activity.

9. BrdU proliferation assay

The proliferation rate was measured using the cell proliferation enzyme-linked immunosorbent assay BrdU kit (Roche Applied Science; Indianapolis, IN, USA) according to the manufacturer's instructions. In brief, HepG2 cells (2×10^4 cells per well) were cultured into 96-well tissue culture plates for 24 hours. In a control group, cells were serum starved in a serum-free medium; respective cells were treated either with DMSO or andrographolide in 10% FBS medium for a period of 48 hours. After that, cells were labeled with 10 mM of BrdU and incubated for 4 hours at 37°C. Cells were fixed and genomic DNA was denatured by adding of FixDenat reagent. Peroxidase-labeled anti-BrdU antibody (1:100) was added and incubated for 90 minutes at room temperature. After washing three times, 3,3′5,5′-tetramethylbenzidine substrate solution was added and incubated until color appearance. The reaction was then stopped by adding 1 M H_2SO_4 and finally optical density was measured using a microplate reader at an absorbance of 450 nm. Proliferation of cells was calculated as percentage of vehicle controls.

10. Immunofluorescence staining and confocal microscopy

HepG2 cells plated onto coverslips were fixed in cold methanol (-20°C) and permeabilized with 0.05% Triton-X in PBS. After blocking, cells were incubated with anti-NF-κB p65. Alexa Fluor 488 anti-rabbit immunoglobulin G (Molecular Probes, Eugene, OR, USA) was used as the

secondary antibody. Nuclei were stained using TOPRO-3 (Molecular Probes). The images were visualized with a Zeiss LSM 510 Meta confocal microscope (Carl Zeiss, Thornwood, NY, USA). All images were captured using the same settings and processed with Adobe PhotoshopCS 6 (Moutainview, CA, USA).

11. Statistical Analysis

All data are expressed as mean values ± standard deviation (SD). Prism 6 software (GraphPad, La Jolla, CA, USA) was used for data analysis. Statistical analyses were performed using two-tailed unpaired Student's t-test or One-way ANOVA when three or more groups were compared and followed by Dunnett's multiple comparison test. Differences with $p < 0.05$ were considered to be statistically significant. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ versus ANIT treatment.

Experimental Design and Results:

Part I. To investigate the effects of andrographolide on ANIT-induced cholestatic liver injury

Previous reports have been shown that ANIT-induced hepatotoxicity in experimental animals is a useful model for studying mechanisms of intrahepatic cholestasis. ANIT administration to rats also mimics to drug-induced cholestasis in human. Models of cholestasis are well-established in Wistar rats. Therefore, we induced intrahepatic cholestasis in adult male Wistar rats and animals were treated with andrographolide, which is kindly provided by Prof. Dr. Apichart Suksamrarn (Department of Chemistry, Faculty of Science, Ramkhamhaeng University). In this study, the animals were randomly divided into 4 major groups that were given one of the following treatments: 1) solvent control, 2) andrographolide control, 3) ANIT and 4) ANIT treated with andrographolide. Rats orally received daily with solvent control, 1% carboxymethyl cellulose (CMC) or andrographolide varying doses from 50 to 200 mg/kg body weight for 2 days prior to ANIT injection. Rats were intraperitoneally injected with ANIT at a dose of 75 mg/kg body weight. Andrographolide was continuously given to rats with and without ANIT injection for 2 days. Twenty-four hours after the last dose of andrographolide (on the fourth day, 48 hours after ANIT administration), rats were sacrificed. Blood and the liver were collected for biochemical analyses, including bile acid and bilirubin levels. Serum was collected for determination of liver enzyme activities, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyl

transpeptidase (GGT), and part of livers were fixed in 10% buffered formalin for histological analyses to examine whether the treatment of andrographolide improves liver functions/injury markers and hepatic and bile duct conditions. Liver histology was assessed for inflammation, necrosis, bile duct proliferation and fibrosis. The remaining part of livers were removed and stored at -80°C for mRNA and protein expression.

We found that the level of liver enzyme activities, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyltransferase (GGT) were increased in ANIT-treated rats. The levels of ALT, AST, ALP and GGT were higher in andrographolide (50 mg/kg BW)/ANIT-treated rats than in control group. In contrast, treatment with andrographolide at a dose of 100 mg/kg BW resulted in marked decreases in ALT, AST, ALP and GGT levels compared with ANIT-treated rats (**Figure 1**, **Figure 2**). Serum and hepatic bile acid levels were also increased after ANIT injection. Pretreatment of andrographolide at a dose of 50 mg/kg BW to ANIT-treated rats still exhibited the high levels of serum and hepatic bile acids. However, pretreatment with andrographolide at a dose of 100 mg/kg BW to ANIT-treated rats significantly reduced both serum and hepatic bile acid levels (**Figure 3**). In addition, the levels of total bilirubin, direct bilirubin, and indirect bilirubin were significantly increased in ANIT-treated rats. Andrographolide at a dose of 50 mg/kg BW did not show alterations in serum bilirubin levels compared to ANIT-treated rats. However, the administration of andrographolide at a dose of 100 mg/kg BW to ANIT-treated rats significantly decreased the levels of total bilirubin, direct bilirubin, and indirect bilirubin (**Figure 4**). Histological examination and analysis of liver sections stained with hematoxylin followed by eosin (H & E) revealed that liver sections of normal rats treated with and without andrographolide exhibited normal structure and no morphological changes. The liver specimen of ANIT-treated rats demonstrated the liver injuries characterized by the proliferation of bile duct and infiltration of inflammatory cells. Pretreatment of andrographolide at a dose of 50 mg/kg BW to ANIT-treated rats also showed liver damages around portal areas. In contrast, the administration of andrographolide at a dose of 100 mg/kg BW to ANIT-treated rats exhibited less histological damages than rats treated with ANIT alone (**Figure 5**). Taken together, our present findings demonstrated that andrographolide at a dose of 100 mg/kg BW could protect ANIT- induced cholestatic liver injury in rats.

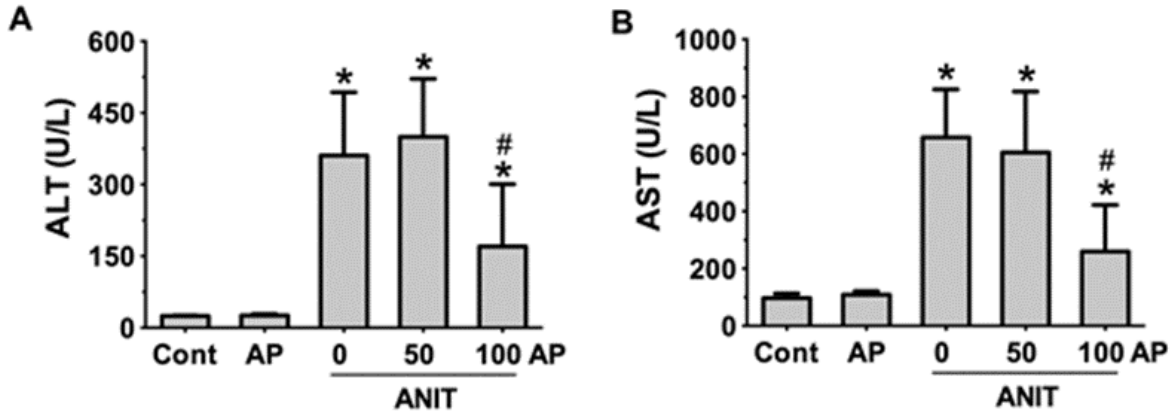

Figure 1. Effect of andrographolide (AP) treatment on serum **(A)** alanine aminotransferase (ALT) and **(B)** aspartate aminotransferase (AST) in ANIT-induced cholestatic liver injury in rats. *$p < 0.05$ significantly different from control rats. $^{\#}p < 0.05$ significantly different from rats treated with ANIT alone.

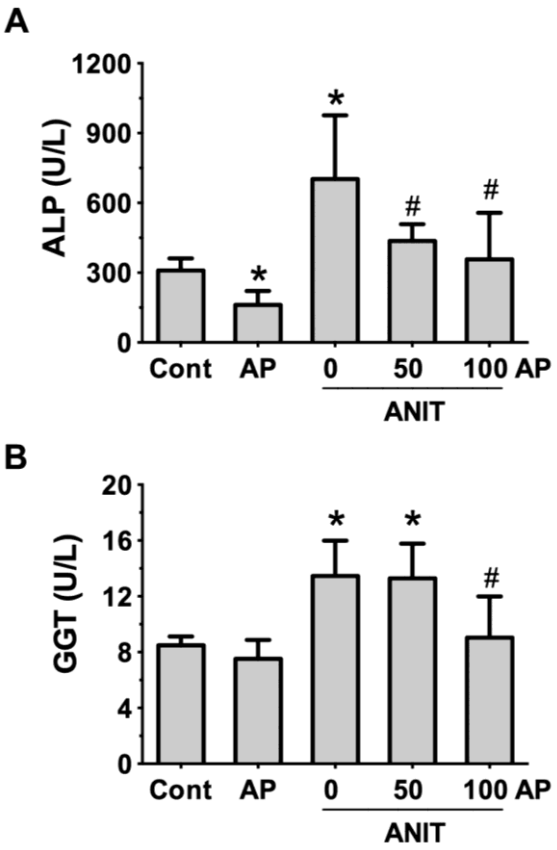

Figure 2. Effect of andrographolide (AP) treatment on serum **(A)** alkaline phosphatase (ALP) and **(B)** gamma-glutamyltransferase (GGT) in ANIT-induced cholestatic liver injury in rats. *$p < 0.05$ significantly different from control rats. $^{\#}p < 0.05$ significantly different from rats treated with ANIT alone.

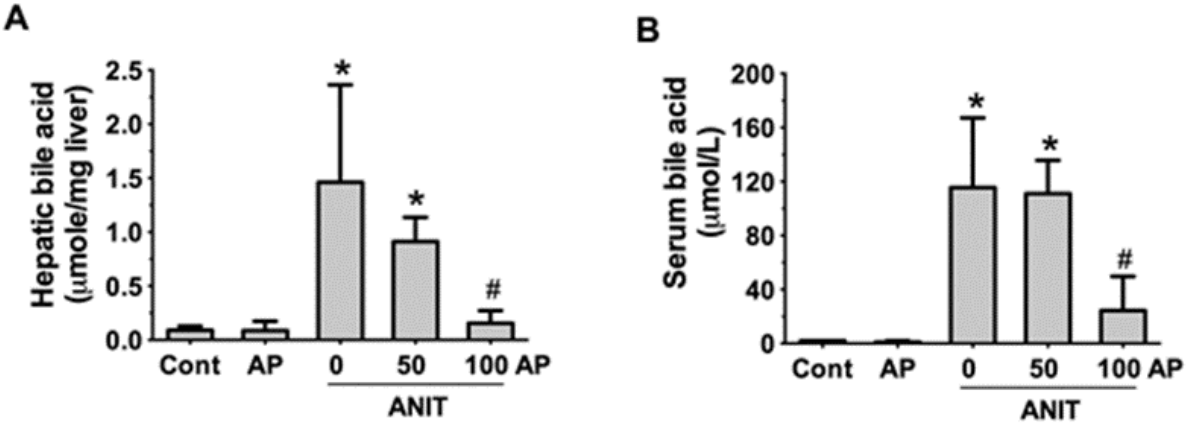

Figure 3. Effect of andrographolide (AP) treatment on **(A)** hepatic and **(B)** serum bile acid levels in ANIT-induced cholestatic liver injury in rats. *$p < 0.05$ significantly different from control rats. $^{\#}p < 0.05$ significantly different from rats treated with ANIT alone.

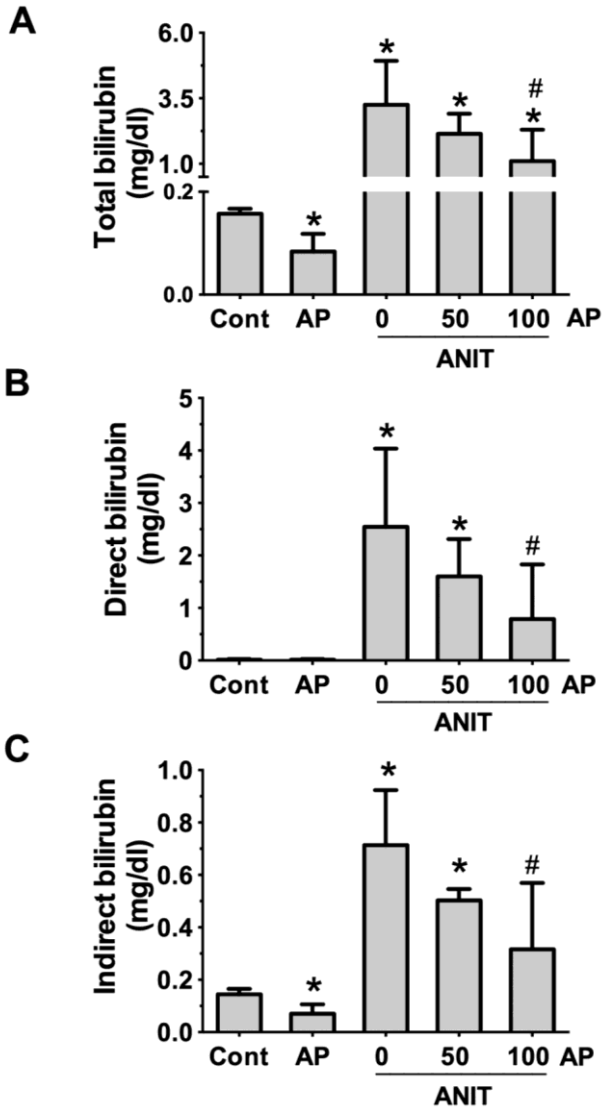

Figure 4. Effect of andrographolide (AP) on **(A)** total bilirubin, **(B)** direct bilirubin, and **(C)** indirect bilirubin levels in ANIT-induced cholestatic liver injury in rats. $^{*}p < 0.05$ significantly different from control rats. $^{\#}p < 0.05$ significantly different from rats treated with ANIT alone.

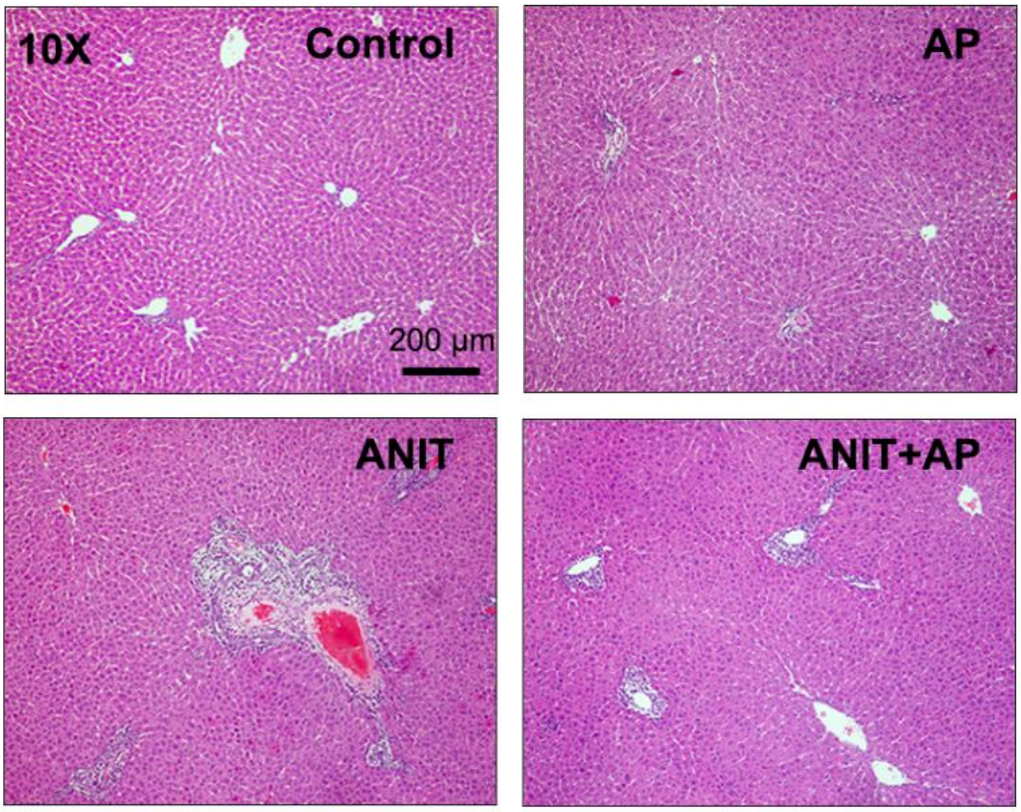

Figure 5. Representative photomicrographs of livers stained with hematoxylin and eosin (H&E) from control and ANIT-treated rats pretreated with solvent control, 1% carboxymethyl cellulose (CMC) or andrographolide (AP). Andrographolide/ANIT treated rats showed less severity of histological damage and less bile duct proliferation. (Original magnification, 10X and scale bar = 200 μM.

Part II: Determine the molecular mechanisms underlying the therapeutic effects of andrographolide on intrahepatic cholestasis

2.1. Investigate whether the adaptive defense mechanism is associated with the therapeutic effect of andrographolide to ANIT-induced liver injury with cholestasis

Because, in cholestasis, the induction of alternative efflux transporters, MRP3, MRP4 and OSTα/OSTβ, takes into account the adaptive defense mechanisms in response to cholestatic liver injury and plays a vital role in protecting the liver from obstructive cholestasis. Stimulation of orthograde biliary secretion and induction of alternative efflux transporters to retrograde secretion of bile acids and other toxic cholephils into the systemic circulation for excretion via the kidneys to reduce their retention in hepatocytes is one of the pharmacological targets for treatment of intrahepatic cholestasis. We, therefore, examined whether induction of

alternative efflux routes contributes to the therapeutic effects of andrographolide. We examined the protein expression of hepatic efflux transporters on sinusoidal and canaliculi membranes in andrographolide-fed ANIT rats. As shown in **Figure 6**, ANIT administration increased MRP3 and MRP4 protein expression. However, less pronounced induction of both MRP3 and MRP4 was observed in andrographolide-fed ANIT rats (**Figure 6A, 6B**). Protein expression of the bile salt export pump (BSEP) was unchanged by ANIT administration (**Figure 6C**), while MRP2 expression was significantly decreased by ANIT (**Figure 6D**).

