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Alisma orientale extract exerts the reversing cholestasis effect by activation of farnesoid X receptor

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ABSTRACT

Background: Cholestasis is a clinical syndrome of liver damage that is caused by accumulation of bile acids in the liver and systemic circulation. Farnesoid X receptor (FXR) can regulate synthesis, metabolism, and excretion of bile acids. The rhizomes of *Alisma orientale* is a well-known traditional Chinese medicine to treat edema, obesity, gonorrhea, leukorrhea, diarrhea, hyperlipidemia, and diabetes in China.

Hypothesis/purpose: We hypothesized *Alisma orientale* extract (AOE) to exert hepatoprotective effect against α-naphthylisothiocyanate (ANIT) induced cholestasis in rat. We aimed to investigate the mechanism of AOE.

Study design: Male Sprague Dawley rats with intrahepatic cholestasis induced by ANIT were treated with AOE (150, 300, or 600 mg/kg). Rats receiving vehicle (0.5% CMC-Na) served as control.

Methods: 48 h after ANIT administration, rats were sacrificed. Blood was collected to obtain serum and livers were removed for histopathology and protein preparation. Biochemical indicators in serum were determined using commercial kits and triterpenoids were determined by liquid chromatography tandem Qtrap mass spectrometry. Proteomics was analyzed by liquid chromatography tandem ion-trap mass spectrometry. The differently expressed proteins were analyzed via the network database and verified by western blotting. The interaction between triterpenoids and FXR were evaluated by luciferase assay and molecular docking.

Results: AOE treatment significantly decreased the serum AST, ALT, TBIL, and intrahepatic TBA and improved the liver pathologic change induced by ANIT. Proteomics analysis indicated that AOE regulated proteins related to bile acid homeostasis via activating farnesoid X receptor (FXR) signaling pathway. Luciferase assay and molecular docking results indicated that triterpenoids could activate FXR, which resulting in ameliorative accumulation of bile acids in the liver by increase of metabolism and transportation for bile acids, and decrease of synthesis for bile acids.

Conclusion: AOE protected against rat liver injury and cholestasis induced by ANIT by activation of farnesoid X receptor, suggesting that *A. orientale* could be regarded as a potential hepatoprotective drug.

Introduction

Cholestasis is a clinical syndrome of liver damage that is caused by accumulation of bile acids in the liver and systemic circulation. Abnormal metabolism of bile acids is considered as an important risk factor to induce cholestasis, and it would lead to primary biliary cirrhosis, primary sclerosing cholangitis, and hepatic failure due to long-term cholestasis. Farnesoid X receptor (FXR) was the first nuclear receptor mainly expressed in the liver, intestine, kidney, and adrenal gland (Parks et al., 1999). FXR regulated synthesis, metabolism, and excretion of bile acids by controlling the expression of genes involved in their transport and metabolism (Forman et al., 1995; Makishima et al., 1999). Therefore, FXR was considered as a target to cure cholestasis and liver injury (Jonker et al., 2012).

Abbreviations: AOE, *Alisma orientale* extract; ANIT, α-naphthylisothiocyanate; FXR, farnesoid X receptor; AST, aspartate aminotransferase; ALT, alanine aminotransferase; TBIL, total bilirubin; TBA, intrahepatic total bile acids; CMC-Na, sodium carboxymethyl cellulose; H&E, hematoxylin and eosin; ESI, electrospray ionization; Cyp7a1, cholesterol 7-alpha-monooxygenase; Slc10a1, sodium/bile acid cotransporter; Ugt1a1, UDP-glucuronosyltransferase 1A1; Sult2a1, sulfotransferase 2A1; BSEP, bile salt export pump

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Alisma orientale (Sam.) Juzep. is a perennial plant of the genus *Alisma* (Alismataceae), in which comprises 11 species and mainly distributed in north temperate zone and Oceania (Flora of China Committee, 1992). Its rhizoma is a well-known traditional Chinese medicine, known as "*Ze-Xie*" in Chinese, and has been used to treat edema, obesity, gonorrhea, leukorrhea, diarrhea, hyperlipidemia, and diabetes in China according to Pharmacopoeia of the People's Republic of China (Pharmacopoeia Commission of People's Republic of China, 2010). Phytochemical investigations of the genus *Alisma* have indicated that it mainly possesses protostane-type triterpenoids and guaiane-type sesquiterpenes (Mai et al., 2015a, 2015b; Zhang et al., 2017). To our knowledge, the protostanes were considered as chemotaxonomic markers of the genus *Alisma* due to the only occurrence of this genus. At present, triterpenes isolated from the genus *Alisma* include alisol A-P and their derivatives (Tian et al., 2014; Wang et al., 2017; Yu et al., 2017; Xin et al., 2017, 2018). They showed a wide variety of bioactivities, such as hepatoprotective (Hong et al., 2010), antilipemic (Dan et al., 2011; Wu et al., 2007), NO production (Matsuda et al., 1999; Zhao et al., 2017), antiproliferative (Law et al., 2010), immunomodulatory (Kubo et al., 1997; Lee et al., 2012) activities, which attracted attention from pharmacists, botanists, and chemists. In our previous studies, twenty-six protostane-type triterpenoids were isolated and obtained from the rhizome of *A. orientale* and biotransformation by *Penicillium janthinellum* AS 3.510, respectively, which exhibited inhibitory activity on human carboxylesterase 2 (Mai et al., 2015a, 2015b; Zhang et al., 2017).

