SUPPLEMENTARY MATERIAL

Hepatoprotective Activity of Isostrictiniin from *Nymphaea candida* on Con A-Induced Acute Liver Injury in Mice

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Abstract

This study is to investigate hepatoprotective activity of isostrictiniin from *Nymphaea candida*. Hepatic injury in mice was induced immunologically by caudal vein injecting Con A (20 mg/kg) on tenth day of isostrictiniin (25, 50, or 100 mg/kg) intragastric administration. The results demonstrated that pretreatment with isostrictiniin significantly and dose-dependently prevented increase of serum AST and ALT induced by Con A (P<0.05). Isostrictiniin significantly reduced the levels of MDA and NO in the liver tissue and restored activities of antioxidant enzymes SOD and GSH compared with model group (P<0.05). Furthermore, the increase of pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and IL-18 levels were significantly suppressed by isostrictiniin pretreatment compared with model group (P<0.05). Histopathological analysis showed that isostrictiniin attenuated the hepatocellular necrosis and reduction of inflammatory cells infiltration. The results indicates that preventive effect of isostrictiniin on acute liver injury may be attributed to its antioxidative and immunomodulatory activities.

Keywords: *Nymphaea candida*; isostrictiniin; hepatoprotective activity; Concanavalin A.

Experimental

Chemicals and Reagents

Concanavalin A (Con A) were purchased from Sigma Chemical Co.(St Louis, MO, USA). Biphenyl dicarboxylate (DDB) was obtained from Dezhou Deyao Pharmaceutical Co. Assay kits for aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were provided by Zhongsheng Tech. (Beijing, China). Commercial kits used for determining (MDA), superoxide dismutase (SOD), glutathione peroxidase (GSH) and nitric oxide (NO) activity were obtained from the Jiancheng Institute of Biotechnology (Nanjing, China). Elisa kits for interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) were supplied by Boshide Co. (Wuhan, China), and elisa kits for interleukin-18 (IL-18) was obtained from Jianglai Co. (Shanghai, China) All other chemicals were of analytical grade and were purchased from a local reagent retailer.

Plant material and preparation of isostrictiniin

The dried *N. candida* flower buds was obtained from Traditional Uygur pharmaceutical Co in Xinjiang of China, and identified by associate researcher Jiang He Institute of Materia Medica of Xingjiang in China. A voucher specimen (20160502) was deposited at the Institute of Materia Medica of Xinjiang in China.

Ten kilogram of this plant were extracted with 70% ethanol under reflux for 1 h at three times, and 70% ethanol extracts was evaporated under vacuum. The 70% ethanol extracts were purified by D101 resin to obtain the extracts as follows: water, 30%, 50% and 95% ethanol eluates, of which 30% ethanol eluates were applied to polyamide column and eluted with 70% ethanol after water removing impurities. Isostrictiniin was obtained from 30% methanol eluates by MCI gel CHP 20P chromatography with a high purity (more than 97.0% as determined by HPLC, Figure S1).

Animals

Kunming mice, weighing 20.0 ± 2.0 g, supplied by the Experimental Animal Centre of Xinjiang Medical University in China [No. SYXK (xin) 2011-0004]. The mice were housed in plastic cages with room temperature of 25 ± 1 ℃, under a 12 h light–dark cycle, with free access to food and water. All animal studies were performed in accordance with the guidelines for care and use of laboratory animals by Institute of Materia Medica of Xinjiang,.

Con A induced hepatotoxicity

Mice were randomly divided into six groups (n=10): control group, model group, DDB group (150 mg/kg) and isostrictiniin groups (25, 50 and 100 mg/kg). DDB was suspended in 0.5% sodium carboxymethylcellulose (CMC-Na) solution for administration, and isostrictiniin was dissolved in water. Mice in the DDB and isostrictiniin groups received DDB (150 mg/kg, ig, once daily) and isostrictiniin (25, 50 and 100 mg/kg, ig, once daily) except that mice in the control and model groups were given distilled water (0.2 mL/10 g, ig, once daily), respectively. All administrations were conducted for ten consecutive days. One hour after the last administration on the tenth day, mice in the control group received saline (0.1mL/10 g, iv) while mice in other groups were injected Con A (20 mg/kg, iv). Mice were sacrificed after fasted for 8 h, blood samples were collected and serum

was isolated for further tests; the livers were removed for biochemical studies and histopathological analysis.