2.2. Examine if andrographolide treatment affects the hepatic expression of pro-inflammatory and pro-fibrogenic genes related to cholestasis

It has been known that hepatocellular injury activates the innate immune system, thereby leading to release of growth factors and cytokines that stimulate extracellular matrix (ECM) synthesis. As the positive therapeutic effects of andrographolide have been ascribed to the anti-inflammatory property, we further examined if andrographolide treatment could affect the hepatic expression of pro-inflammatory and pro-fibrogenic genes related to cholestasis. We measured the mRNA expression of pro-inflammatory (*TNF-α)* and key genes involved in hepatic fibrogenesis (α-SMA). We found the mRNA expression of *TNF-α* was increased in ANIT-treated rat liver, and this induction was decreased in response to andrographolide treatment (**Figure 7**). ANIT-treated rats exhibited a marked increase in hepatic α-SMA protein expression (**Figure 8**). In addition, as shown in **Figure 9**, we found that the IHC stained liver sections of ANIT-treated rats exhibited a marked increase in the number of α-SMA-positive cells. In comparison, less α-SMA-positive cells and less periductal fibrosis were seen throughout in andrographolide/ANIT-treated rats. These findings suggest that andrographolide reduced periductal inflammation and fibrosis. Together, these findings suggest that andrographolide exerts an anti-inflammatory effect.

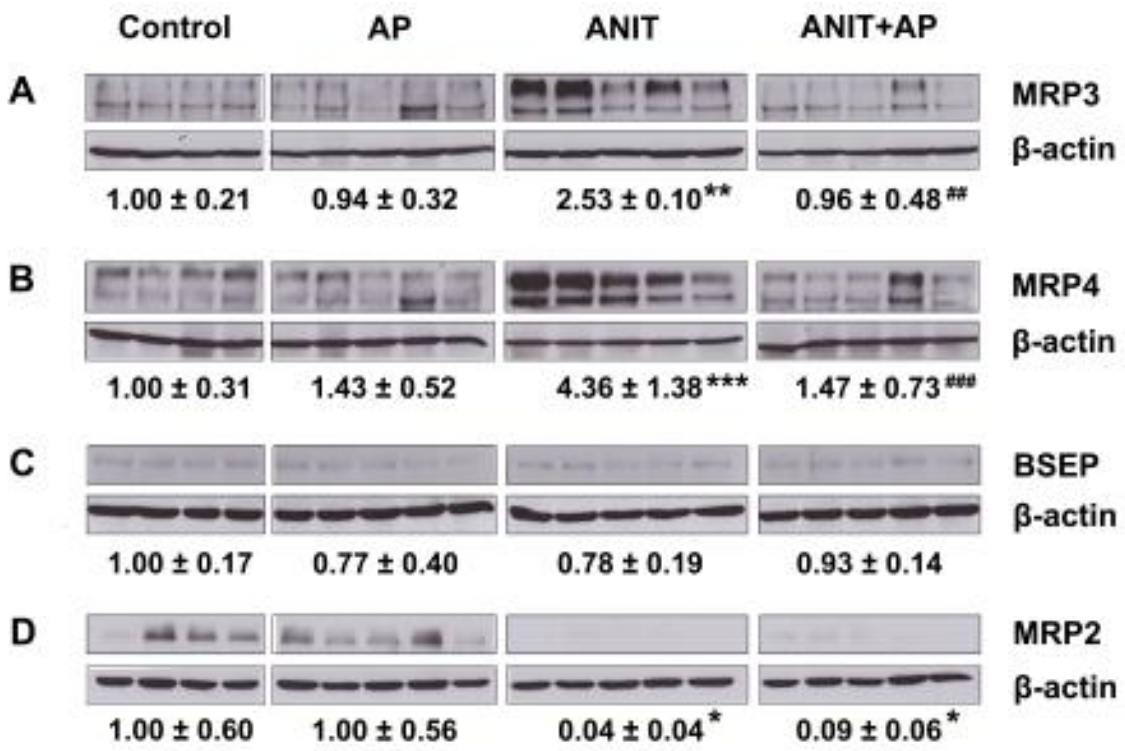

Figure 6. Effect of andrographolide on protein expression of hepatic bile acid transporters. Immunoblot analysis showed the expression of the basolateral membrane transporters **(A)** MRP3 and **(B)** MRP4, and the canalicular membrane transporters **(C)** BSEP and **(D)** MRP2 in control and ANIT-treated rats pretreated with CMC or andrographolide. $^{*}p < 0.05$ significantly different from control rats. $^{\#}p < 0.05$ significantly different from rats treated with ANIT alone. AP, andrographolide.

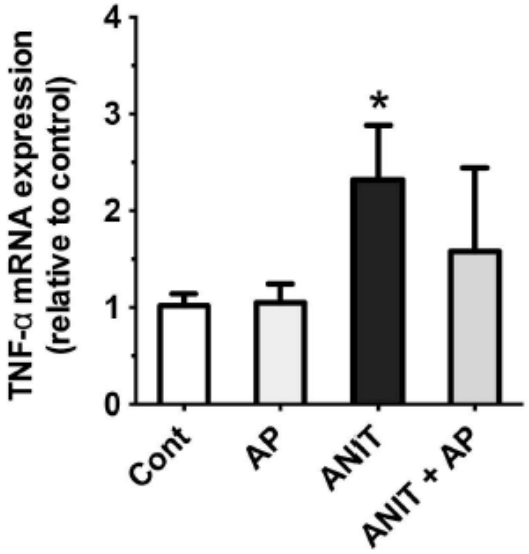

Figure 7. Effect of andrographolide on pro-inflammatory gene expression. Relative mRNA expression of *TNF-α* in livers of control and ANIT-treated rats pretreated with CMC or andrographolide. $^{*}p < 0.05$ significantly different from control rats. AP, andrographolide.

Figure 8. Effect of andrographolide on the expression of protein involved in hepatic fibrogenesis. Immunoblot analysis of α-SMA normalized to β-actin in control and ANIT-treated rat livers pretreated with and without andrographolide. $^{*}p < 0.05$ significantly different from control rats. $^{\#}p < 0.05$ significantly different from rats treated with ANIT alone. AP, andrographolide.

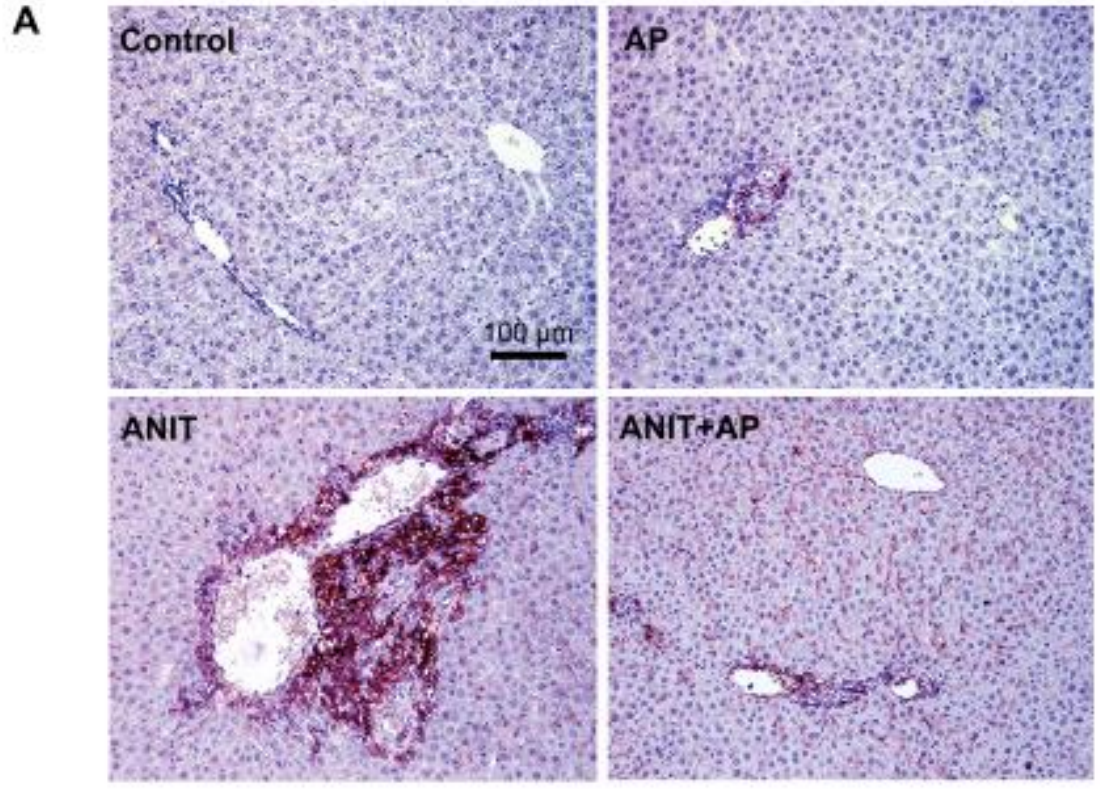

Figure 9. Representative immunohistochemical staining of α-SMA was visualized with NovaRED. Sections stained were from control and ANIT-treated rat livers pretreated with CMC or andrographoide. α-SMA-positive cells are red; Magnification, 20x. Scale bar = 100 μM.

3. Investigate whether the protective effect of andrographolide is associated with inhibition of the NF-κB signaling pathway

It has been known that nuclear factor-kappaB (NF-κB) plays a key role in inflammation and proliferation. A previous study also showed that andrographolide modulates inflammation by inhibition of NF-κB activation. As we found that andrographolide treatment is related to a reduction of pro-inflammatory genes, we further investigated whether the protective effect of andrographolide is associated with inhibition of the NF-κB signaling pathway. We measured the protein expression of the NF-κB (p50), NF-κB (p65) and IκB-α, a negative regulator in the NF-κB signaling pathway. The protein expression of NF-κB p50, p65, and inhibitors of NF-κB (IκB-α) were significantly induced by ANIT injection (**Figure 10**). In contrast, andrographolide treatment significantly reduced the expression of NF-κB p50, p65, and IκB-α compared to ANIT-treated rats.

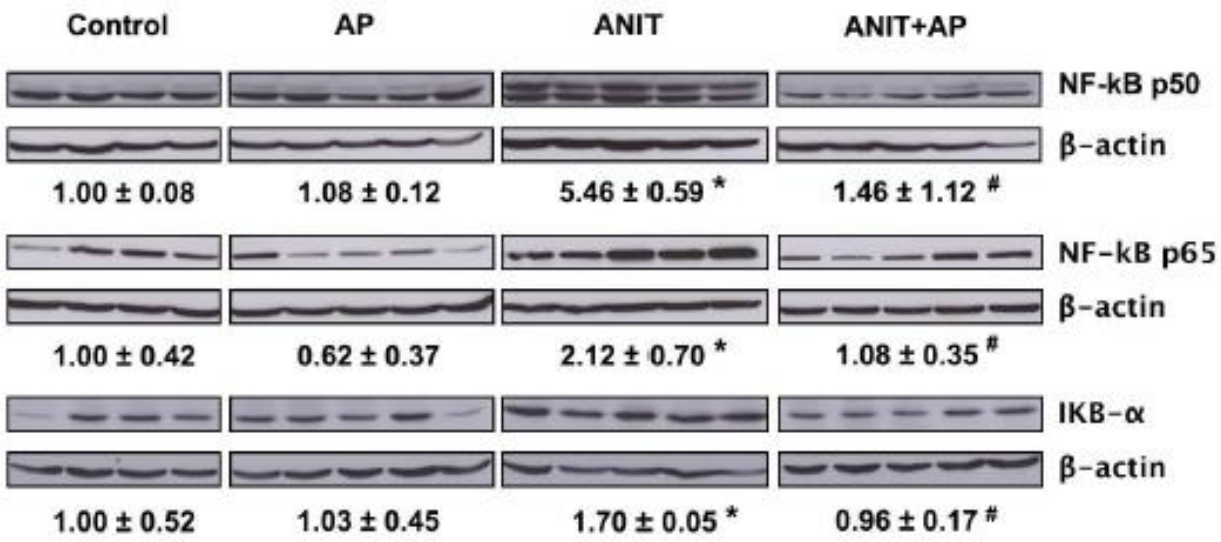

Figure 10. Effect of andrographolide on the NF-κB signaling pathway. Immunoblot analysis of NF-κB p50, p65, and IκB-α from control and ANIT-treated rat livers pretreated with CMC or andrographolide. *$p < 0.05$ significantly different from control rats. #$p < 0.05$ significantly different from rats treated with ANIT alone. AP, andrographolide.

Next, we verified that andrographolide also represses the expression of NF-κB p65 expression in human cells. We treated HepG2 cells with andrographolide. Immunofluorescence staining of HepG2 cells treated with andrographolide showed a dose dependent reduction in NF-κB p65 expression (**Figure 11**), thus suggesting that andrographolide protects the liver by diminishing NF-κB activation.

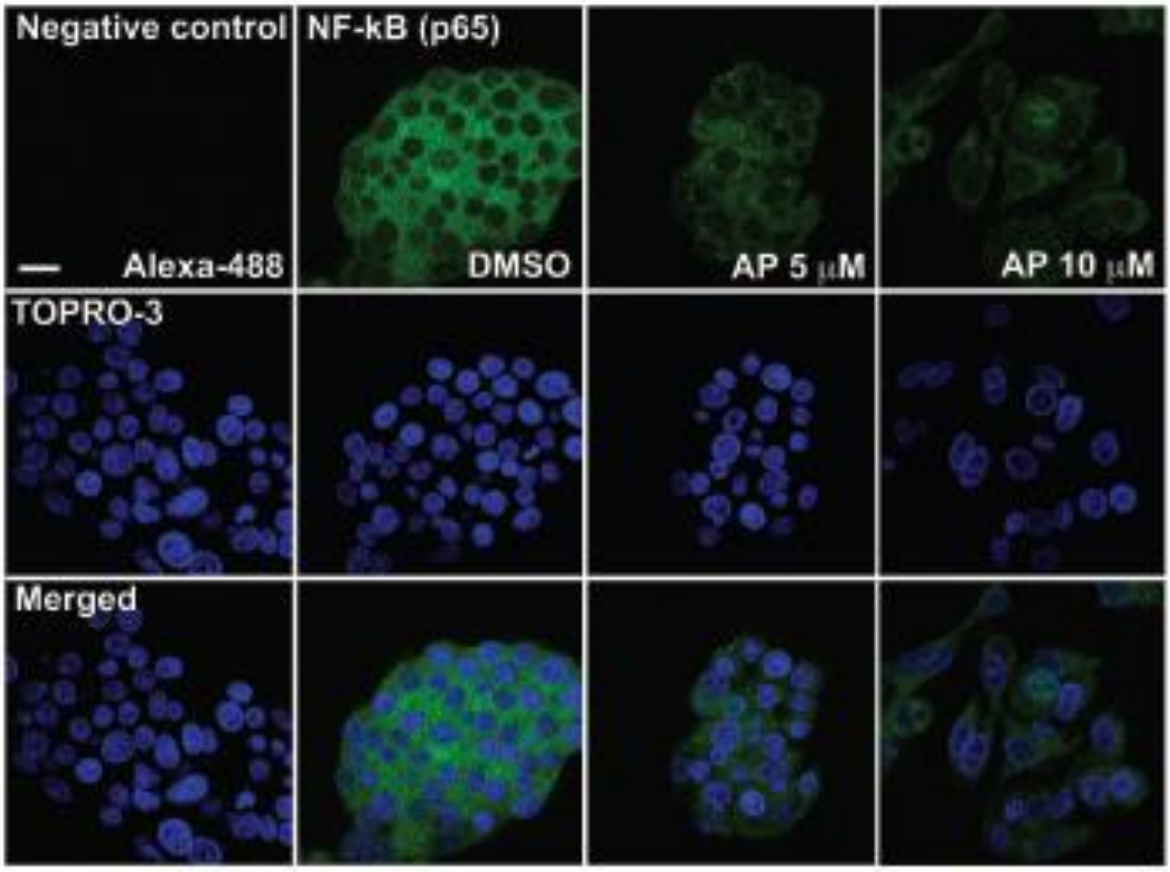

Figure 11. Confocal immunofluorescence images of HepG2 cells treated with DMSO (control) or andrographolide with labeling of NF-κB p65 (*green*). Nuclei were labeled with TOPRO-3 (*blue*).

We further investigated if andrographolide indeed reduces NF-κB activation. As shown in **Figure 12**, in the absence of andrographolide, luciferase promoter activity was increased compared with the pGL4 vector alone. In contrast, addition of andrographolide to transfected HepG2 cells with NF-κB-Luc and NF-κB p65 resulted in a reduction in NF-κB promoter activity.

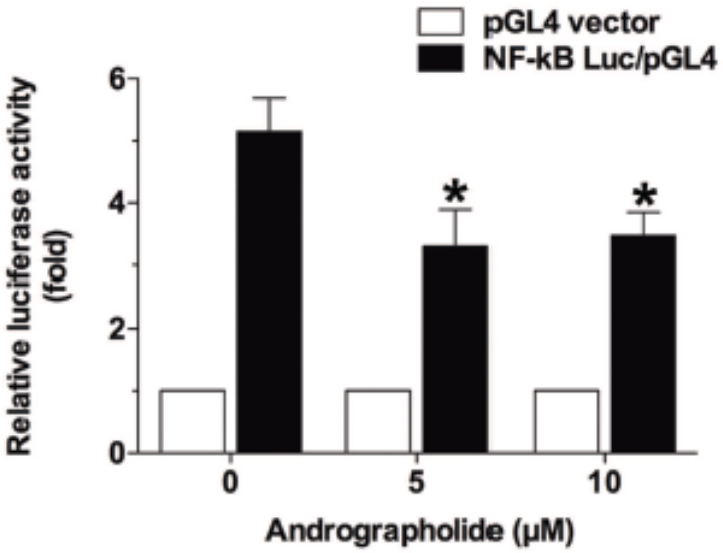

Figure 12. Andrographolide inhibited NF-κB promoter activity. HepG2 cells were co-transfected with the NF-κB p65 and NF-κB luciferase reporter construct and treated with and without andrographolide. Luciferase activity was determined 48 hours after andrographolide treatments. The results are presented as fold changes relative to pGL4 empty vector, n = 4 experiments; *$p < 0.05$ relative to pGL4.

