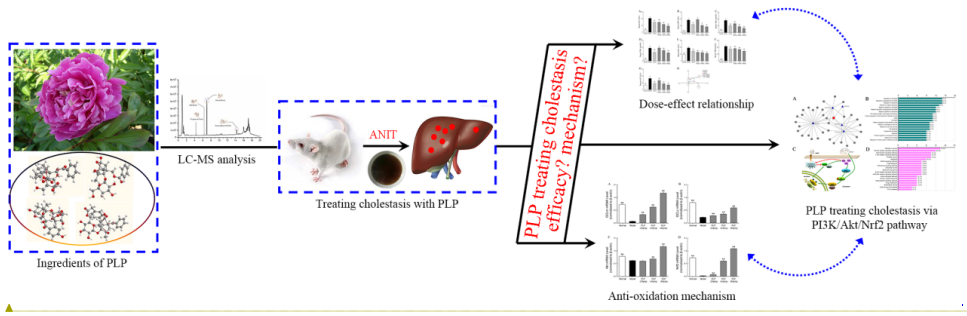

~~Figure 1~~ ~~Work flow of present study.~~

~~2.~~ Materials and ~~M~~methods

~~2.1~~ Chemicals and reagents

~~–~~In this ~~experiment~~study, the dried root of ~~*Paeonia lactiflora* Pall~~PLP~~.~~ was used. PLP was purchased from Beijing Lvye Medicinal Materials Company (Beijing, People's Republic of China). ANIT was purchased from Sigma-Aldrich Co.~~Sigma Chemical Company~~ (St~~.~~ Louis, MO, USA). Total bilirubin (TBIL), direct bilirubin (DBIL), aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), γ-glutamyltranspeptidase (γ-GT), total bile acid (TBA), and GSH assay kits were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, People's Republic of China). All ~~of~~ the other chemicals of analytical grade were purchased from commercial sources~~ with analytical grade~~.

~~2.2 Compounds information of PLP identified~~Identification of compounds present in PLP by UPLC-Q-TOF

The dried root of ~~*Paeonia lactiflora* Pall~~PLP~~.~~ was cut into small pieces, mixed, and soaked in water (1/10 w/v) for 0.5 hours~~;~~ then the mixture was boiled ~~with~~ and reflux extraction was performed two times~~ twice~~ (2 hours for the first time, 1.5 hours for the second time). The ~~water~~ aqueous extract of PLP was decanted, filtered through six~~6~~-layer gauze, and evaporated to dryness under reduced pressure. The weight ratio (w/w) of powder to raw herb was 25.8%. The powder was dissolved ~~to the concentration of 0.02g/mL with~~ in purified water to obtain a final concentration of 0.02 g/mL, which was ~~as~~used as the injection sample.

UPLC-Q-TOF was performed with the following conditions~~:~~; ~~C~~column: 2.1×100 mm, 1.8 μm (Zorbax Eclipse Plus C18, Agilent, Santa Clara, CA, USA)~~;~~; ~~I~~injection volume: 2 μL; ~~F~~flow rate: 0.3 mL/min~~;~~; ~~S~~solvents: A, H_2O with 0.1% H_3PO_4; and B, 100% acetonitrile~~;~~; ~~G~~gradient: 0~~–~~2 minutes, 95% A; 2~~–~~5 minutes~~:~~, 85% A; 5–10 minutes, 75% A; 10~~–~~12 minutes, 70% A; 12–14 minutes, 62% A; 14–16 minutes, 20% A; 16–20 minutes, 95% A.

For operation in MS/MS mode, a mass spectrometer (MS) fitted with an orthogonal Z-spray ion interface was used for all the analyses. Ionization was achieved using electrospray technique. For analysis, the electrospray source parameters were fixed as follows: electrospray capillary voltage was 3.0 kV in negative ionization mode and 4.0 kV in positive ionization mode~~;~~ ~~T~~the

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mass range was set from ~~m/z~~ 80 to 1,000 m/z~~.~~; ~~G~~gas temperature was 200°C in negative ionization mode and 225-°C in positive ionization mode~~.~~; ~~G~~gas flow was 11 L/min~~.~~; ~~N~~nebulizer was set to 35 pisg (negative) and 45 pisg (positive)~~.~~; ~~S~~sheath gas temperature was 350~~–~~°C and sheath gas flow was 12 L/min~~.~~; ~~N~~nozzle voltage was 500 V in both negative and positive modes.

~~2.3~~ Animals and treatments

~~–~~Male Sprague~~–~~ Dawley rats weighing 200±20 g were obtained from the laboratory animal center of The Military Medical Science Academy of the PLA (Permission No~~.~~ SCXK-(A) 2012-0004). All ~~of~~ the animals were acclimated for 1 week prior to the experiment and were kept under the same temperature (25±2~~–~~°C) and lighting (12:12~~–~~hour light:dark cycle) conditions. Water and food were available for rats ad libitum. ~~The whol~~All the studies were performed in accordance with the guidelines of the Council on Animal Care of Academia Sinica. The animals were randomly divided into six groups with ~~10~~ten rats in each group. ~~Water extraction~~The aqueous extract of PLP was dissolved in normal saline (PLP group) and was ~~intragastrically~~ given to the rats intragastrically at doses of 80, 40, or 20 g/kg~~.~~ daily ~~respectively~~ for ~~five~~5 days, respectively. Rats in PLP groups were intragastrically treated with 60 mg/kg ANIT (dissolved in ~~the~~ olive oil) (ANIT group) on the third day. PLP doses adopted in this study were based on preliminary experiments, and were proved ~~with no toxicity~~to have no toxic effects.[13,14] ANIT group was administrated normal saline each day compared with PLP groups. On the third day, ANIT group was also treated with 60 mg/kg ANIT. The rats serving as control ~~was~~ were administrated normal saline ~~each~~ every day and intragastrical treatment with the vehicle (olive oil) alone on the third day. Ursodeoxycholic acid (UDCA), the positive control, was ~~treated~~ given to rats at a dose~~a~~age of 60 mg/kg~~.~~ ~~d~~daily for 5 days with the same condition as PLP or ANIT groups.