Determination of the serum AST, ALT, TNF-α, IL-1β and IL-6 activities

The serum AST and ALT levels were measured using AST and ALT test kits according to the manufacturer's instructions, and the results were shown in Figure S2-I. ELISA kits were used to measure the serum TNF-α, IL-1β and IL-6 levels according to the manufacturer's instructions, and the results were shown in Figure S4.

Measurement of Liver Homogenate MDA, SOD, GSH, NO and IL-18 Contents

Each liver tissue sample was homogenized in nine volumes of ice cold saline solution and centrifuged at 3000 rpm for 10 min at 4 °C. Supernatants were used to determine the MDA, GSH, SOD and NO concentrations by using the commercially kits. The levels of MDA, GSH, SOD, and NO were normalized with the total protein content. Liver homogenate IL-18 levels were determined using ELISA kits according to introduction. The above determination results were shown in Figure S3 and S4.

Histopathological examination

For the histological investigations, a middle portion in the left lobe of the liver in each mouse was removed and then perfused in 4% paraformaldehyde for at least 24 h. After fixation, they were embedded in paraffin, sliced in 5 μm sections, and stained with hematoxylin and eosin (H&E) according to standard protocols for observing the tissue change under light microscopy (Figure S2-II).

Statistical analysis

Statistical analysis was conducted using SPSS 13.0 (SPSS Inc., Chicago IL, USA). All data were expressed as the means ± standard error (S.E.M.). The differences between different groups were analyzed using one-way analysis of variance (ANOVA). $P < 0.05$ were taken as statistically significant.

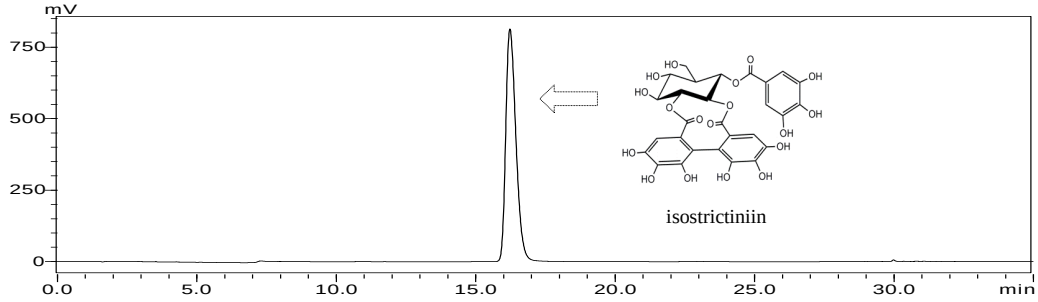
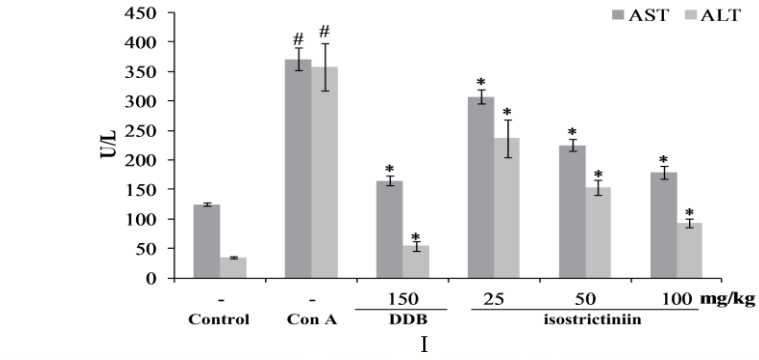


Figure S1. HPLC chromatogram of. A Shimadzu LC-10ATvp and Phenomenex Gemini C18 column (250 mm × 4.6 mm, 5 μm); the mobile phase composed of A (acetonitrile) and B (0.2% phosphoric acid, v/v) with a gradient elution: 0~20~25~30~35 min, 11% ~ 11% ~ 30% ~ 30% ~ 11%A; the flow rate at 1.0 mL/min, the column temperatures at 30 ℃ and detection wavelength at 266 nm.