There is evidence that activation of NF-κB is associated with proliferation. Analysis of PCNA immunohistochemical liver staining from rats received ANIT alone showed that ANIT markedly increased PCNA expression (**Figure 13A**). A more profound induction in hepatic PCNA protein expression was also seen in ANIT-treated rats than in the control rats (**Figure 13B**). ANIT treatment increased PCNA protein expression compared to control rats. In contrast, andrographolide treatment resulted in a significant decrease in the number of PCNA-positive cells observed in the portal areas of andrographolide/ANIT-treated rats (**Figure 13A**). Western blotting of hepatic proteins confirmed that andrographolide decreased protein expression of PCNA compared to ANIT-treated rats (**Figure 13B**), suggesting that andrographolide inhibited bile duct cell proliferation.

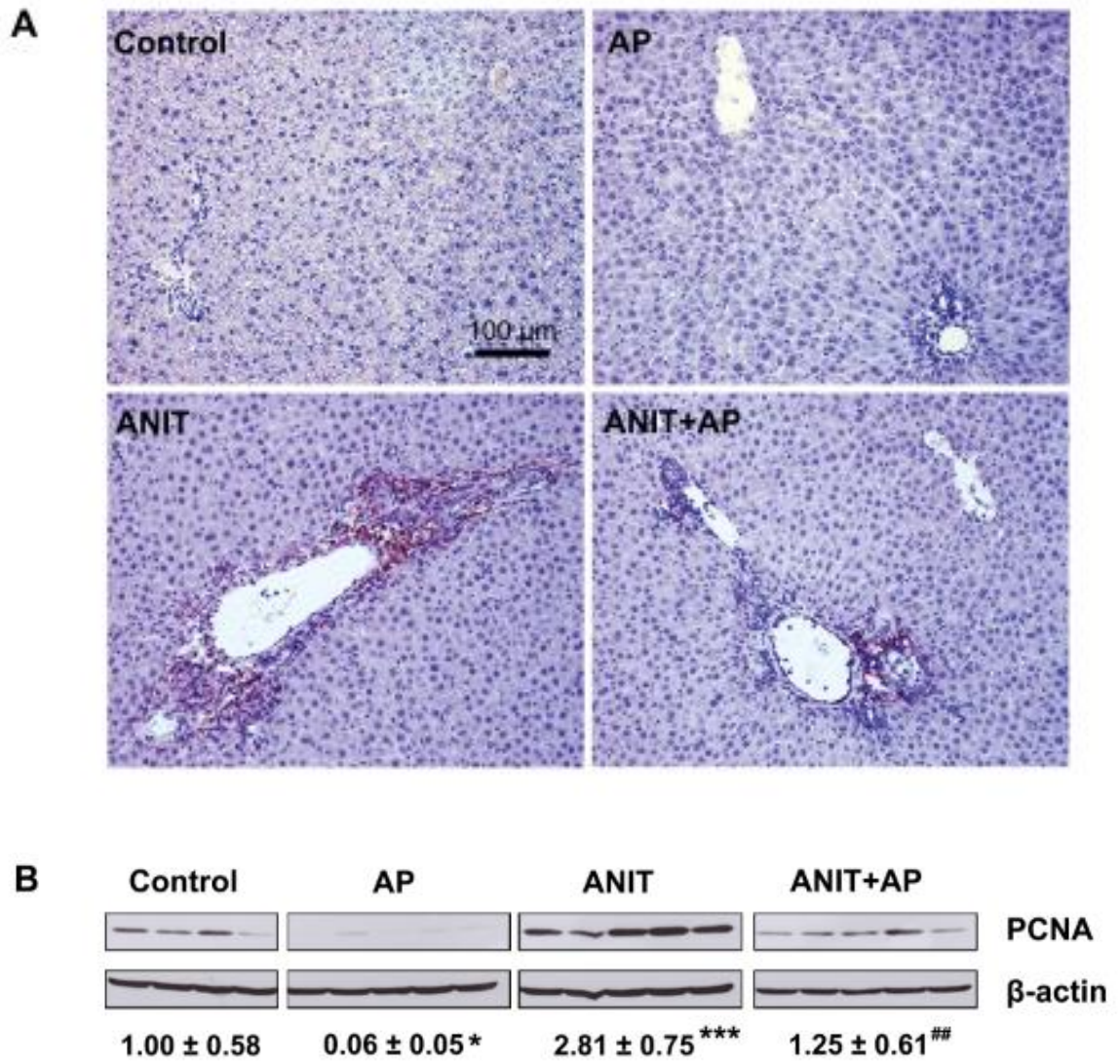

Figure 13. (A) Immunohistochemical staining of PCNA was visualized with NovaRED. PCNA-positive cells are red. Sections stained were from control and ANIT-treated rat livers pretreated with CMC or andrographoide. Magnification, 20x. **(B)** Western blot analysis of hepatic PCNA expression *$p < 0.05$ significantly different from control rats. $^{\#}p < 0.05$ significantly different from rats treated with ANIT alone.

As andrographolide treatment reduced bile duct proliferation in ANIT-treated rats, we investigated whether andrographolide would have similar anti-proliferative effects in HepG2 cells. We found that BrdU uptake was dose dependently reduced in HepG2 cells treated with andrographolide (**Figure 14**), confirming that andrographolide inhibited cell proliferation.

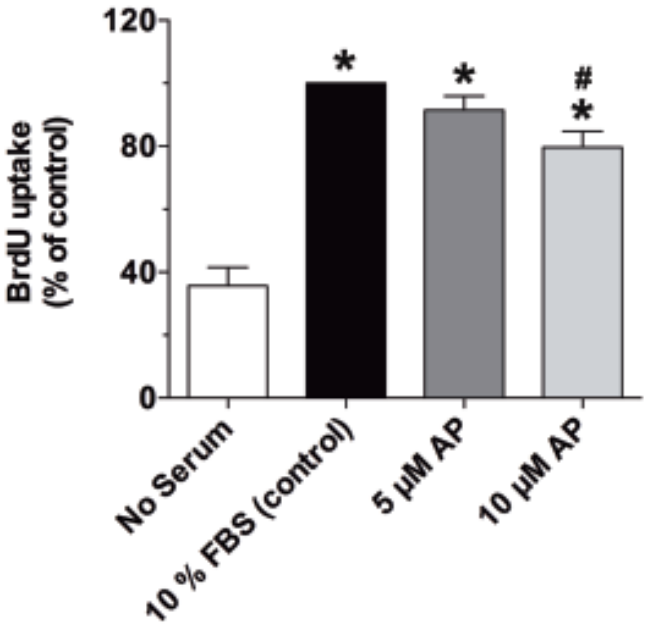

Figure 14. The effect of andrographolide on cell proliferation in HepG2 cells was examined by DNA incorporation of BrdU. Data represent average ± SD, n = 4 experiments; *$p < 0.05$ vs no serum control, $^{\#}p < 0.05$ vs treated cells with DMSO in 10% FBS medium.

Therapeutic concepts targeting nuclear receptor in cholestasis are established. In particular, the pregnane X receptor (PXR), a classical xenobiotic receptor, has been reported to have anticholestatic properties in cholestatic animals. We, therefore, further examined whether andrographolide could increase *PXR* mRNA expression and related *PXR* genes. We found that treatment with andrographolide also gave rise to induction of *Pxr* and also led to increase *Cyp3a2* (also known as *Cyp3a11*), which promotes bile acid metabolism (**Figure 15A, 15B**). Additionally, we checked the expression of key gene involved in bile acid synthesis, *Cyp7a1*. Andrographolide repressed *Cyp7a1* mRNA expression, thus suggesting that andrographolide blocks bile acid biosynthesis. This finding was in agreement with the hepatic bile acid levels (**Figure 3A**). Andrographolide/ANIT-treated rats had decreased levels of hepatic bile acids.

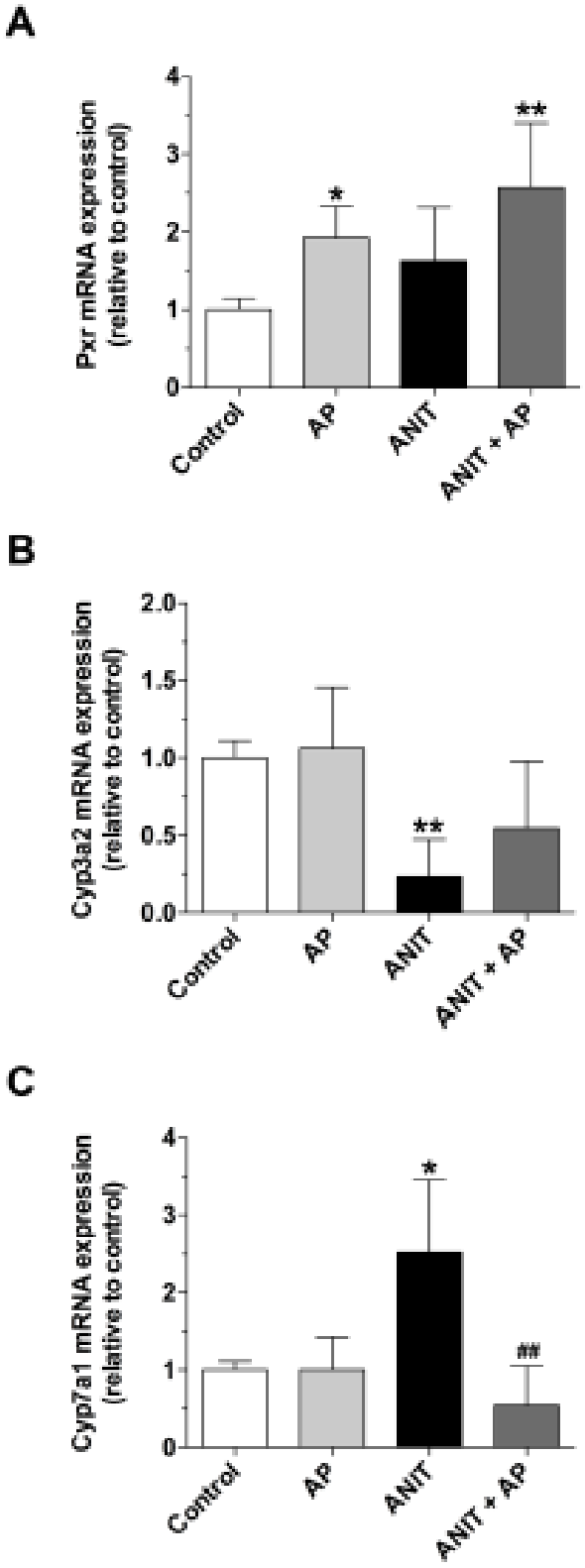

Figure 15. Relative mRNA expression of **A)** *Pxr*, **B)** *Cyp3a2*, and **C)** *Cyp7a1* in livers of control and ANIT-treated rats pretreated with CMC or andrographolide. Data represent mean ± SD, n = 4 - 8 animals per group, *$p < 0.05$ versus control, #$p < 0.05$ versus ANIT-treated rats. AP, andrographolide.

Discussion

Cholestasis leads to liver fibrosis and cirrhosis, which eventually results in liver failure and cancer. So far, effective pharmacologic agents and therapeutic approaches are limited. In this study, we investigated whether andrographolide could protect against cholestatic liver injury. We have shown that andrographolide reduced serum biomarkers of cholestasis and protected bile duct damage against ANIT-induced intrahepatic cholestasis in rats.

ANIT has long been employed to induce cholestatic liver injury (Goldfarb et al., 1962). ANIT is conjugated to glutathione in the hepatocytes and transported across the canalicular membrane by MRP2, resulting in damage to the cholangiocytes lining the bile ducts and the accumulation of bile acids in the liver (Kossor et al., 1995). Adaptive responses to cholestasis to compensate for the loss of biliary excretory function and the accumulation of potentially toxic biliary constituents have been reported in ANIT-induced cholestatic liver injury (Yang et al., 2012). Basolateral bile acid influx transporters, the sodium taurocholate co-transporting polypeptide (NTCP) and the organic anion-transporting polypeptide (OATP), are down-regulated to reduce the uptake of bile acids and other organic anions in cholestasis. Basolateral bile acid efflux transporters, MRP3, MRP4 and organic solute transporters (OST-α/OST-β) are up-regulated in cholestatic liver injury (Paumgartner, 2006). In the present study, profound compensatory changes in the expression of basolateral bile acid transporters, particularly MRP3 and MRP4, were decreased in andrographolide/ANIT-treated rats compared to ANIT-treated rats (Fig. 6). Having less up-regulation of MRP3 and MRP4 could imply that andrographolide/ANIT-treated rats would have less severe cholestatic liver injury. This idea was consistent with the remarkable improvement observed in serum liver enzyme markers and liver histology (Fig. 1- Fig. 5).

How does andrographolide protect against ANIT-induced cholestatic liver injury? A widely appreciated pharmacological property of andrographolide is anti-inflammation, and a link between inflammation and cholestasis has been established (O'Brien et al., 2013). Miyake and coworkers reported that bile acids are capable of inducing Kupffer cells to release proinflammatory cytokines. The hydrophobic bile acid, chenodeoxycholate, induces the mRNA expression of interleukin-1 and tumor necrosis factor-α (TNF-α) (Miyake et al., 2000). In our study, TNF-α mRNA expression also was found to be induced in ANIT-treated rat liver, and this induction was decreased in response to andrographolide treatment (Fig. 7). A reduction in TNF-α expression by andrographolide also correlates with reduction in serum markers of hepatic damage and with severity of liver histological damage (Fig. 1-Fig. 5) consistent with the

idea that andrographolide alleviates cholestatic liver injury through an anti-inflammatory mechanism.

In addition, it was found that andrographolide exerted an anti-inflammatory effect through inhibition of the NF-κB pathway. This impression was supported by the following findings: i) andrographolide treatment inhibited the protein expression of NF-κB p50 and NF-κB p65; and ii) as the activation of hepatic stellate cells is associated with NF-κB activation (Baghdasaryan et al., 2010; Kim et al., 2012), we found that andrographolide inhibited the activation of hepatic stellate cells as seen by a reduction in protein expression of α-SMA (Fig. 8 and Fig. 9). In addition, the critical requirement of NF-κB in the expression of genes involved in proliferation, typified by Cyclin D1, has been reported (Sethi et al., 2008). The inhibition of NF-κB activation led to down-regulation of the Cyclin D1 gene. We found the protein level of Cyclin D1 was decreased in andrographolide/ANIT-treated rats, thus further supporting the idea that andrographolide exerted an anti-inflammatory and anti-proliferative effect through inhibition of the NF-κB pathway.

One of the potential targets for the medical therapy of cholestasis is stimulation of the conversion of hydrophobic bile acids and other toxic compounds to more hydrophilic, less toxic metabolites (Paumgartner, 2006). Therapeutic concepts targeting nuclear receptor in cholestasis are also established. In particular, the pregnane X receptor (PXR), a classical xenobiotic receptor, has been reported to have anticholestatic properties in cholestatic animals (Kliewer and Willson, 2002). Staudinger and coworkers revealed that activation of PXR protects against severe liver damage induced by lithocholic acid, and PXR agonists may be useful in the treatment of human cholestatic liver disease (Staudinger et al., 2001). Studies in humans showed that the PXR ligand rifampicin induces genes involved in bile acid and bilirubin metabolism (Marschall et al., 2005), and indeed rifampicin has been used in the treatment of jaundice and pruritus associated with intrahepatic cholestasis (Wagner et al., 2010). Our findings showed that treatment with andrographolide also gave rise to induction of Pxr expression and led to increased expression of Cyp3a2 (also known as Cyp3a11), which promotes bile acid metabolism (Fig. 15). Furthermore, andrographolide repressed Cyp7a1 expression, which blocks bile acid biosynthesis (Fig. 15). In addition, andrographolide/ANIT-treated rats had decreased levels of hepatic bile acids (Fig. 3A). Induction of Pxr and Cyp3a11 and repression of Cyp7a1 would prevent the accumulation of bile acids to toxic levels and render the bile composition to be less toxic for the injured epithelium. Our data are consistent with the idea that the anti-cholestatic effects of andrographolide may be mediated through PXR. Because andrographolide, like rifampicin, is a potent PXR agonist, our findings may have implications

for the development of new therapeutic agents for the treatment of human cholestatic liver disease.

Conclusion:

This present study indicates that andrographolide ameliorates liver injury in ANIT-induced cholestasis. The underlying mechanisms involve (i) decreasing bile duct proliferation, (ii) suppressing activation of hepatic stellate cells, and (iii) diminishing NF-κB activation. These findings suggest andrographolide could be a promising therapeutic option in the treatment of cholestatic liver disease. This study is the first report providing *in vivo* evidence of hepatoprotection by andrographolide in an animal model of intrahepatic cholestatic liver disease. Data obtained from this research suggest that andrographolide may be an effective alternative agent and support the potential utility of andrographolide for the treatment of intrahepatic cholestasis. Our findings together with the results of earlier studies indicate that andrographolide may be desirable not only for anti-tumorigenesis but also for treatment of cholestatic liver disease. Future work may focus on confirmation of andrographolide's effect in other cholestatic models, and may culminate in clinical trials with andrographolide for patients with intrahepatic cholestatic liver diseases.

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Yang, F., Xu, Y., Xiong, A., He, Y., Yang, L., Wan, Y.J., Wang, Z., 2012. Evaluation of the protective effect of Rhei Radix et Rhizoma against alpha-naphthylisothiocyanate induced liver injury based on metabolic profile of bile acids. Journal of ethnopharmacology 144, 599-604.