~~2.4~~ Sample collection and liver functional assays

Rats were sacrificed after the last administration. Blood samples were collected and centrifuged at 3,000~~–~~× *g* for 10 minutes ~~to obtain~~and serum was separated. ~~All of t~~The serum samples thus obtained were sterile, hemolysis-free, and stored at ~~–~~70~~–~~℃ before determining the biochemical parameters. The serum levels of TBIL, DBIL, AST, ALT, ALP, γ-GT, and TBA were measured by Synergy ~~h~~Hybrid ~~r~~Reader (Biotek, Winooski, VT, USA) ~~with~~ using assay kits, wherein assays were performed according to the manufacturer's protocol.[15]

~~2.5~~ Histological assessment of liver damage

Liver tissues were excised and fixed in 10% phosphate-buffered saline (PBS)~~–~~buffered formalin. Three or four paraffin-embedded sections (4–5 μm thick) per specimen were prepared and stained with hematoxylin-eosin (HE)~~ staining)~~. The stained sections were examined under Nikon microscope and analyzed by image Pro-Plus 7200 software.

~~2.6~~

Measurement of GSH content in liver tissue

~~–~~A portion of the liver tissue was homogenized with a buffer containing 0.15 M KCl to obtain a

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1:10 (w/v) solution. The homogenates were then centrifuged at 12,000-× *g* (4-°C) for 20 minutes ~~to collect~~ and the supernatants were collected. The supernatant was measured using the assay kit. All the steps were performed according to the manufacturer's instructions for the determination of GSH content.[16]

~~2.7~~ Western blot for detecting GLCc, GCLm, pAkt, and Nrf2 in liver tissue

~~–~~Rat liver tissue (0.1 g) was homogenized and subsequently lysed in ice-cold lysis buffer containing 1 mM phenylmethylsulfonyl fluoride and a protease inhibitor mixture. The sample was subjected to centrifugation at 8,000~~–~~× *g* for 10 minutes at 4~~–~~°C to remove any debris. After centrifugation, the supernatant was aliquoted and stored at ~~–~~80~~–~~°C. These samples were used to ~~for~~perform ~~w~~Western blot assay to detect the presence of GLCc, GCLm, and pAkt. The nuclear and cytoplasmic extractions were performed using the nuclear and cytoplasmic extraction kits (Biosynthesis Biotechnology Company, Beijing, People's Republic of China) following manufacturer's instructions. Then, nuclear and cytoplasmic proteins were assayed by ~~w~~Western blot analysis for the presence of Nrf2. Fifty micrograms of total liver protein was separated by 12% SDS–polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. Immunodetection was performed using a rabbit anti-GCLc antibody (1:5,000), anti-GCLm antibody (1:5,000), anti-Akt antibody (1:2,000), anti-Nrf2 antibody (1:2,000), and mouse anti-GAPDH antibody (1:1,000) in a solution of 5% milk, Tris-buffered saline (TBS), and 0.05% Tween-20 to detect GCLc, GCLm, Akt, Nrf2, and GAPDH, respectively. After incubation with the appropriate ~~secondary~~ peroxidase-conjugated secondary antibody, the membrane was washed in TBST (TBS with Tween 20) for 60 minutes, and the immunoreactive bands were visualized with chemiluminescence.

~~2.8~~

Quantitative RT-PCR analyses for GCLc, GCLm, Akt, Nrf2, Nqo1, and HO-1 in liver tissue

The effect of PLP on mRNA expressions of GCLc, GCLm, Akt, Nrf2, NAD(P)H/quinone oxidoreductase 1 (Nqo1), and heme oxygenase-1 (HO-1) ~~mRNA expressions of~~ in the liver tissue ~~from~~ of ANIT-induced cholestasis rats were determined by quantitative reverse transcription polymerase chain reaction (RT-PCR). Total RNA was extracted from the liver tissues of each group using Trizol reagent following the manufacturer's protocols ~~using Trizol reagent~~. RNA concentration was determined by optical density measurement at 260 nm on a spectrophotometer. RNA (2 µg) was reverse-transcribed using a PrimeScript™ RT reagent kit, and 2 µL cDNA was used for the PCR reaction. List of ~~P~~primers used in our ~~paper~~ study are listed in Table 1. The subsequent PCR amplification was carried out by ABI Step One Plus (Applied Biosystems Inc, Carlsbad, CA, USA) PCR machine, ~~using~~running 40 cycles ~~of~~at 95-°C for 5 seconds and 60°C for 60 seconds. Fold changes were calculated for comparison through $2^{-\triangle\triangle CT}$ method.

~~**Table 1** Primers sequences for RT-PCR.~~

~~Gene~~	~~Sense Primer (5'–3')~~	~~Anti-Sense Primer (5'–3')~~

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