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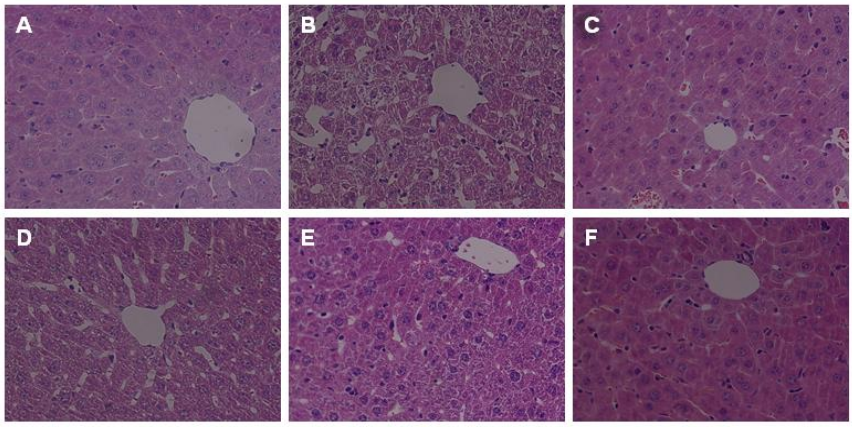

II

Figure S2. I. Effects of isostrictiniin on the serum AST and ALT in Con A- intoxicated mice. Values are mean ± S.E.M., n = 10. $^{\#}P < 0.05$ compared with control group. $^{*}P < 0.05$ compared with Con A group. II. Pathological changes of liver tissues in hematoxylin–eosin (HE) staining. **A,** Control group; **B,** Con A treated group; **C,** Con A + DDB (150 mg/kg) treated group. **D,** Con A + isostrictiniin (25 mg/kg) treated group; **E,** Con A + isostrictiniin (50 mg/kg) treated group; **F,** Con A and isostrictiniin (100 mg/kg) treated group.