Research Outputs:

1. **Human Resource Development (HRD):** this research, in part, produces a graduate student in an academic institution. One graduate student, Tanaporn Khamphaya, Toxicology Graduate Program, Faculty of Science, Mahidol University, obtained a Master Degree in Toxicology in July 2014.

2. **Knowledge:**

   2.1 National Proceeding:

   Tanaporn Khamphaya, Pawinee Piyachaturawat and Jittima Weerachayaphorn. Effect of *Andrographis paniculata* on alpha-naphthylisothiocyanate-induced cholestatic liver injury in rats.
   Kasetsart University Annual Conference (52nd Annual meeting, February 4-7, 2014)

   2.2 International Publication:

   Tanaporn Khamphaya, Piyachat Chansela, Pawinee Piyachaturawat, Apichart Suksamrarn, Michael H. Nathanson and Jittima Weerachayaphorn. Protective effect of andrographolide isolated from *Andrographis paniculata* against alphanaphthylisothiocyanate-induced cholestatic liver injury. (*Submitted to Journal of Ethnopharmacology* (*please see Appendix/attachment*)

Appendix:

1. Submitted manuscript to Journal of Ethnopharmacology (Elsevier),

Elsevier Editorial System(tm) for Journal of Ethnopharmacology
Manuscript Draft

Manuscript Number: JEP-D-15-02404

Title: Protective effect of andrographolide isolated from Andrographis paniculata against alpha-naphthylisothiocyanate-induced cholestatic liver injury

Article Type: Research Paper

Keywords: alpha-naphthylisothiocyanate, andrographolide, cholestasis, bile duct, liver injury

Corresponding Author: Prof. Jittima Weerachayaphorn, Ph.D.

Corresponding Author's Institution: Yale University

First Author: Tanaporn Khamphaya, M.Sc.

Order of Authors: Tanaporn Khamphaya, M.Sc.; Piyachat Chansela, Ph.D.; Pawinee Piyachaturawat, Ph.D.; Apichart Suksamrarn, Ph.D.; Michael H Nathanson, MD., Ph.D.; Jittima Weerachayaphorn, Ph.D.

Abstract: Ethnopharmacological relevance: Andrographolide is a diterpene lactone, which is the main bioactive compound isolated from the leaves of Andrographis paniculata. This medicinal plant Andrographis paniculata has been widely used as a traditional medicine for treatment of fever, common cold, diarrhea and other infectious ailments in many Asian countries.
Aim of the study: This present study aims to investigate the hepatoprotective effect and possible mechanism of andrographolide on alpha-naphthylisothiocyanate (ANIT)-induced cholestatic liver injury.
Materials and Methods: Intrahepatic cholestasis was induced by intraperitoneal injection of ANIT. Rats orally received andrographolide or a control solvent for a total period of 4 days. On day 2 of the treatment, rats were injected with ANIT. Serum biochemistry and liver histology were evaluated at 48 hours after ANIT injection. Hepatic transporter expression, markers of inflammation, hepatic stellate cells activation, and bile duct proliferation were examined.
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Conclusions: Andrographolide ameliorates liver injury in ANIT-induced cholestasis. This involves (i) decreasing bile duct proliferation, (ii) suppressing activation of hepatic stellate cells and (iii) diminishing NF-kB expression. Taken together, these findings suggest andrographolide could be a promising therapeutic option in the treatment of cholestatic liver disease.

Cover Letter

Professor R. Verpoorte
Editor-in-Chief, Journal of Ethnopharmacology
Gorlaeus Lab., HB024,
Universiteit Leiden,
Einsteinweg 55, 2333 CC,
Leiden, Netherlands

August 12, 2015

Dear Professor Verpoorte,

Please consider the enclosed manuscript entitled "Protective effect of andrographolide isolated from *Andrographis paniculata* against alpha-naphthylisothiocyanate-induced cholestatic liver injury" for publication as an original research article in Journal of Ethnopharmacology. Andrographolide is a natural diterpenoid lactone, which is the main bioactive compound isolated from the leaves of *Andrographis paniculata*. This medicinal plant *Andrographis paniculata* has been widely used as a traditional oriental medicine for treatment of fever, common cold, diarrhea and other infectious ailments. There is also recent evidence that andrographolide may be a useful anticancer agent for breast, ovarian, lung and pancreatic cancer. Cholestasis is a cardinal manifestation of liver disease. If left untreated, cholestasis leads to liver fibrosis and cirrhosis and eventually liver failure and cancer. So far, therapeutic approaches for cholestasis are still limited and relate to lack of effective treatment. In light of the lack of effective treatment for cholestasis, this study examines whether and how andrographolide could protect against cholestatic liver injury. In this manuscript, we report for the first time that andrographolide ameliorates liver injury in a commonly used animal model of intrahepatic cholestasis. This study is significant because it indicates that andrographolide may be desirable not only for anti-tumorigenesis but also for treatment of cholestatic liver disease. Thus, andrographolide may be a new promising therapeutic approach for patients with intrahepatic cholestasis.

We declare that all applicable institutional and national guidelines for the care and use of animals were followed, all the listed authors have read and approved the submitted manuscript, and this work has not been published for publication elsewhere.

Thank you very much for considering this work for publication in Journal of Ethnopharmacology. We look forward to hearing from you soon.

Respectfully yours,

Jittima Weerachayaphorn, Ph.D.
Assistant Professor
Faculty of Science, Mahidol University
Department of Physiology
Bangkok 10400, THAILAND
Tel: +66-2201-5514
Fax: +66-2354-7154
Email: jittima.wee@mahidol.ac.th

Journal of Ethnopharmacology
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*Graphical Abstract

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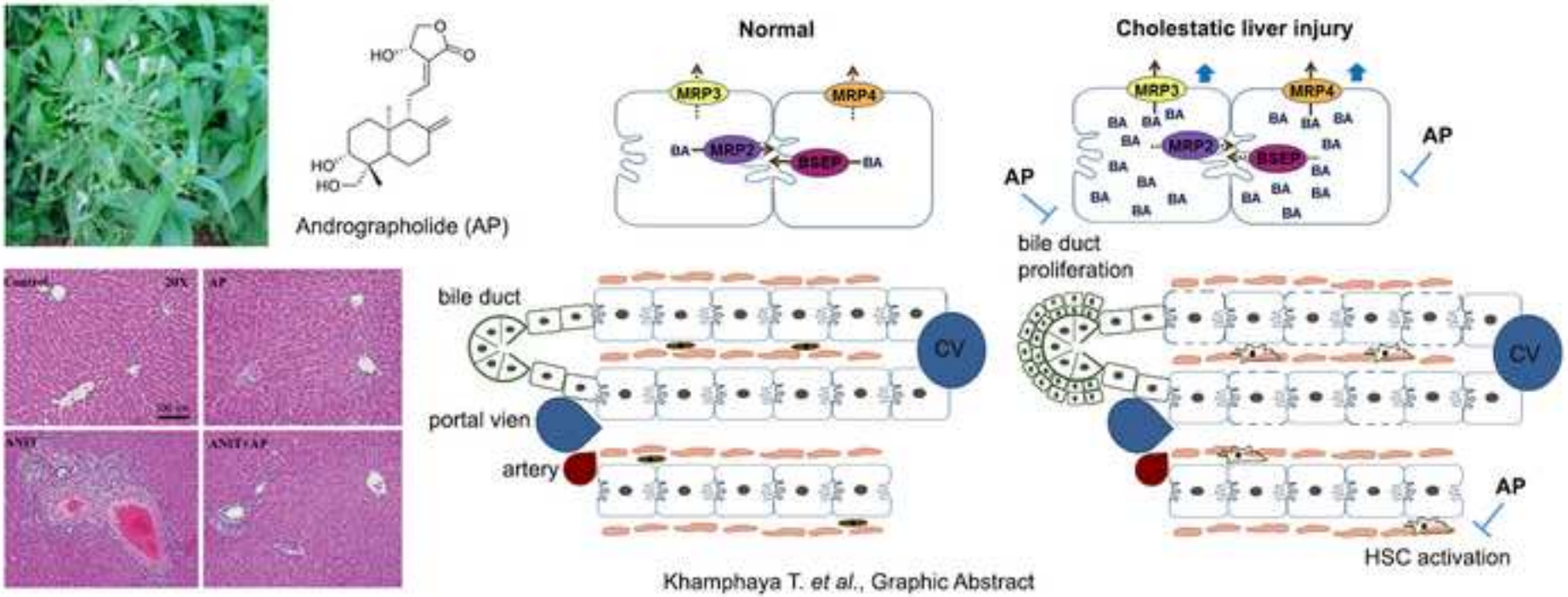

Khamphaya T. *et al.*, Graphic Abstract

ABSTRACT

Ethnopharmacological relevance: Andrographolide is a diterpene lactone, which is the main bioactive compound isolated from the leaves of *Andrographis paniculata*. This medicinal plant *Andrographis paniculata* has been widely used as a traditional medicine for treatment of fever, common cold, diarrhea and other infectious ailments in many Asian countries.

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Conclusions: Andrographolide ameliorates liver injury in ANIT-induced cholestasis. This involves (*i*) decreasing bile duct proliferation, (*ii*) suppressing activation of hepatic stellate cells and (*iii*) diminishing NF-κB expression. Taken together, these findings suggest andrographolide could be a promising therapeutic option in the treatment of cholestatic liver disease.

Protective effect of andrographolide isolated from *Andrographis paniculata* against alpha-naphthylisothiocyanate-induced cholestatic liver injury

Tanaporn Khamphaya[a], Piyachat Chansela[b], Pawinee Piyachaturawat[a,d], Apichart Suksamrarn[c], Michael H. Nathanson[e] and Jittima Weerachayaphorn[d,e]*

[a]Toxicology Graduate Program, Faculty of Science, Mahidol University, Bangkok, Thailand

[b]Department of Anatomy, Phramongkutklao College of Medicine, Bangkok, Thailand

[c]Department of Chemistry, Faculty of Science, Ramkhamhaeng University, Bangkok, Thailand

[d]Department of Physiology, Faculty of Science, Mahidol University, Bangkok, Thailand

[e]Department of Internal Medicine, Liver Center, Yale University, New Haven, Connecticut, USA

*Corresponding author: Jittima Weerachayaphorn, Ph.D., Department of Physiology, Faculty of Science, Mahidol University, 272 Rama VI Road, Ratchathevi, Bangkok 10400, Thailand

Tel: +66-2201-5514

Fax: +66-2354-7154

E-mail: jittima.wee@mahidol.ac.th.

jittima.weerachayaphorn@yale.edu

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Key words: alpha-naphthylisothiocyanate, andrographolide, cholestasis, bile duct, liver injury

Chemical compounds studied in this article: Alpha-naphthylisothiocyanate (PubChem CID: 11080); Andrographolide (PubChem CID: 5318517); Carboxymethyl Cellulose Sodium (PubChem CID: 6328154)

1. INTRODUCTION

Cholestasis is a cardinal manifestation of liver diseases. It is associated with impaired hepatocellular secretion of bile, resulting in intrahepatic accumulation of bile acids and bilirubin with progressive liver injury (Trauner et al., 1998; Wagner et al., 2009). Prolonged, chronic cholestasis results in liver cirrhosis and eventually liver failure and cancer (Carey and Lindor, 2012). Nearly one in five liver transplants in the United States is for chronic cholestatic conditions in patients with disorders such as primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC), and cholestatic disorders are the most common indication for transplant among pediatric patients (Carbone and Neuberger, 2011). Currently, ursodeoxycholic acid (UDCA) is the only drug approved by the Food and Drug Administration for the treatment of cholestatic disorders. UDCA has been particularly successful for the treatment of primary biliary cirrhosis (Poupon et al., 1997). However, not all cholestatic patients respond well to UDCA, the effectiveness of UDCA is limited to the early stages of PBC, and the risk for disease progression still remains (Lindor et al., 2009). UDCA has no proven benefit in PSC and may even be harmful when administered in high doses to these patients (Lindor et al., 2009). Furthermore, UDCA could be deleterious in obstructive cholestasis by increasing biliary pressure and liver necrosis, as shown in mouse models of bile duct ligation (Fickert et al., 2002). Although the molecular basis of cholestasis and the pathophysiology of the associated hepatic fibrosis have been extensively studied,

there is no uniformly effective treatment for cholestasis. Therefore, additional therapeutic approaches for patients with cholestatic liver diseases are needed.

Andrographolide is a diterpene lactone isolated from the leaves of *Andrographis paniculata*, which is an indigenous medicinal plant widely used in many Asian countries for cold, influenza and other infectious ailments (Akbar, 2011). Andrographolide has been reported to possess several pharmacological activities, including anti-inflammatory (Lim et al., 2012), immunostimulatory (Kumar et al., 2004), antiviral (Lee et al., 2013) and cardioprotective actions (Woo et al., 2008). In the last several years, increasing attention has been paid to andrographolide because of its diverse therapeutic properties, including its anticancer activity in breast (Chao et al., 2013), and colon cancer models (Chao et al., 2010; Jada et al., 2008). Andrographolide also has potential as a chemotherapeutic agent for treating human pancreatic cancer as it causes cell cycle arrest in pancreatic cancer cells through inhibition of STAT3 and Akt activation (Bao et al., 2013). The pharmacological value of andrographolide's liver protective property has been increasingly recognized as well. Andrographolide can protect against hepatocellular damage caused by carbon tetrachloride (Ye et al., 2011), acetaminophen (Roy et al., 2013), concanavalin-A (Burgos et al., 2005) and hexachlorocyclohexane (Trivedi et al., 2007). However, little is known about its exact mechanism of action. Combining data from multiple experiments regarding potential therapeutic value of andrographolide, particularly in the liver, raises the question of whether it would be useful for therapy of cholestatic liver diseases. Whether andrographolide has a protective effect in cholestatic liver injury is unknown. Therefore, this study was undertaken to investigate the hepatoprotective effect of andrographolide on ANIT-induced cholestatic liver injury, and to determine how andrographolide could protect against cholestatic liver injury.

2. MATERIALS AND METHODS

2.1 Preparation and isolation of andrographolide

The aerial parts of *Andrographis paniculata* (*A. paniculata*) used in this study were

collected from Kampaengsaen District, Nakhon Pathom Province, Thailand. A voucher specimen (PBM 05153) has been deposited at Faculty of Pharmacy, Mahidol University, Bangkok, Thailand. In a part of extraction and isolation, the air-dried aerial parts of *A. paniculata* (6.5 kg) were milled to small pieces and extracted successively with n-hexane, CH2Cl2 and MeOH at room temperature and the solvents were removed under reduced pressure to give the hexane (30.50 g), CH2Cl2 (211.70 g) and MeOH (350.80 g) extracts. The CH2Cl2 extract (211.00 g) was subjected to column chromatography (Merck silica gel 60, 0.063-0.200 mm, 1,400 g), using a gradient solvent system of n-hexane-EtOAc, EtOAc, EtOAc-MeOH and MeOH with increasing amount of the more polar solvent. The eluates were examined by thin-layer chromatography and 4 groups of eluting fractions were obtained. The group 4 was rechromatographed over silica gel and eluted under isocratic conditions (60% EtOAc in n-hexane) to afford 4 subfractions. Subfraction 3 was obtained as white solid (16.50 g) and was identified as andrographolide. The ^{1}H and ^{13}C NMR spectroscopic data of andrographolide were identical to those previously reported for andrographolide (Matsuda et al., 1994). A fingerprint of andrographolide was verified using HPLC-UV and LC-QTOF-MS with commercial andrographolide ($C_{20}H_{30}O_5$, PubChem CID: 5318517) purchased from Sigma-Aldrich (St. Louis, MO) (Suppl. Fig. S1-S2).

2.2 Reagents

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO). BSEP antibody was purchased from Kamiya Biomedical (Seattle, WA). Monoclonal antibody to MRP2 was obtained from ALEXIS Biochemicals (San Diego, CA). Anti-MRP3 and anti-MRP4 antibodies were purchased from Abcam (Cambridge, MA) and Everest Biotech (Oxfordshire, UK), respectively. Monoclonal antibodies to β-actin and alpha-smooth muscle actin (α-SMA) were obtained from Sigma-Aldrich. Polyclonal antibodies to NF-κB p50, NF-κB p65 and proliferating cell nuclear antigen (PCNA) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

2.3 Animals and experimental design

The animal study (protocol no. MUSC54-027-273) was approved by the Institutional Animal Care and Use Committee, Mahidol University, in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health. Adult male Wistar rats weighing between 170 and 200 grams were obtained from the National Laboratory Animal Center, Bangkok, Thailand. Animals were housed in animal facility under a 12-hour light: 12-hour dark cycle at temperature 22°C, and allowed free to food and water. Animals were acclimatized for at least 5 days prior to the experiments. Rats were randomized and pretreated orally with andrographolide at a dose of 50 and 100 mg/kg body weight, which are pharmacological doses and in the safe dose range, or the control solvent (1% carboxymethylcellulose, CMC) once daily for 4 days prior to receive the ANIT. On the second day of treatment, rats were intraperitoneally administered a single dose of ANIT (75 mg/kg body weight in olive oil) to induce cholestasis. A second set of vehicle-treated rats was given only olive oil to serve as the solvent control. At 48 hours after ANIT administration, animals were fasted overnight prior to sacrifice. Serum and liver samples were collected. Serum liver enzyme measurements for clinical chemistry parameters indicative of liver damage were made immediately after processing of blood samples. Liver tissues were snap frozen in liquid N_2 and stored in a −80°C freezer. A part of the liver from each rat was fixed in 10% neutral buffered formaldehyde for histological study.

2.4 Biochemical analysis

The levels of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), total bilirubin (TBIL), direct bilirubin (DBIL) and indirect bilirubin (IDBIL) levels were measured using a commercial kit (Human Gesellschaft für Biochemica und Diagnostica mbH, Wiesbaden, Germany). Bile acids were extracted from liver homogenates as previously described

(Mennone et al., 2006). Bile acid concentrations in the serum and liver were determined using a commercial total bile acid kit (Diazyme Laboratories, Poway, CA).

