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Protective effect of a polysaccharide from *Anoectochilus roxburghii* against carbon tetrachloride-induced acute liver injury in mice

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ABSTRACT

Ethnopharmacological relevance: *Anoectochilus roxburghii* (Wall.) Lindl. is traditionally used for the treatment of various types of chronic and acute hepatitis in China. Considering that *Anoectochilus roxburghii* polysaccharide (ARPT) is the main constituent of *Anoectochilus roxburghii*, the present study was designed to investigate the hepatoprotective effect of ARPT and its possible mechanism in carbon tetrachloride (CCl_4)-induced mice.

Material and methods: The hepatoprotective activity of ARPT (150, 300 and 500 mg/kg) were investigated on CCl_4-induced acute liver damage in mice. The activities of alanine transaminase (ALT), aspartate transaminase (AST) were determined in serum. The hepatic levels of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and malondialdehyde (MDA) were measured in liver homogenates. The levels of cytochrome P450 sub family 2E1 (CYP2E1), tumor necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β), interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-2 (MIP-2), KC (Murine IL-8 ortholog), transforming growth factor-beta1 (TGF-β1), Bcl-2 and Bax were measured by quantitative real-time polymerase chain reaction (qRT-PCR). The expressions of CYP2E1, nuclear factor-kappa B (NF-κB) p65 and caspase-3 were evaluated by western blot assays. The hepatic levels of TNF-α, IL-6, MIP-2 and TGF-β1 were measured by enzyme-linked immunosorbent assay (ELISA). Furthermore, histopathological observation and terminal-deoxynucleoitidyl transferase mediated nick end labeling assay (TUNEL) were carried out on the separated livers of mice.

Results: ARPT significantly decreased serum ALT and AST activities, hepatic MDA level, and markedly enhanced antioxidant enzyme (SOD, CAT and GSH-Px) activities and GSH level in hepatic tissue, in a dose-dependent manner, when compared to the model group. Histopathological observation revealed the hepatoprotective effect of ARPT against the damage. Furthermore, ARPT remarkably inhibited CYP2E1 mRNA expression, decreased NF-κB p65 expression and therefore to prevent the secretion of pro-inflammatory cytokines (TNF-α and IL-6) and chemokines (MCP-1, MIP-2 and KC), suppressed TGF-β1 expression and hepatocytes apoptosis. Moreover, ARPT could prevent DNA fragmentation based on TUNEL assay results.

Conclusion: These findings suggested that ARPT possessed hepatoprotective effect against CCl_4-induced hepatotoxicity in mice and the action might in part be through reducing oxidative stress, inflammation and apoptosis.

1. Introduction

The liver, an important organ, performs critical functions such as production of serum proteins and hormones, metabolism of endogenous and exogenous substances, detoxification and so on (Sun and Karin, 2008). Exogenous substances such as drugs, infections, alcohol and chemical may induce hepatotoxicity, causing an overall decline in metabolic functions of the liver. Carbon tetrachloride (CCl_4) is widely used as hepatotoxin in experimental animal models. Increasing studies showed that oxidative stress was an important mechanism for hepatotoxicity induced by CCl_4 (Shim et al., 2010; Xiao et al., 2012). CCl_4 was metabolized by cytochrome P450 system (CYP2E1) to form trichloromethyl free radical (CCl_3•) and trichloromethyl peroxy radical (CCl_3OO•), which induced membrane lipid peroxidation (Nada et al., 2010). Inflammation was thought to be another important mechanism in CCl_4-induced hepatic injury. Hepatic macrophages were activated, which induced the release of inflammatory mediators, leading to liver injury (Shim et al., 2010; Xiao et al., 2012). Several lines of evidence

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suggest that the liver damage may be prevented by inhibiting oxidative stress and inflammation response (Tipoe et al., 2010; Aldaba-Muruato et al., 2012).

Currently, growing attentions have been paid to the use of phytomedicines for low toxicity and good therapeutic performance. *Anoectochilus roxburghii*, belongs to the genus *Anoectochilus* (Orchidaceae), is a traditional herb used in China. *A. roxburghii* is widely distributed in the tropical regions from India through the Himalayas and from southeast Asia to Hawaii (Zhang et al., 2015). Both *A. formosanus* and *A. roxburghii* are called 'king medicine' (Liu et al., 2014). These plants were traditionally used for the treatment of hepatitis, tumor, diabetes, hyperliposis, cardiovascular diseases, respiratory infections, snake bites and so on (Zhang et al., 2007; Liu et al., 2014; Shao et al., 2014). Pharmacological studies showed that these plants had a wide range of biological activities including antioxidant (Shih et al., 2003), hepatoprotective (Lin et al., 1993, 2000; Du et al., 2003; Shih et al., 2004, 2005; Wu et al., 2007; Fang et al., 2008; Hsieh et al., 2011), antihyperglycemic (Zhang et al., 2007; Cui et al., 2013), immunostimulatory and antitumor (Tseng et al., 2006) effects and so on. Recently, polysaccharides, the major constituents of *A. roxburghii*, have been studied for their antidiabetic (Zhang et al., 2015), renal protective (Li et al., 2016) and hepatoprotective (Zeng et al., 2016) effects. However, there is still no report on the related mechanisms underlying the hepatoprotective effect of the polysaccharides. Therefore, the present study was designed to evaluate the hepatoprotective activity of polysaccharide from *A. roxburghii* and then to explore the related mechanisms of this action.