In this paper, *A. orientale* extract (AOE) was evaluated for its protective activity against liver injury and intrahepatic cholestasis in ANIT-induced rats through histopathology observation, proteomics analysis, western blot, and determination of biochemical indicators including serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin (TBIL), and intrahepatic total bile acids (TBA).

Materials and methods

General

An ultrasonic cleaner (KQ5200DV, Kunshan Ultrasonic Instruments Co., Ltd., China) at 40 kHz frequency and 200 W power was used for the ultrasound-assisted extraction of total triterpenoids from *A. orientale*. Standard substances 17-epoalisol A (**1**), alisol P (**2**), alisol F (**3**), alisol A (**4**), 25-anhydro alisol F (**5**), 25-anhydro alisol A (**6**), alisol B (**7**), alisol B 23-acetate (**8**), 16-oxo-alisol A (**9**), 24-deacetyl alisol O (**10**), and 16-oxo-11-anhydro alisol A (**11**) were separated from *A. orientale* by the authors (Fig. 1), their purities were more than 98% analyzed by HPLC. ANIT was purchased from Sigma-Aldrich (St. Louis, MO). All biochemical indicator kits and other chemicals were commercially available.

Plant material

Dried rhizomes of *A. orientale* were purchased in January 2013 from Beijing Tongrentang Co., Ltd., China, and identified by Prof. Jing-Ming Jia, Shenyang Pharmaceutical University. A voucher specimen (301114120P) has been deposited in the herbarium of the Department of Medicinal Chemistry, Dalian Medical University.

Preparation of AOE

A. orientale powder (capable of passing a 4-mm sieve) was untrosonicly extracted at the following condition: ultrasound power 200 W, extraction time 90 min, extraction temperature 70 °C, and material ratio 1:15. The extract was evaporated in *vacuo* at 45 °C, then it was stored at 4 °C.

In vivo evaluation of reversing cholestasis effect

Treatments and experimental protocol

Male Sprague Dawley rats (200–220 g) were obtained from the Experimental Animal Center of Dalian Medical University (Dalian, China; permit number SCXK 2008-0002). All animal maintenance and treatment protocols were in accordance with local institutional guidelines for the care and use of laboratory animals. Rats were randomly divided into six groups ($n = 6$). Control group was given 0.5% CMC-Na for 7 days, and received the vehicle (olive oil) alone on the 5th day. ANIT group was treated with 0.5% sodium carboxymethyl cellulose (CMC-Na) for seven days, and administrated ANIT (100 mg/kg, dissolved in olive oil) on the 5th day. The AOE groups were administrated by oral gavage of AOE (150, 300, or 600 mg/kg, dissolved in 0.5% CMC-Na) for seven days, and administrated with or without ANIT (100 mg/kg, dissolved in olive oil) on the 5th day.

Serum biochemical and intrahepatic total bile acids analyses

Blood was collected from postcava to obtain serum, and AST, ALT, TBIL, and intrahepatic TBA were detected by commercial kits (Nanjing Jiancheng Institute of Biotechnology, Nanjing, China).

Histopathology

The livers were removed after rats were euthanized, fixed in 10% neutral buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin (H&E).

Determination of triterpenoids in rat serum

A 50 μL aliquot of serum and 100 μL of acetonitrile were mixed and vortexed for deproteinization. After centrifugation at 20,000 g for 10 min, 10 μL aliquot was injected into the LC-MS/MS for determination of triterpenoids in rat serum.

Separation experiment was performed using Shimadzu Prominence UFLC with Waters XBridge C_{18} column (4.6 × 150 mm, 2.5 μm). Mobile phase consisted of acetonitrile (A) and aqueous buffer solution of 0.1% methanoic acid (v/v) with the gradient program as 0/10/12/20/25/31 min, 50%/50%/65%/65%/94%/94% (A). The flow rate was 0.5 ml/min and the injection volume was 10 μL. The column temperature was maintained at 30 °C. The mass spectrometer was operated on AB Sciex Qtrap® 4500 tandem mass spectrometer (Foster City, CA, USA) with an electrospray ionization source (ESI) in the positive ion mode. The dynamic multiple reaction monitoring (DMRM) method was used for the multiple components quantification. The optimized conditions were as follows: ion spray voltage, 4.5 kV (positive mode); gas source (1), 30 psi; gas source (2), 40 psi; turbo temperature, 500 °C; entrance potential (EP), 10 V; and collision cell exit potential (CXP), 13 V; the curtain gas pressure at 20 psi. And the information for DMRM parameters together with the related optimized declustering potential (DP), and collision energy (CE) for the different analytes were shown in the quantitative analysis results section.

Proteomics analysis

Sample preparation

The isolated liver tissues of ANIT group rats (vehicle + ANIT) and AOE (600 mg/kg) group rats were cut into pieces and washed with PBS. Equal weight liver tissue pieces of two groups were transferred to new tubes, homogenized in an ice-cold lysis buffer containing 8 M urea, 50 mM Tris-HCl (pH 7.4), 65 mM DTT, 1% protease cocktail (v/v), 1% Triton X-100 (v/v), 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, sonicated for 400 W × 120 s within ice bath and centrifuged at 25,000 g for 1 h, respectively. The supernatants were collected, and the protein concentrations were determined by BCA assay (Beyotime Institute of Biotechnology, Jiangsu, China). Equal amount of two protein samples were purified by ultrafiltration with two 10 KDa tubular ultrafiltration membrane, then reduced by 10 mM DTT at 60 °C for 1 h and alkylated