2.5 Liver histology and immunohistochemical analysis

Formalin-fixed liver was embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Immunohistochemical analysis of α-SMA and PCNA was performed on paraffin embedded rat liver sections. The liver tissues were deparaffinized in xylene and rehydrated through a graded alcohol series. Antigen retrieval using 10 mM sodium citrate (pH 6.0), in the microwave oven for 20 min (750 watts) was done prior to overnight incubation with anti-α-SMA or anti-PCNA antibodies. The tissue sections were followed by incubation with horseradish peroxidase-linked anti-mouse IgG for 1 hour at room temperature. Finally, tissue sections were developed for 60 second using the NovaRED staining kit (Vector Laboratories, Burlingame, CA), and counterstained with hematoxylin. Tissue slides were examined and imaged using a Nikon Eclipse E600 microscope-fitted with Nikon digital camera DXM1200 (Nikon Inc., Melville, NY). All images were captured using the same settings, and images were processed with Adobe Photoshop CS6 (Moutainview, CA).

2.6 Western blot analysis

The rat liver was homogenized using a TissueLyser LT (QIAGEN, Valencia, CA) in RIPA lysis buffer (Amresco, OH) containing Halt protease inhibitor cocktail (Thermo Scientific, IL). The protein concentration of each sample was determined using a BCA protein assay kit (Pierce, IL). Western blot analysis was performed. The proteins separated by SDS-PAGE were blotted onto nitrocellulose membranes. Blots were probed with the primary antibody at 4°C overnight and followed by incubation with secondary antibody conjugated with HRP for 1 hour. The immunoreactive protein was detected with the SuperSignal West Pico Chemiluminescent Substrate kit (Pierce). Immunoblots were

visualized and the relative quantities of protein expression were analyzed using the ImageJ software developed at the National Institutes of Health (NIH, Bethesda). The β-actin was used as internal control and was used as a reference to normalize data.

2.7 TaqMan reverse transcription quantitative PCR

Total RNA was prepared from rat liver tissues using TRIzol reagent (Invitrogen). The RNA was then cleaned up and purified using the RNeasy MinElute Cleanup kit (Qiagen). RNA was converted to cDNA using RT-PCR kit-RNA to cDNA EcoDry Premix *(*Clontech Laboratories, Mountain View, CA*)*. Quantitative TaqMan real-time PCR was performed on an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster city, CA). All primers were TaqMan Gene Expression Assays and were purchased from Applied Biosystems. The expression of target genes was normalized to *Gapdh* and quantification of relative expression was determined by the Pfaffl's method (Pfaffl, 2001).

2.8 Statistical Analysis

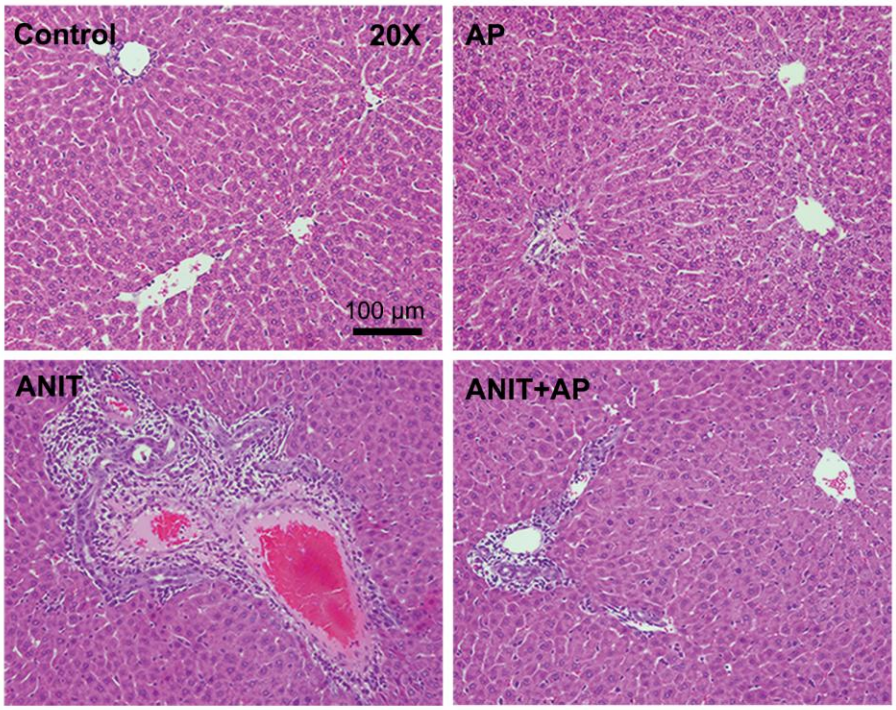
All data are expressed as mean values ± standard deviation (SD). Prism 6 software (GraphPad, La Jolla, CA) was used for data analysis. Statistical analyses were performed using two-tailed unpaired Student's *t*-test or One-way ANOVA when three or more groups were compared and followed by Dunnett's multiple comparison test. Differences with $p < 0.05$ were considered to be statistically significant. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *versus* control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ *versus* ANIT treatment.

3. RESULTS

3.1 Andrographolide ameliorates ANIT-induced cholestatic liver damage

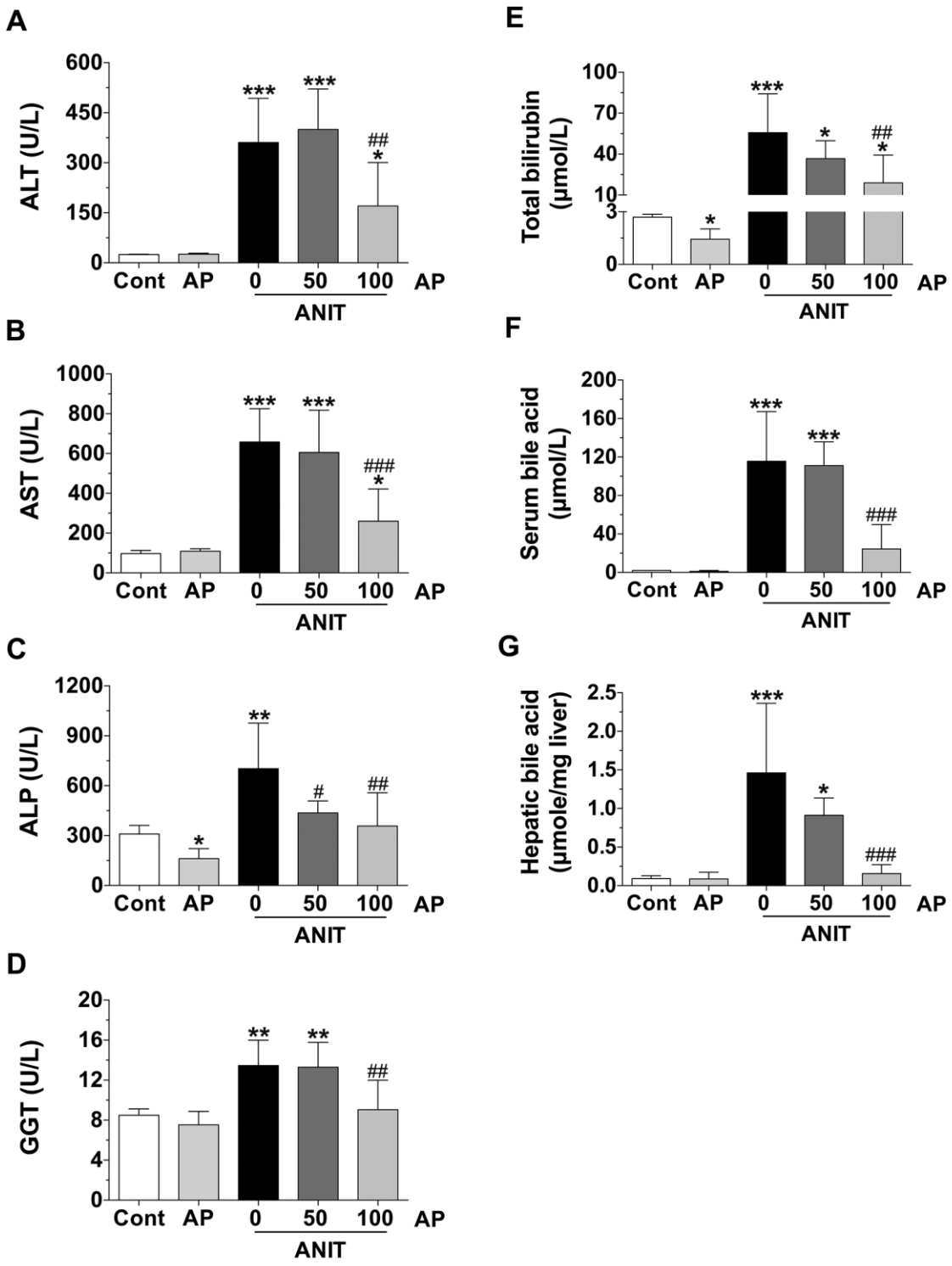
Biliary epithelial cell damage and intrahepatic cholestasis were observed 48 hours after a single dose of ANIT, as has been reported previously (Ohta et al., 1999). To determine if andrographolide protects against ANIT-induced cholestatic liver injury, we pretreated rats with andrographolide and examined the effect of ANIT. Histological analysis

of liver sections from control rats receiving the CMC or andrographolide without ANIT showed no significant changes (Fig. 1). In contrast, liver sections from ANIT-treated rats exhibited large areas of cholangitis with marked bile duct proliferation (Fig. 1).



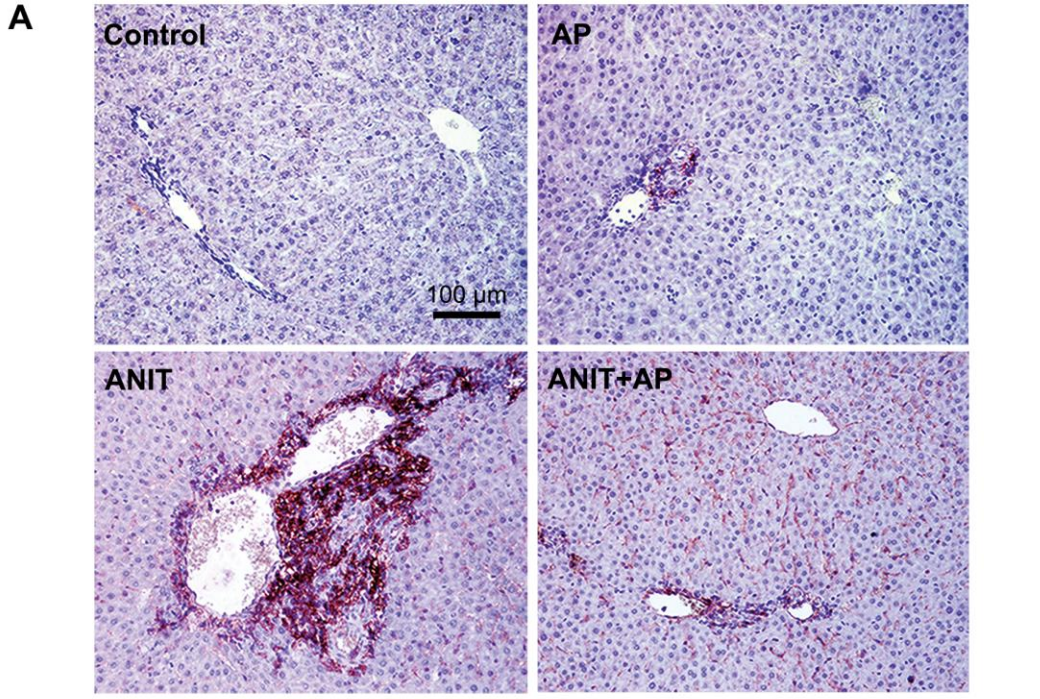
Khamphaya T., *et al.*_Fig. 1

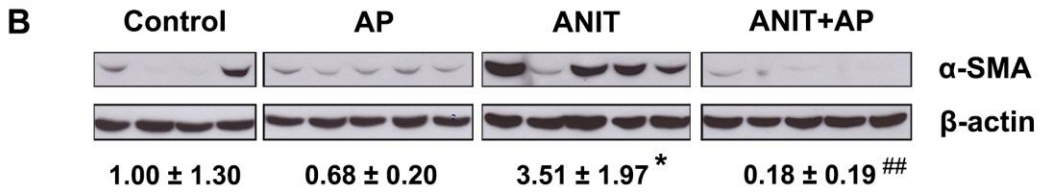
Serum activities of liver injury and cholestatic markers, including ALT, AST, ALP and GGT were all significantly increased by ANIT (Fig. 2A-D). Total bilirubin, serum bile acid and hepatic bile acid levels were also elevated following ANIT treatment (Fig. 2E-G), which are in line with previous studies (Tanaka et al., 2009). Andrographolide treatment showed substantial, profound protection against ANIT-induced liver damage (Fig. 1). Liver sections from the andrographolide/ANIT-treated rats showed smaller areas of cholangitis, less inflammatory cell infiltration, and less bile duct proliferation (Fig. 1), suggesting that andrographolide treatment ameliorated the cholestatic changes. Consistent with the results of liver histology, andrographolide treatment significantly improved the biochemical markers of liver injury ALT, AST, ALP and GGT in the ANIT-treated rats (Fig. 2A-D). A reduction in hepatic bile acids and both serum bile acid and bilirubin levels was also observed in andrographolide/ANIT-treated rats (Fig. 2E-G).



Khamphaya T., *et al.*_Fig. 2

In addition, the H&E stained liver sections of ANIT-treated rats exhibited more periductular inflammation and were accompanied by a marked increase in the number of α-SMA-positive cells and increased hepatic α-SMA protein expression (Fig. 3A-B). In comparison, less α-SMA-positive cells and less periductular inflammation were seen throughout in andrographolide/ANIT-treated rats, which were paralleled by a substantial reduction in α-SMA protein expression (Fig. 3B). Taken together, these findings indicate that andrographolide attenuates liver damage and protects the liver in the ANIT model of intrahepatic cholestasis.

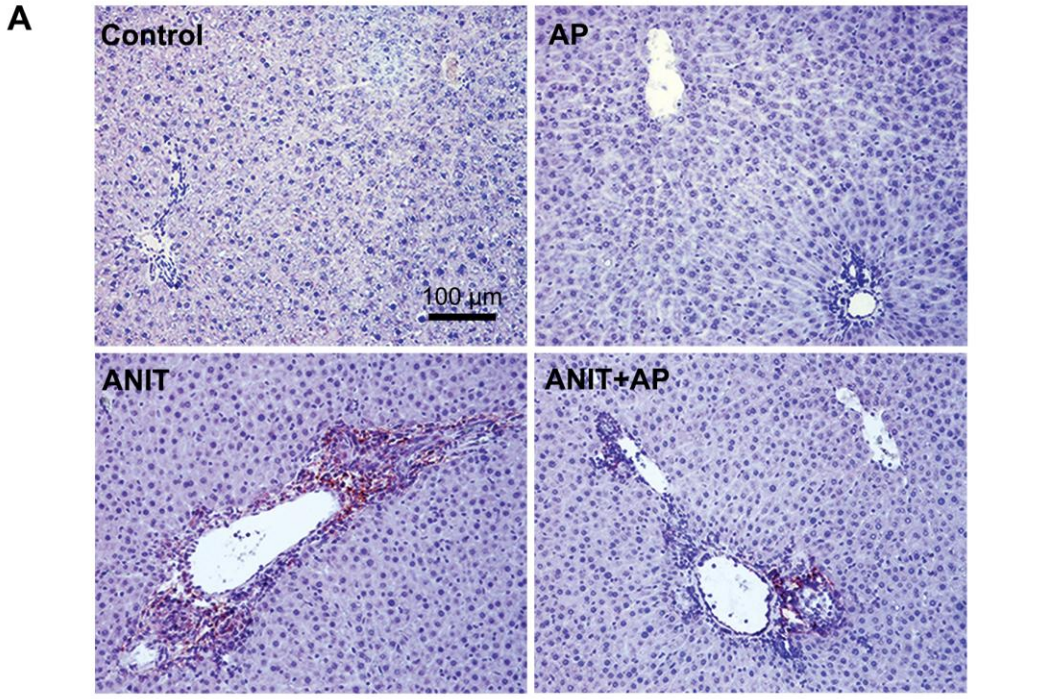




Khamphaya T., *et al.*_Fig. 3

3.2 Andrographolide reduces bile duct proliferation

Biliary disease is often associated with bile duct proliferation, and the ANIT model recapitulates this (Fig. 1). The degree of inhibition of increased serum liver enzyme activities by andrographolide correlated well with less bile duct proliferation (Fig. 2). To quantify this, we analyzed the expression of proliferating cell nuclear antigen (PCNA), a known marker of cell proliferation, in andrographolide/ANIT-treated rats. Analysis of PCNA immunohistochemical liver staining from rats received ANIT alone showed that ANIT injection substantially increased PCNA expression (Fig. 4A). Similarly, a more profound induction in hepatic PCNA protein expression was also seen in ANIT-treated rats than in the vehicle-treated rats (Fig. 4B). ANIT treatment increased PCNA protein expression approximately 3-fold compared to control rats. In contrast, andrographolide treatment resulted in a significant decrease in the number of PCNA-positive cells observed in the portal areas of andrographolide/ANIT-treated rats (Fig. 4A). Western blotting of hepatic proteins also confirmed that andrographolide decreased protein expression of PCNA compared to ANIT-treated rats (Fig. 4B), suggesting that andrographolide inhibited proliferation of bile duct epithelium.