2. Materials and methods

2.1. Materials and reagents

Six months old in vitro-grown plantlets of *A. roxburghii* , native to Fujian Province, were purchased from Geyuan Agriculture and Forestry Science and Technology Co. (Zhangzhou, China). It was identified by Senior Engineer Wenge Lin of Xiamen Diyuan Biological Technology Co. (Xiamen, China). A voucher specimen (NO. ZBY20140926001) was deposited at the Subtropical Plant Institute of Fujian Province (Xiamen, China).

Bicinchoninic acid (BCA) protein assay kit (#23225) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI, #D8417), Proteinase K (#P6556) and DNase I (#D4527) were from Sigma Chemical Co. (St. Louis, MO, USA). In situ cell death detection kit for terminal-deoxynucleoitidyl transferase mediated nick end labeling (TUNEL) assay was obtained from Roche Diagnostics (#12156792910, Roche Diagnostics GmbH, Mannheim, Germany). All other chemicals and reagents were of analytical grade.

2.2. Preparation and characterization of ARPT

The preparation of precipitated polysaccharides (ARCP) from *A. roxburghii* was prepared as previously described (Zeng et al., 2016). ARCP were dissolved and excluded protein with Sevag method (Staub, 1965), followed by dialysis with running water for 24 h and then with distilled water for 12 h. The polysaccharide solution was precipitated by 80% ethanol, collected after centrifugation, washed sequentially with 95% ethanol, anhydrous ethanol, acetone and ether, and then dried under reduced pressure and named ARPT. The characterization (carbohydrate content, uronic acid content, protein content, molecular weights, monosaccharide composition, UV and FT-IR spectra) of ARPT was described in a previous study (Zeng et al., 2016).

2.3. Animal grouping and experimental design

Male Kunming mice were obtained from the Experimental Animal Centre of Southern Medical University (Guangzhou, China). Animal experiment was conducted according to the previous method (Zeng et al., 2016). Briefly, after acclimation for 1 week, mice were randomly divided into seven groups consisting of eight animals per group. (I) normal control group: mice not given CCl_4, ARPT and silymarin; (II) ARPT control group: mice treated with ARPT at a dose of 300 mg/kg BW alone; (III) CCl_4 model control group: mice treated with CCl_4 alone; (IV–VI) ARPT treated groups: mice treated with CCl_4 and administered with ARPT at a dose of 150, 300 and 500 mg/kg BW, respectively; (VII) silymarin treated group (positive control group): mice treated with CCl_4 and administered with silymarin at a dose of 200 mg/kg BW. Animal treatment was administered by intragastric gavage and continued for 14 consecutive days. At 2 h after the last treatment, mice in groups III-VII were intraperitoneal injected with CCl_4 (10 mL/kg BW 0.125% CCl_4 solution in soybean oil). All procedures involving animals were conducted in strict accordance with the Chinese legislation on the use and care of laboratory animals, which approved by the State Council on October 31, 1988 and published on State Scientific and Technological Commission on November 14, 1988.

2.4. Liver index, serum biomarkers analysis and histopathological evaluation

All mice were weighed at 24 h after the injection with CCl_4 or soybean oil vehicle. Blood samples were collected, mice were sacrificed and livers were excised then weighted to calculate liver index (liver weight/body weight ×100). Portions of liver were fixed for histopathological evaluation according to the previous study (Zeng et al., 2016). Another portions of liver were stored at - 80 ℃ for further analysis. Serum alanine amino transferase (ALT, #C009-2) and aspartate amino transferase (AST, #C010-2) activities were measured using commercial reagent kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) according to manufacturer's instructions.

2.5. Assessment of the oxidative stress parameters

The levels of superoxide dismutase (SOD, #A001-1), catalase (CAT, #A007-1), glutathione peroxidase (GSH-Px, #A005), reduced glutathione (GSH, #A006-1) and malondialdehyde (MDA, #A003-1) in livers were determined using commercial reagent kits from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Protein content was determined by BCA protein assay kit. All values were normalized by the protein content of the same sample.

2.6. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from liver tissue with Trizol reagent (#15596-018, Invitrogen, Carlsbad, CA, USA). The purity and concentration of RNA were determined using a nucleic acid protein detector (BioPhotometer plus, Eppendorf, Hamburg, Germany). Complementary DNA (cDNA) was synthesized using PrimeScript® RT reagent Kit (Perfect Real Time) (#DRR037A, TaKaRa Biotechnology (Dalian) Co., Ltd., Dalian, China) according to the manufacturer's instructions. Messenger RNA (mRNA) expression levels of cytochrome P450 sub family 2E1 (CYP2E1), tumor necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β), interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-2 (MIP-2), KC (Murine IL-8 ortholog), transforming growth factor-beta1 (TGF-β1), Bcl-2 and Bax were quantified by qRT-PCR with SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) (#RR820A, TaKaRa Biotechnology (Dalian) Co., Ltd., Dalian, China) and Bio-Rad CFX96 Real-Time PCR System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Reactions were performed in a total volume of 20 μL, containing 10 μL of 2× SYBR Premix Ex Taq II (Tli RNaseH Plus), 0.5 μL each of the primer (10 μM), 0.5 μL cDNA templates and 8.5 μL dH_2O. The primers used for qRT-PCR are listed in Table 1. Thermal cycling