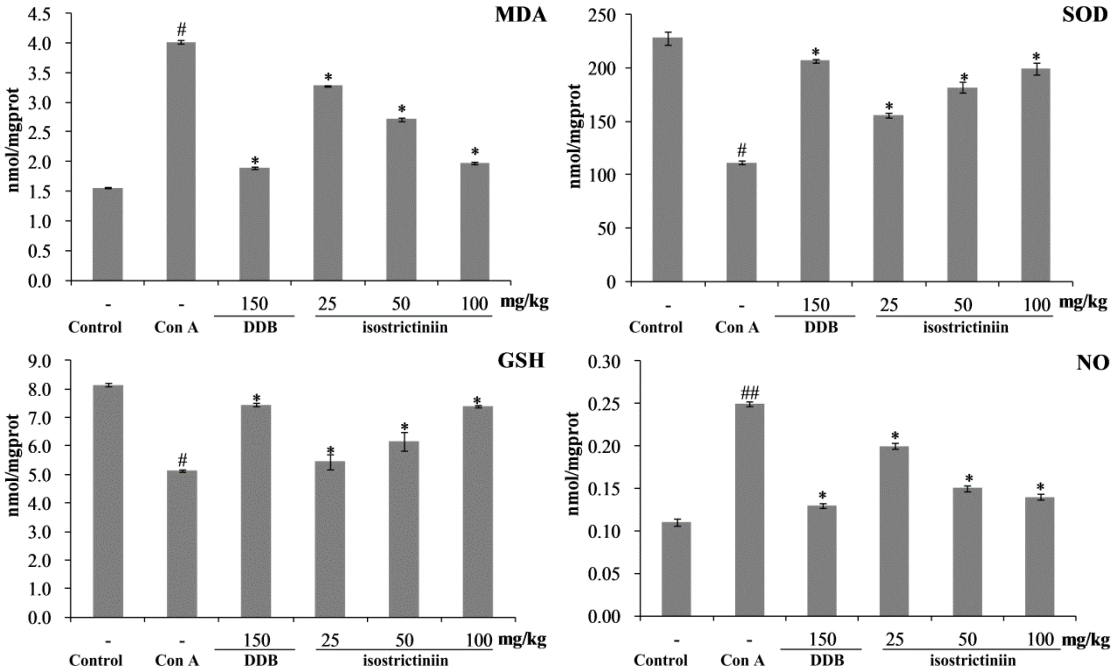

Figure S3. Effects of isostrictiniin on the serum MDA, SOD, GSH and NO in Con A- intoxicated mice. Values are mean ± S.E.M., n = 10. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with control group. $^{*}P < 0.05$ compared with Con A group.

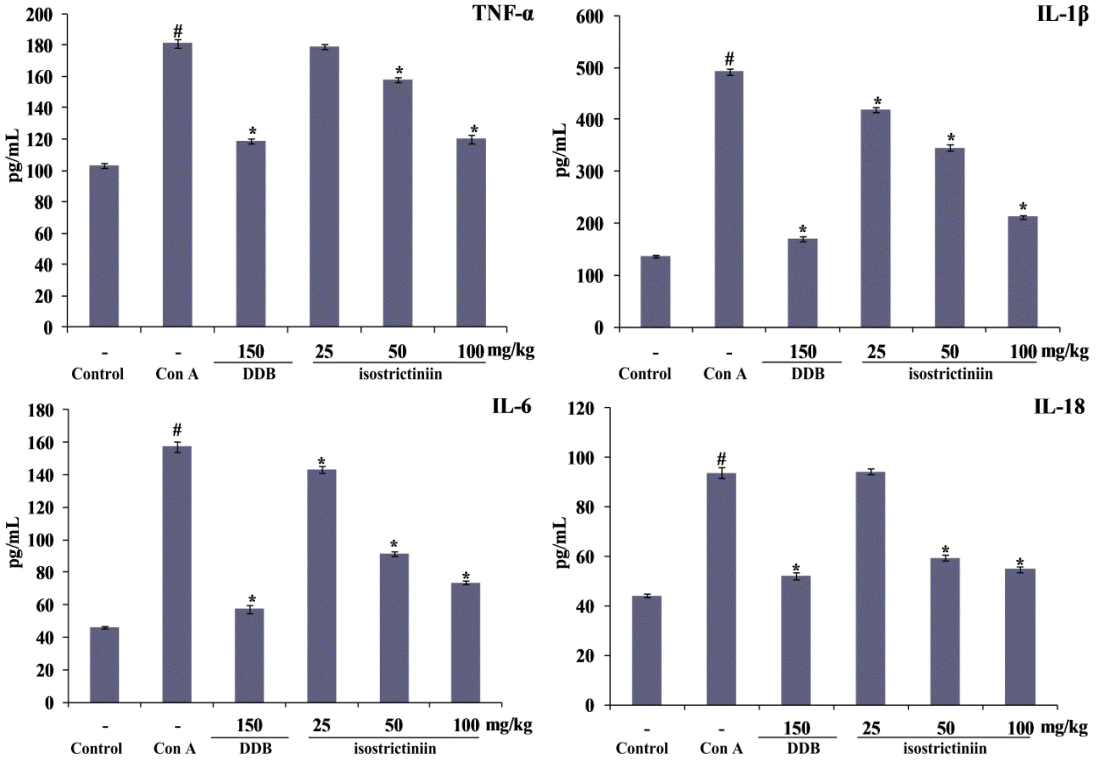

Figure S4. Effects of isostrictiniin on the serum TNF-α, IL-1β, IL-6 and liver homogenate IL-18 in Con A- intoxicated mice. Values are mean ± S.E.M., n = 10. $^{\#}P < 0.05$ compared with control group. $^{*}P < 0.05$, compare with Con A group.