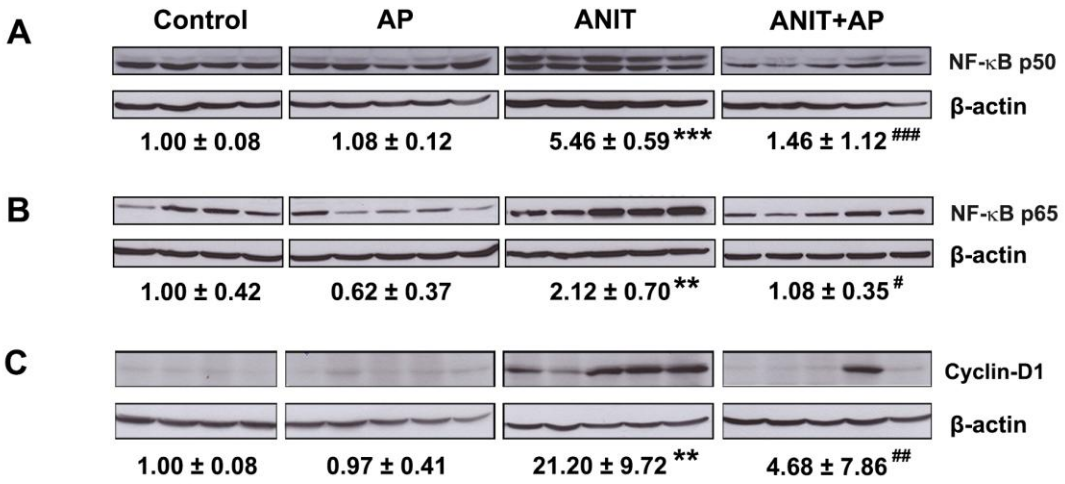


Khamphaya T., *et al.*_Fig. 4

3.3 Andrographolide suppresses the expression of NF-κB

There is increasing evidence that NF-κB is important for the development of liver injury, including inflammation and proliferation (Sethi et al., 2008). We investigated whether the protective effect of andrographolide is associated with inhibition of the NF-κB pathway. The protein expression of NF-κB p50 and NF-κB p65 were significantly induced by ANIT injection (Fig. 5A and B). In contrast, andrographolide treatment significantly reduced the expression of NF-κB p50 and NF-κB p65 compared to ANIT-treated rats.

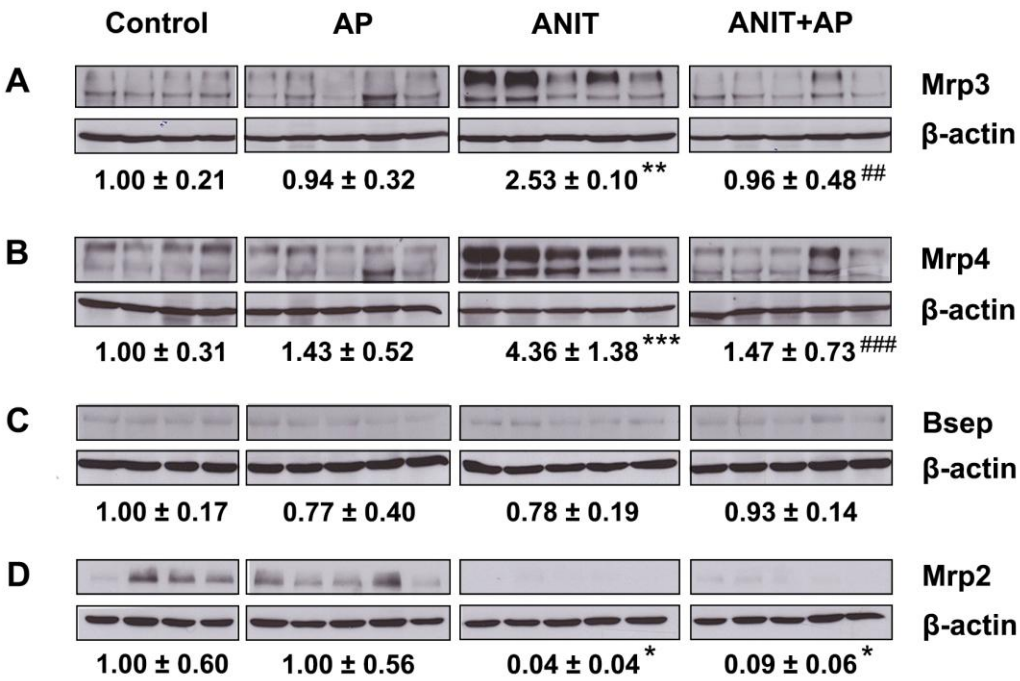
Because inhibition of NF-κB activation leads to down-regulation of gene products involved in proliferation, most notably Cyclin D1 (Hinz et al., 1999), the expression of Cyclin D1 was examined. Protein expression of Cyclin D1 was substantially increased in ANIT-treated rats, while andrographolide markedly decreased Cyclin D1 protein expression in ANIT-treated rats (Fig. 5C), consistent with the idea that andrographolide may suppress the proliferation of bile duct epithelium through suppression of NF-κB-regulated activation of Cyclin D1. Taken together, these results provide evidence that andrographolide exerts its hepatoprotective and anti-proliferative effects in ANIT-induced cholestatic liver injury through modulation of NF-κB activation.



Khamphaya T., *et al.*_Fig. 5

3.4 Effects of andrographolide on hepatocellular transporter expression

Cholestasis causes intrahepatic accumulation of toxic bile acids (Allen et al., 2011). Down-regulation of hepatic basolateral influx transporters represents one defense mechanism by which hepatocytes shut off bile acid and bilirubin influx during cholestasis. Up-regulation of hepatic basolateral efflux transporters provides an alternative mechanism for eliminating bile acids in an attempt to prevent hepatocellular accumulation of toxic bile acids (Trauner and Halilbasic, 2011). To determine if induction of alternative efflux routes contributes to the observed favorable effects of andrographolide, we examined protein expression of alternative basolateral efflux transporters in andrographolide-fed ANIT rats. ANIT administration increased MRP3 and MRP4 protein expressions approximately 2.5-fold and 4-fold, respectively. However, much less pronounced induction of both MRP3 and MRP4 was observed in andrographolide-fed ANIT rats (Fig. 6A-B). In addition, we examined protein expression of the key canalicular transporters implicated in the pathogenesis of intrahepatic cholestasis. Protein expression of the bile salt export pump (BSEP) was unchanged by ANIT administration (Fig. 6C), while MRP2 expression was significantly decreased by ANIT (Fig. 6D), consistent with what is observed in patients with hepatitis (Hartmann et al., 2002). Andrographolide had no significant effects on protein expression of either MRP2 or BSEP. These findings suggest that a less adaptive response in up-regulation of the alternative basolateral efflux transporter for potentially toxic bile acids observed in andrographolide-fed ANIT rats may reflect the protective effect of andrographolide in intrahepatic cholestasis.



Khamphaya T., *et al.*_Fig. 6

4. DISCUSSION

Cholestasis leads to liver fibrosis and cirrhosis, which eventually results in liver failure and cancer. So far, effective pharmacologic agents and therapeutic approaches are limited. In this study, we investigated whether andrographolide could protect against cholestatic liver injury. We have shown that andrographolide reduced serum biomarkers of cholestasis and protected bile duct damage against ANIT-induced intrahepatic cholestasis in rats.

ANIT has long been employed to induce cholestatic liver injury (Goldfarb et al., 1962). ANIT is conjugated to glutathione in the hepatocytes and transported across the canalicular membrane by MRP2, resulting in damage to the cholangiocytes lining the bile ducts and the accumulation of bile acids in the liver (Kossor et al., 1995). Adaptive responses to cholestasis to compensate for the loss of biliary excretory function and the accumulation of potentially toxic biliary constituents have been reported in ANIT-induced cholestatic liver injury (Yang et al., 2012). Basolateral bile acid influx transporters, the sodium taurocholate co-transporting polypeptide (NTCP) and the organic anion-transporting polypeptide (OATP), are down-regulated to reduce the uptake of bile acids and other organic anions in cholestasis. Basolateral bile acid efflux transporters, MRP3, MRP4 and organic solute transporters (OST-α/OST-β) are up-regulated in cholestatic liver injury (Paumgartner, 2006). In the present study, profound compensatory changes in the expression of basolateral bile acid transporters, particularly MRP3 and MRP4, were decreased in andrographolide/ANIT-treated rats compared to ANIT-treated rats (Fig. 6). Having less up-regulation of MRP3 and MRP4 could imply that andrographolide/ANIT-treated rats would have less severe cholestatic liver injury. This idea was consistent with the remarkable improvement observed in serum liver enzyme markers and liver histology (Fig. 1).

How does andrographolide protect against ANIT-induced cholestatic liver injury? A widely appreciated pharmacological property of andrographolide is anti-inflammation, and a link between inflammation and cholestasis has been established (O'Brien et al., 2013). Miyake and coworkers reported that bile acids are capable of inducing Kupffer cells to release proinflammatory cytokines. The hydrophobic bile acid, chenodeoxycholate, induces

the mRNA expression of interleukin-1 and tumor necrosis factor-α (TNF-α) (Miyake et al., 2000). In our study, TNF-α mRNA expression also was found to be induced in ANIT-treated rat liver, and this induction was decreased in response to andrographolide treatment (Suppl. Fig. S3). A reduction in TNF-α expression by andrographolide also correlates with reduction in serum markers of hepatic damage and with severity of liver histological damage (Fig. 1) consistent with the idea that andrographolide alleviates cholestatic liver injury through an anti-inflammatory mechanism.

In addition, it was found that andrographolide exerted an anti-inflammatory effect through inhibition of the NF-κB pathway. This impression was supported by the following findings: *i)* andrographolide treatment inhibited the protein expression of NF-κB p50 and NF-κB p65; and *ii)* as the activation of hepatic stellate cells is associated with NF-κB activation (Baghdasaryan et al., 2010; Kim et al., 2012), we found that andrographolide inhibited the activation of hepatic stellate cells as seen by a reduction in protein expression of α-SMA (Fig. 3). In addition, the critical requirement of NF-κB in the expression of genes involved in proliferation, typified by Cyclin D1, has been reported (Sethi et al., 2008). The inhibition of NF-κB activation led to down-regulation of the Cyclin D1 gene. We found the protein level of Cyclin D1 was decreased in andrographolide/ANIT-treated rats, thus further supporting the idea that andrographolide exerted an anti-inflammatory and anti-proliferative effect through inhibition of the NF-κB pathway.

One of the potential targets for the medical therapy of cholestasis is stimulation of the conversion of hydrophobic bile acids and other toxic compounds to more hydrophilic, less toxic metabolites (Paumgartner, 2006). Therapeutic concepts targeting nuclear receptor in cholestasis are also established. In particular, the pregnane X receptor (PXR), a classical xenobiotic receptor, has been reported to have anticholestatic properties in cholestatic animals (Kliewer and Willson, 2002). Staudinger and coworkers revealed that activation of PXR protects against severe liver damage induced by lithocholic acid, and PXR agonists may be useful in the treatment of human cholestatic liver disease (Staudinger et al., 2001).

Studies in humans showed that the PXR ligand rifampicin induces genes involved in bile acid and bilirubin metabolism (Marschall et al., 2005), and indeed rifampicin has been used in the treatment of jaundice and pruritus associated with intrahepatic cholestasis (Wagner et al., 2010). Our findings showed that treatment with andrographolide also gave rise to induction of *Pxr* expression and led to increased expression of *Cyp3a2* (also known as *Cyp3a11*), which promotes bile acid metabolism (Suppl. Fig. S4). Furthermore, andrographolide repressed *Cyp7a1* expression, which blocks bile acid biosynthesis (Suppl. Fig. S4). In addition, andrographolide/ANIT-treated rats had decreased levels of hepatic bile acids (Fig. 2G). Induction of *Pxr* and *Cyp3a11* and repression of *Cyp7a1* would prevent the accumulation of bile acids to toxic levels and render the bile composition to be less toxic for the injured epithelium. Our data are consistent with the idea that the anti-cholestatic effects of andrographolide may be mediated through PXR. Because andrographolide, like rifampicin, is a potent PXR agonist, our findings may have implications for the development of new therapeutic agents for the treatment of human cholestatic liver disease.

In conclusion, this is the first report providing *in vivo* evidence of hepatoprotection by andrographolide in an animal model of intrahepatic cholestatic liver disease. These data suggest that andrographolide may be an effective alternative agent and support the potential utility of andrographolide for the treatment of intrahepatic cholestasis. Our findings together with the results of earlier studies indicate that andrographolide may be desirable not only for anti-tumorigenesis but also for treatment of cholestatic liver disease. Future work may focus on confirmation of andrographolide's effect in other cholestatic models, and may culminate in clinical trials with andrographolide for patients with intrahepatic cholestatic liver diseases.

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CONFLICT OF INTEREST: The authors declare that they have no conflict of interest.

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FIGURE LEGENDS

Figure 1. Andrographolide protects against ANIT-induced liver damage.

Representative photomicrographs of livers stained with hematoxylin and eosin from control and ANIT-treated rats pretreated with CMC or andrographolide. Andrographolide/ANIT treated rats showed less severity of less periductular inflammation and less bile duct proliferation. Magnification, 20x, Scale bars, 100 μm, *AP*, andrographolide.

Figure 2. Andrographolide reduces the level of liver enzymes, bilirubin and bile acids in ANIT-treated rats.

Serum levels of **A)** ALT, **B)** AST, **C)** ALP, **D)** GGT, **E)** total bilirubin and **F)** bile acid were measured in each group to evaluate the protective effect of andrographolide after ANIT treatment. **G)** Hepatic bile acid concentrations, livers from andrographolide-pretreated ANIT-treated rats exhibited decreased hepatic bile acid concentrations. Data represent average ± SD, n = 4 - 8 animals per group; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *versus* control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ *versus* ANIT-treated rats. *Cont*, control; *AP*, andrographolide.

Figure 3. Andrographolide reduces periductular inflammation and activation of hepatic stellate cells.

A) Representative immunohistochemical staining of α-SMA was visualized with NovaRED. Sections stained were from control and ANIT-treated rat livers pretreated with CMC or andrographolide. α-SMA-positive cells are *red*; Magnification, 20x, Scale bars, 100 μm. Andrographolide/ANIT treated rats exhibited less periductular inflammation and were accompanied by a marked decrease in the number of α-SMA-positive cells. **B)** Immunoblot analysis of α-SMA normalized to β-actin in control and ANIT-treated rat livers pretreated with and without andrographolide. Data are expressed in arbitrary units ± SD, n = 4 - 8 animals per group; $^{*}p < 0.05$ *versus* control; $^{\#\#}p < 0.01$ *versus* ANIT-treated rats; *AP*, andrographolide.

Figure 4. Andrographolide decreases bile duct proliferation.

A) Immunohistochemical staining of PCNA was visualized with NovaRED. PCNA-positive cells are *red*. Sections stained were from control and ANIT-treated rat livers pretreated with CMC or andrographolide. Andrographolide decreased bile duct proliferation after ANIT treatment. Magnification, 20x, Scale bars, 100 μm. **B)** Western blot analysis of hepatic PCNA expression demonstrated andrographolide treatment diminished the protein expression of PCNA after ANIT treatment. Data are expressed in arbitrary units ± S.D., n = 4-8 animals per group; $^{*}p < 0.05$, *** $p < 0.001$ *versus* control, $^{\#\#}p < 0.01$ *versus* ANIT-treated rats. *AP*, andrographolide.

Figure 5. Andrographolide inhibits the NF-κB signaling.

Immunoblot analysis of **A)** NF-κB p50, **B)** NF-κB p65 and **C)** Cyclin D1 in control and ANIT-treated rat livers pretreated with CMC or andrographolide. Band intensities were quantitated and expressed as fold-change relative to control rats receiving CMC alone. Values are

normalized to β-actin. Data are expressed in arbitrary units ± SD, *n* = 4 - 5 animals per group; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ *versus* control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ *versus* ANIT-treated rats; AP, andrographolide.

Figure 6. Effect of andrographolide on protein expression of hepatic bile acid transporters.

Immunoblot analysis showed the expression of the basolateral membrane transporters **A)** MRP3 and **B)** MRP4, and the canalicular membrane transporters **C)** BSEP and **D)** MRP2 in control and ANIT-treated rats pretreated with CMC or andrographolide. Band intensities were quantitated and expressed as fold-change relative to control rats receiving CMC alone. Values are normalized to β-actin. Data are expressed in arbitrary units ± SD, *n* = 4 - 5 animals per group; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ *versus* control; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ *versus* ANIT-treated rats; *AP*, andrographolide.

Figure 1

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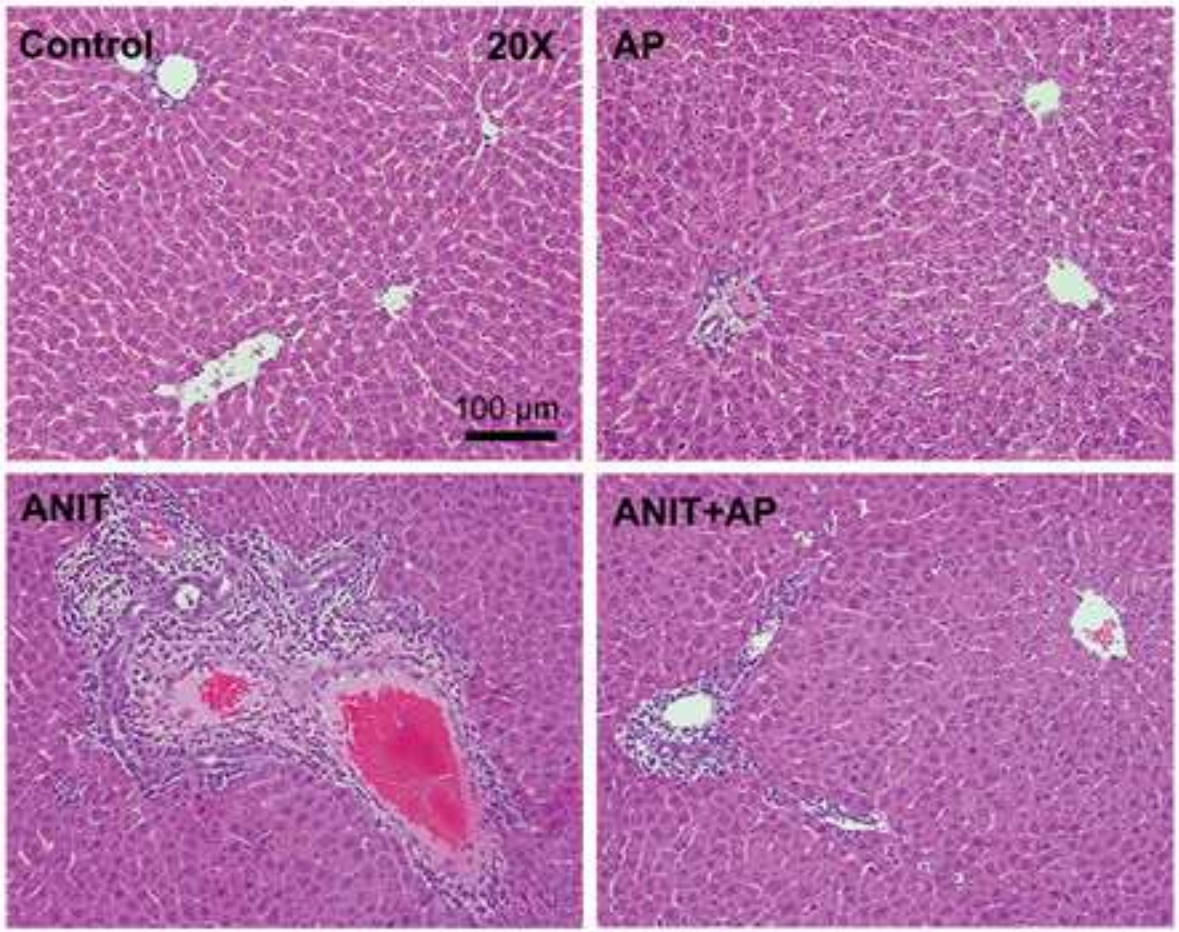

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Figure 2

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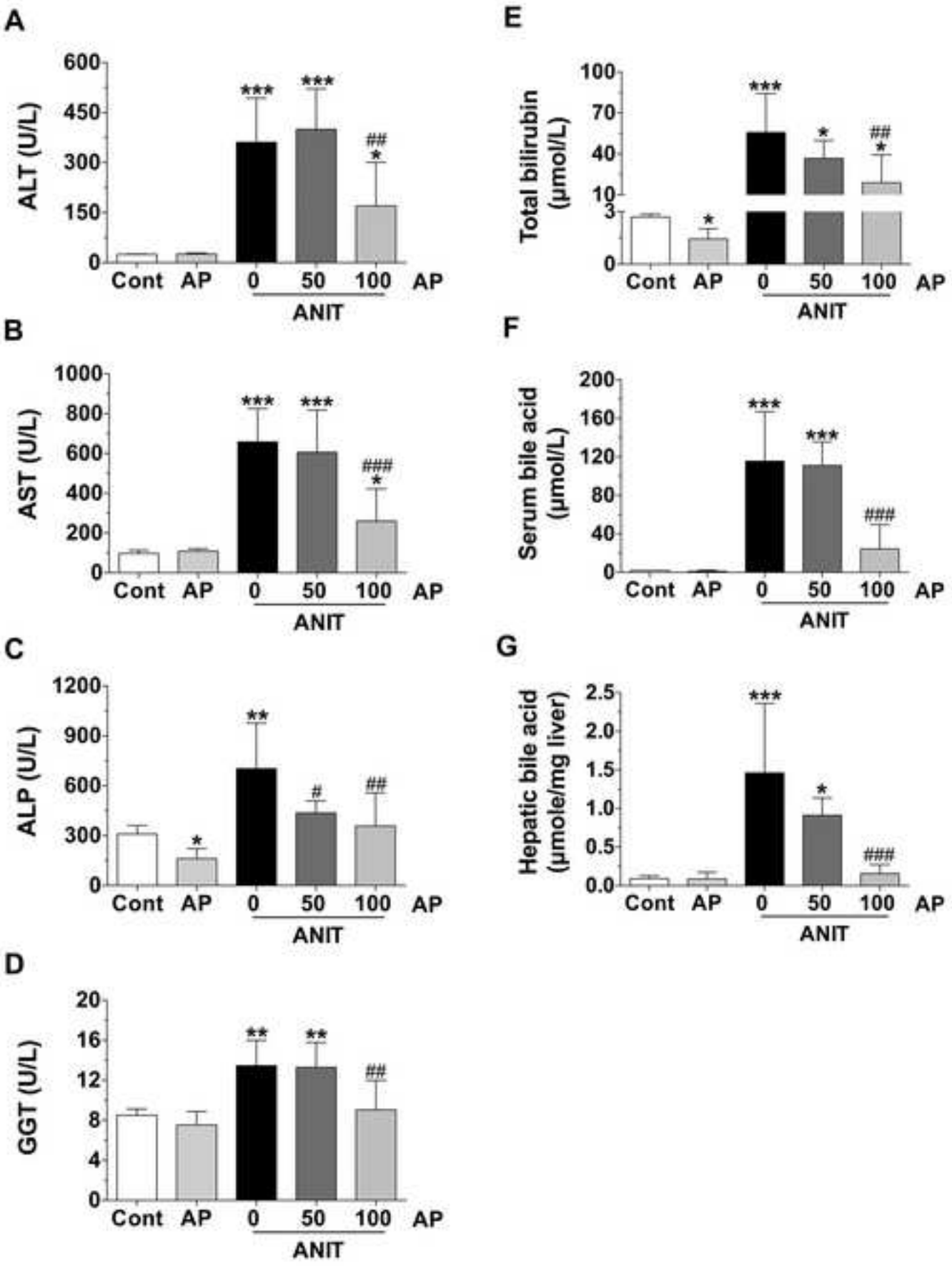

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Figure 3

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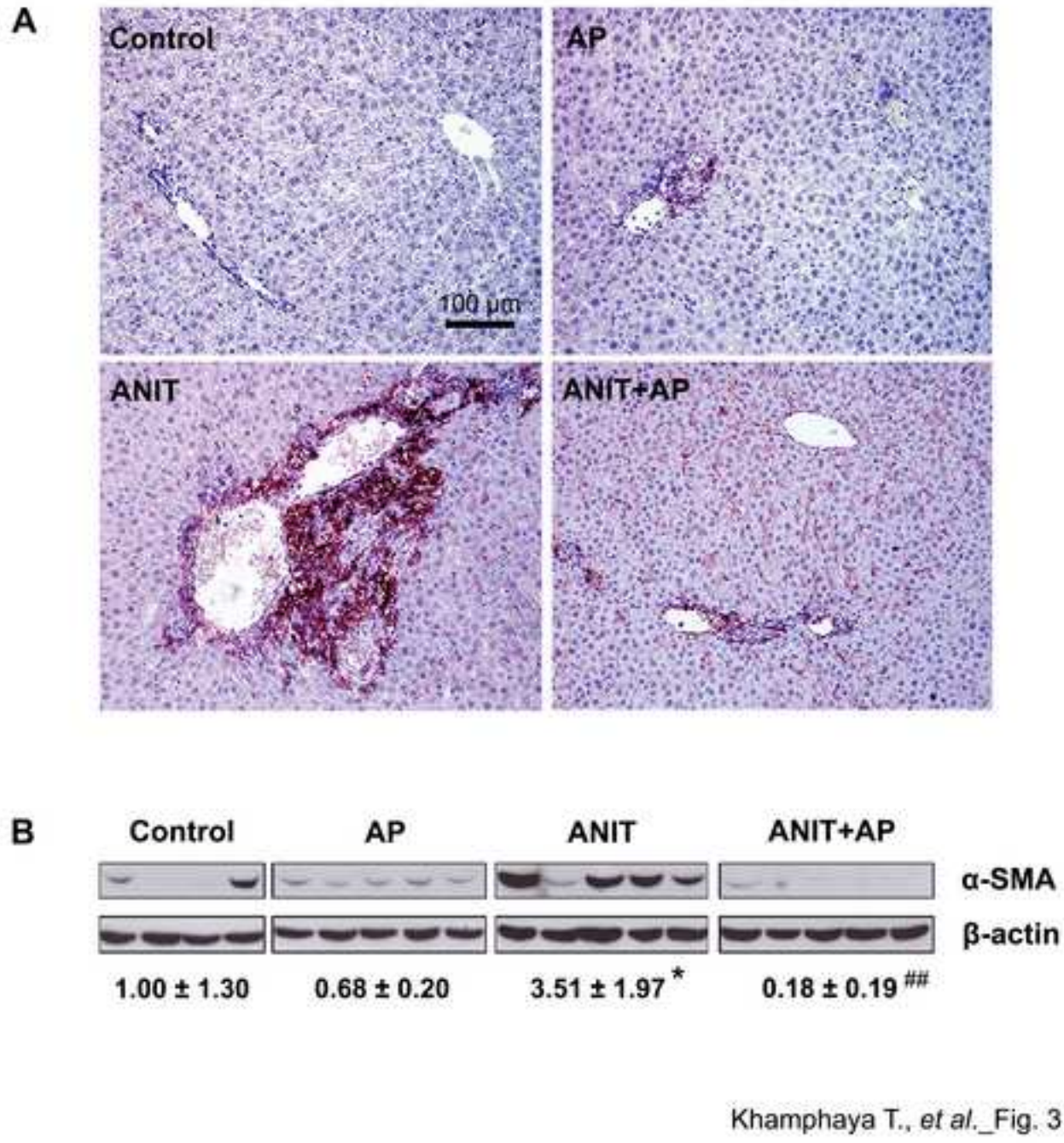

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Figure 4

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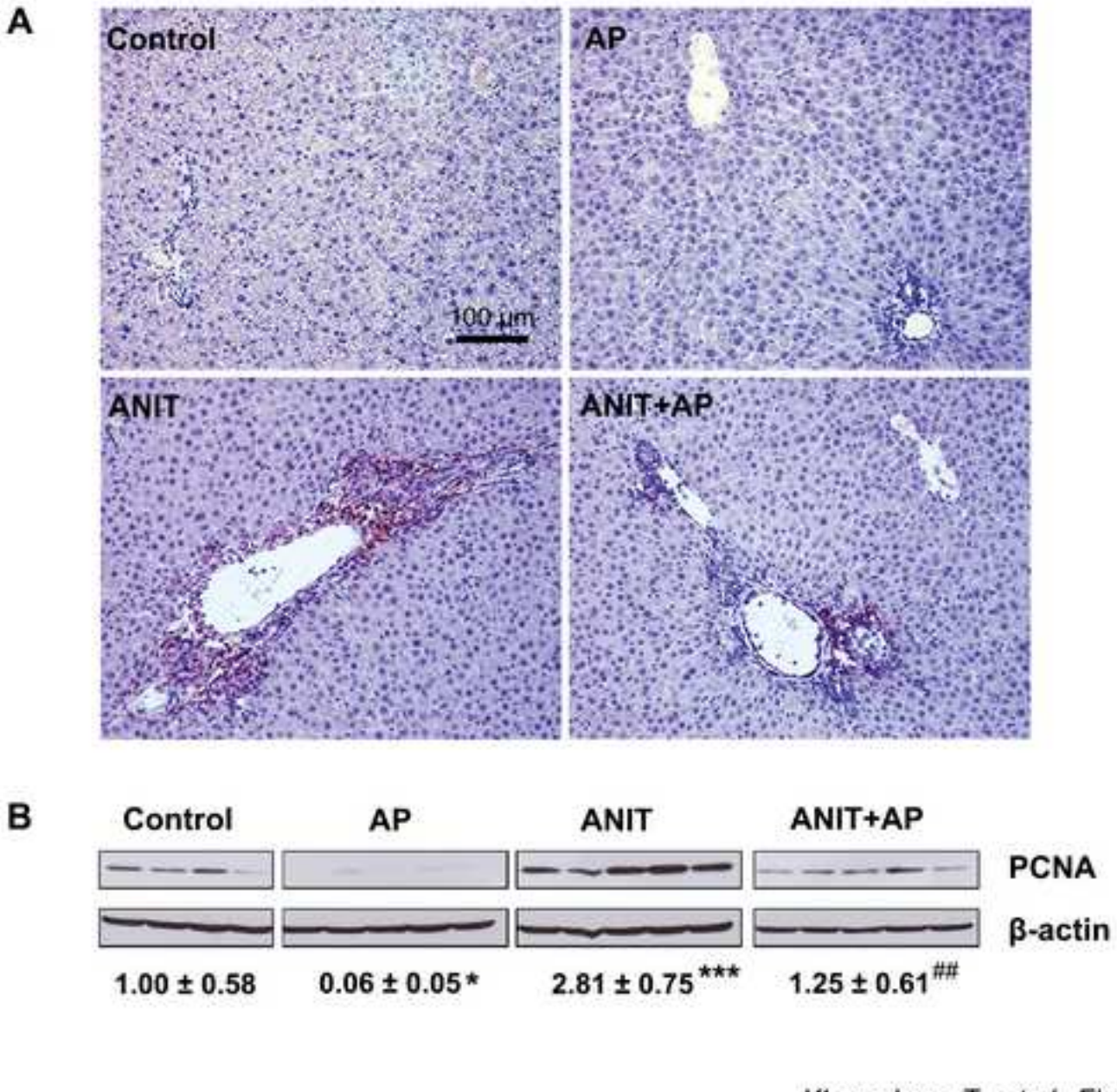

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Figure 5

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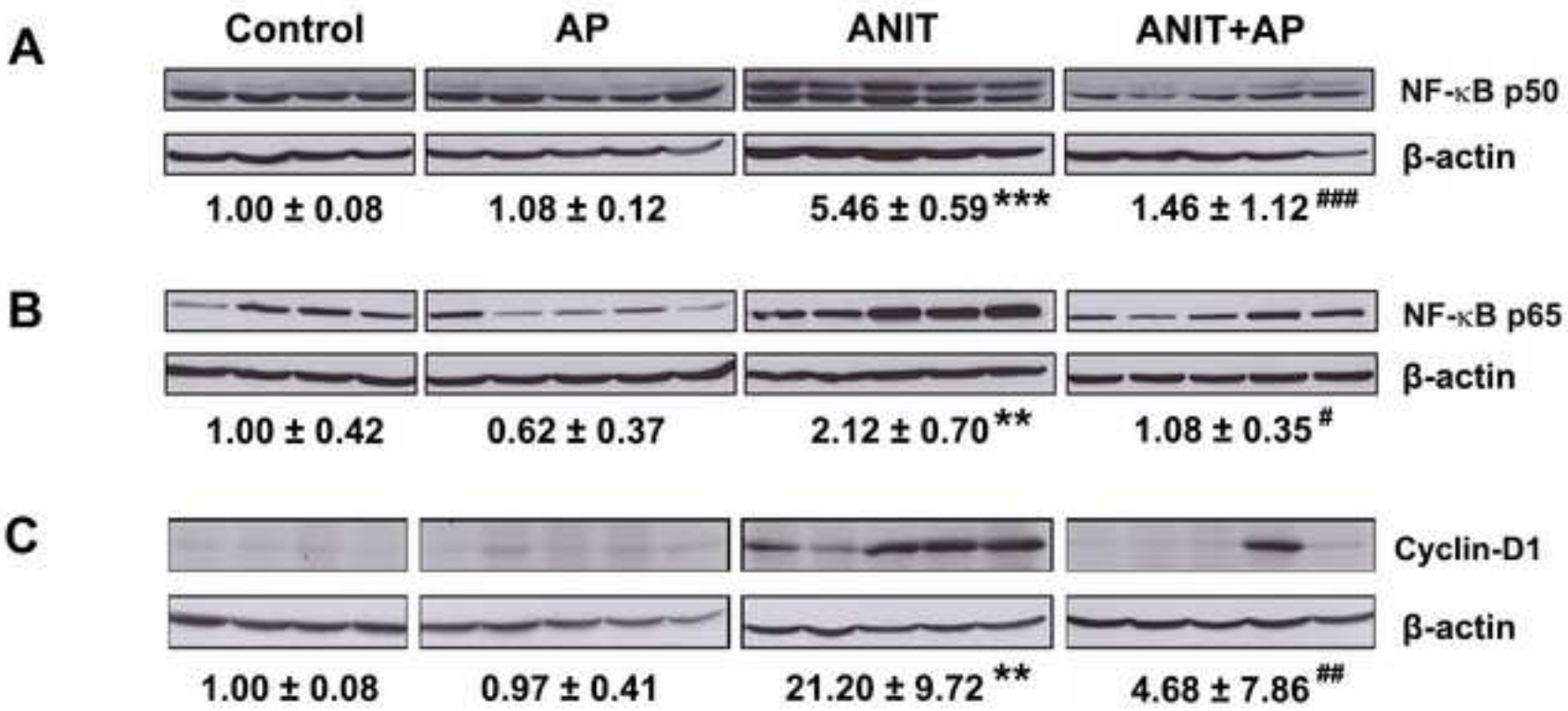

Khamphaya T., *et al.*_Fig. 5

Figure 6

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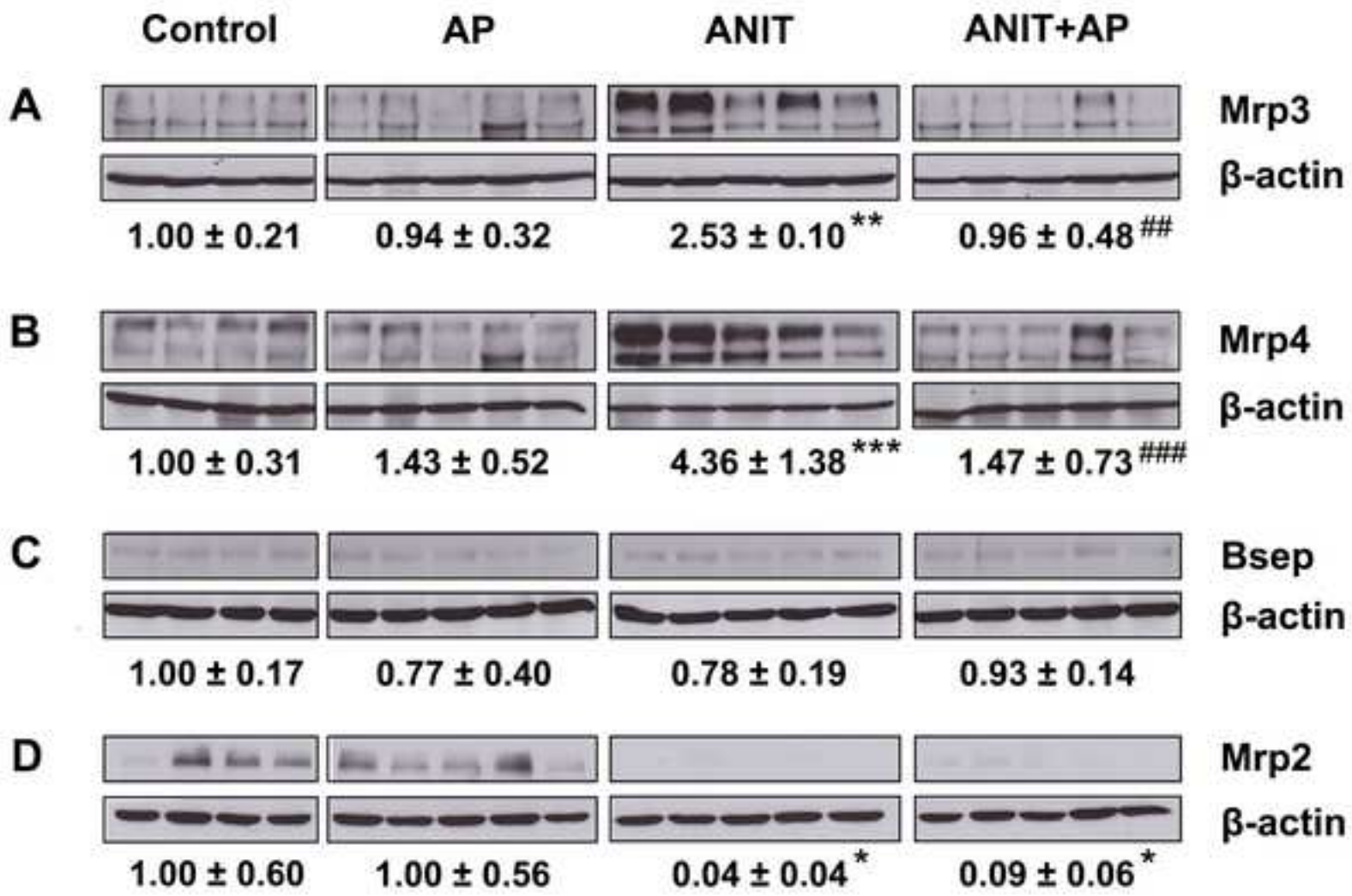

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Supplementary Figure S1

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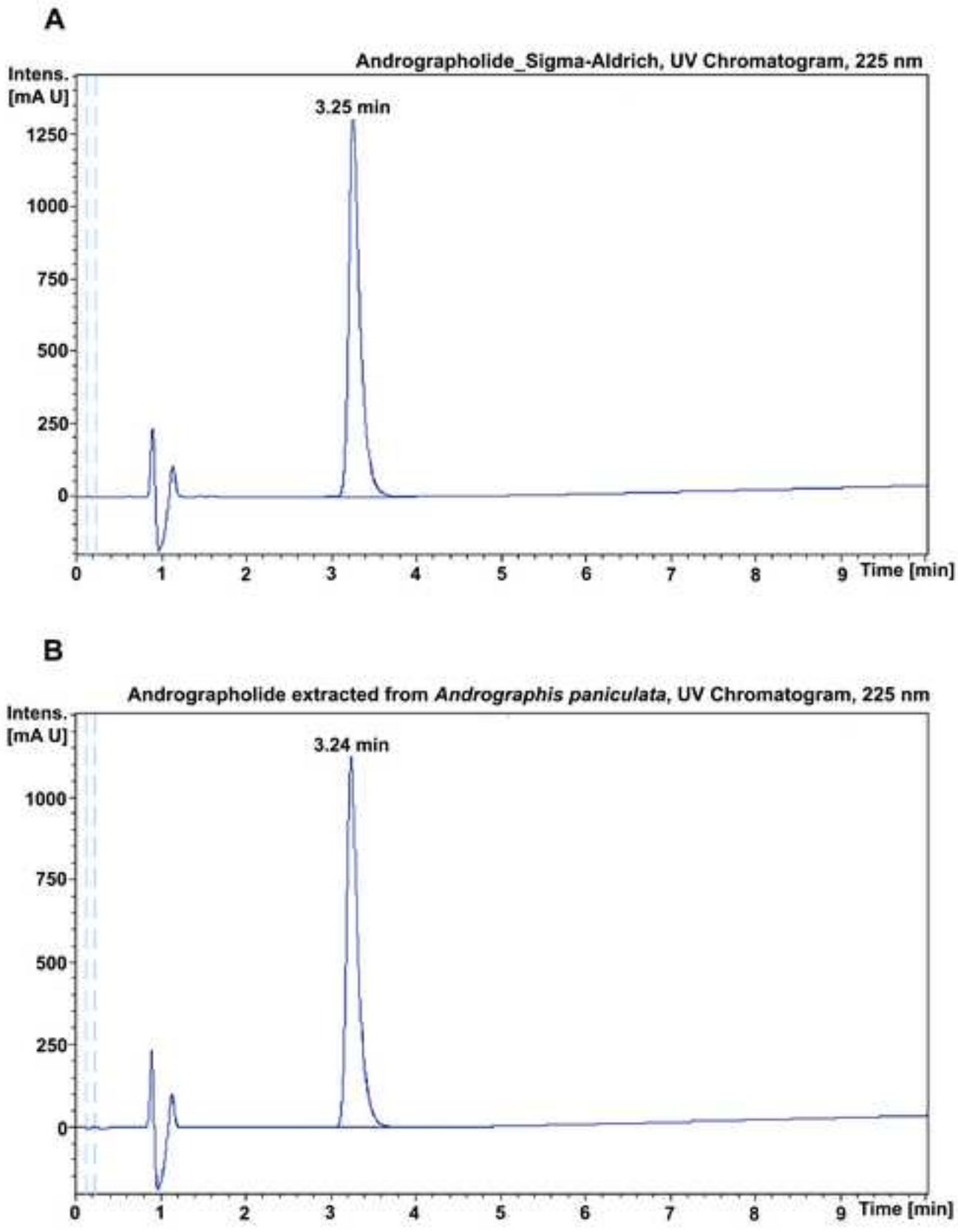

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Supplementary Figure S2

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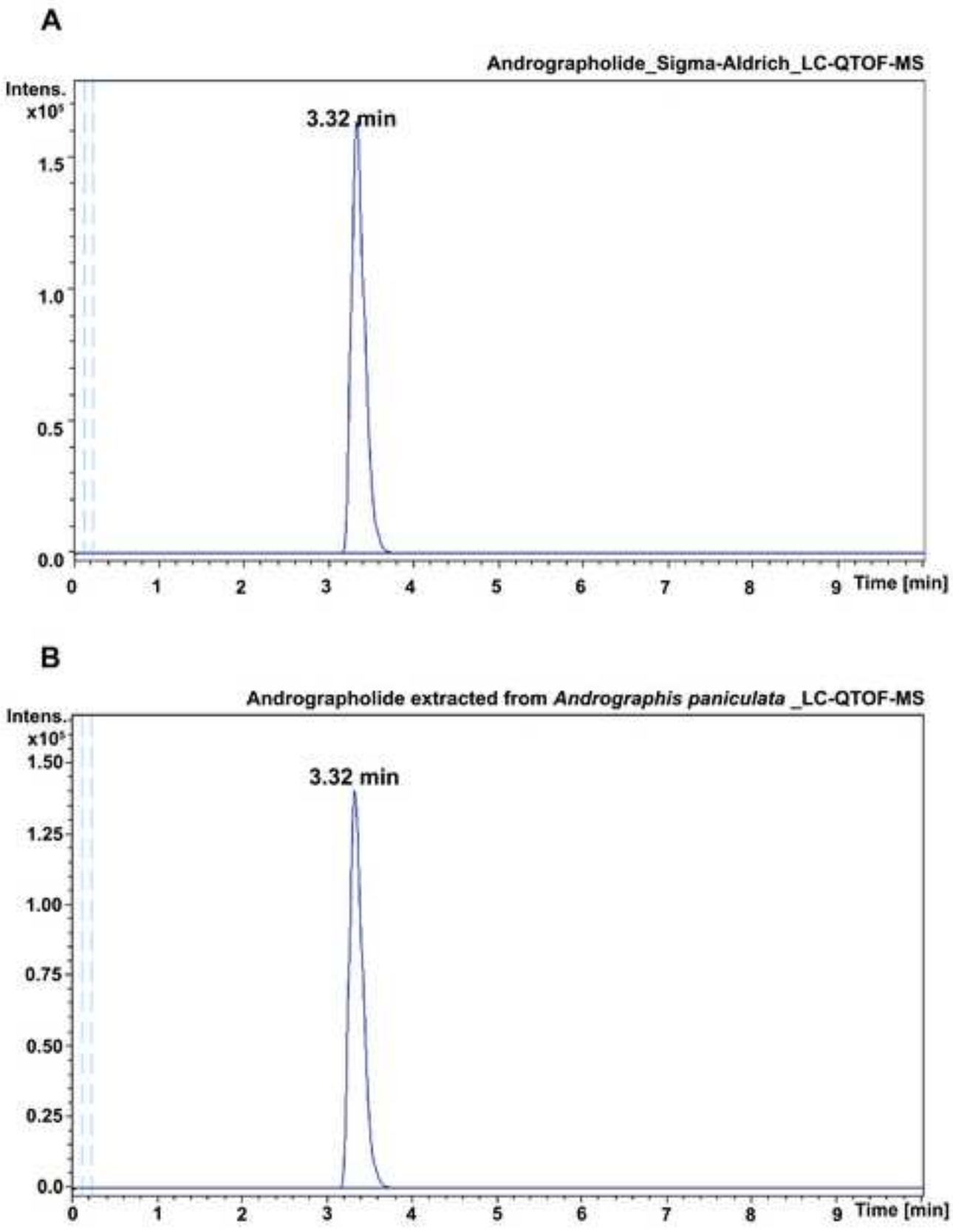

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Supplementary Figure S3

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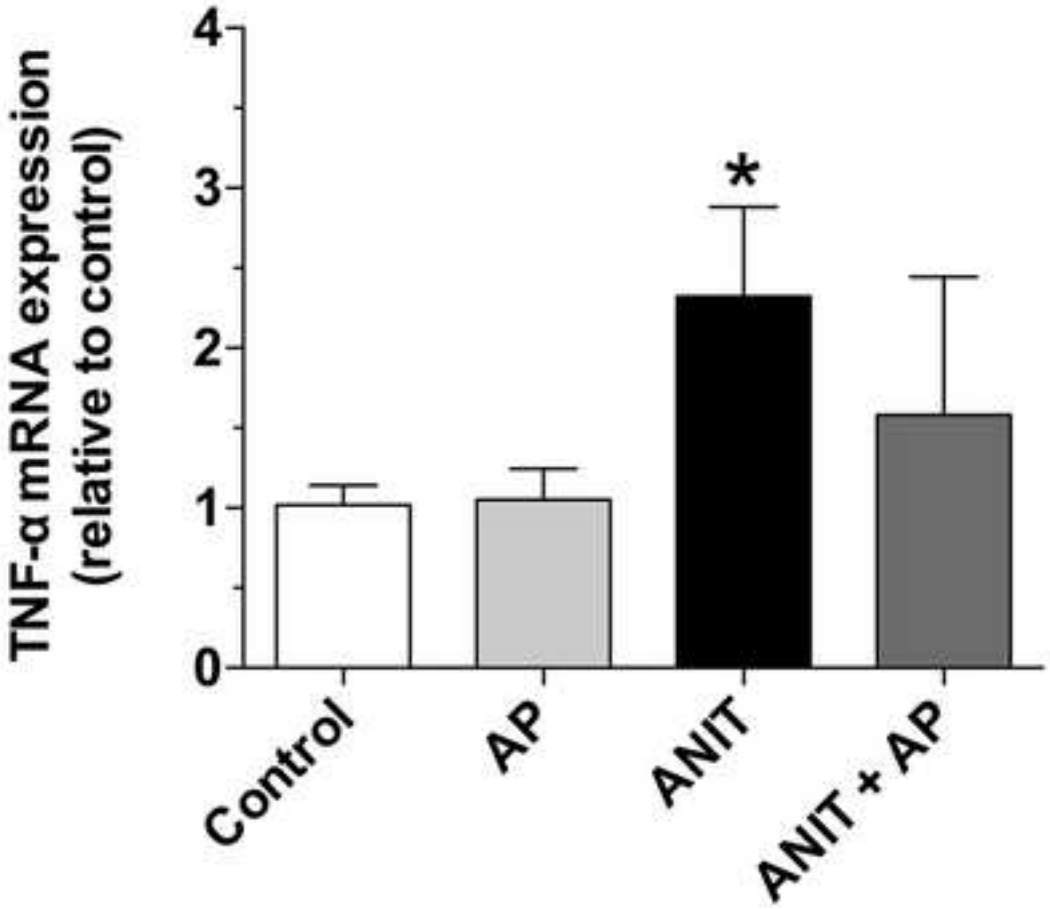

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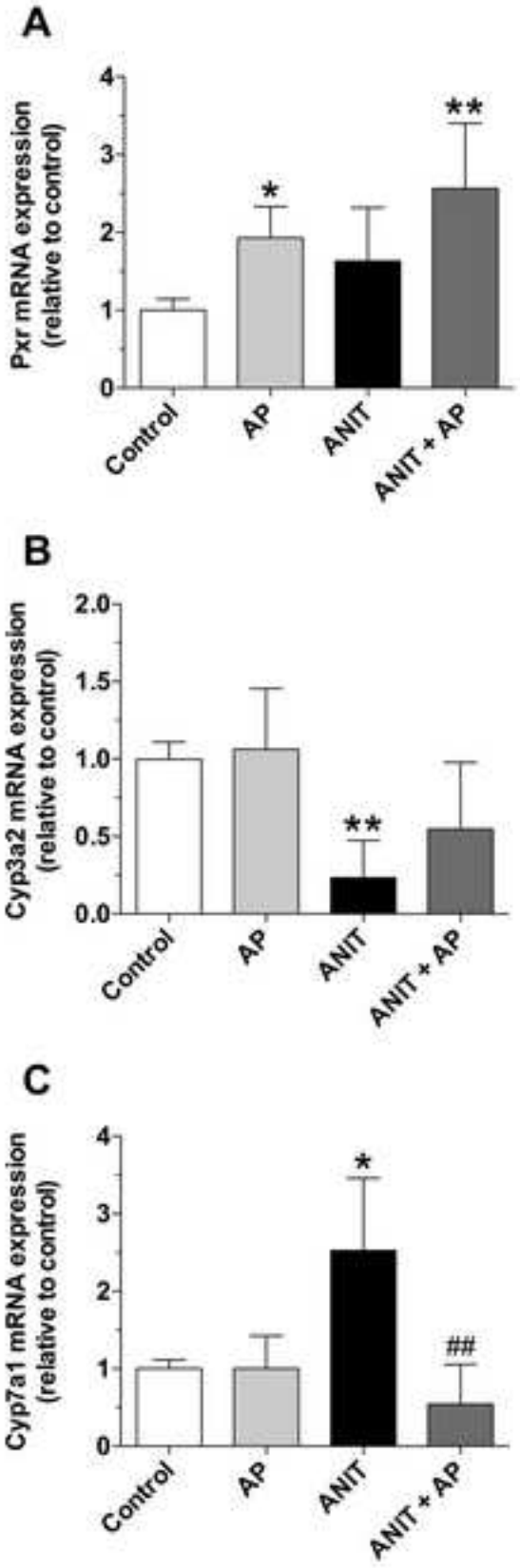

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SUPPLEMENTAL INFORMATION

Supplemental Figure S1. HPLC chromatogram and UV spectra.

HPLC-UV chromatograms of **A)** a standard andrographolide obtained from Sigma-Aldrich (365645-100MG, 98%), **B)** andrographolide extracted from *Andrographis paniculata*. From the absorption spectral data for andrographolide in the gradient mobile phase of 0.1% formic acid in deionized water/acetonitrile (70/30, v/v) with flow rate at 300 μL/min. Both andrographolide from Sigma-Aldrich and andrographolide extracted from *Andrographis paniculata* had the maximal absorption wavelength and detection at 225 nm.

Supplemental Figure S2. Accurate mass measurements by Liquid chromatography quadrupole time-of-flight mass spectrometry (LC-QTOF-MS).

LC-QTOF-MS analysis of **A)** andrographolide obtained from Sigma-Aldrich, **B)** andrographolide extracted from *Andrographis paniculata*. Andrographolide from Sigma-Aldrich extracted from *Andrographis* paniculata gave the identical fragment ion at m/z 701.4259 corresponding to $C_{20}H_{30}O_5$.

Supplemental Figure S3. Andrographolide decreases the expression of tumor necrosis factor (TNF-α).

Relative mRNA expression of TNF-α in livers of control and ANIT-treated rats pretreated with CMC or andrographolide. ANIT induced a marked increase in TNF-α mRNA expression that was diminished by andrographolide. Data represent mean ± S.D., n = 4 - 8 animals per groups, *$p < 0.05$ *versus* control; *AP*, andrographolide.

Supplemental Figure S4. Effect of andrographolide on hepatic expression of genes involved in bile acid and bilirubin metabolism in ANIT-induced cholestasis.

Relative mRNA expression of **A)** *Pxr*, **B)** *Cyp3a2*, and **C)** *Cyp7a1* in livers of control and ANIT-treated rats pretreated with CMC or andrographolide. Data represent mean ± S.D., n = 4 - 8 animals per group, *$p < 0.05$; **$p < 0.01$ *versus* control, $^{\#\#}p < 0.01$ *versus* ANIT-treated rats. *AP*, andrographolide.