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Preliminary characterization and potential hepatoprotective effect of polysaccharides from *Cipangopaludina chinensis*

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ABSTRACT

In the present study, we investigated the preliminary characterization, *in vitro* antioxidant and *in vivo* heptoprotective activities of polysaccharides from *Cipangopaludina chinensis* (CCPS). The results of chemical and gas chromatography analysis indicated that CCPS was mostly composed of glucose with high contents of uronic acid and sulfate. For antioxidant activities *in vitro*, CCPS showed medium lipid peroxidation inhibition effect and high Fe^{2+} chelating and hydroxyl radical scavenging activities. For hepatoprotective activity *in vivo*, the administration of CCPS significantly decreased the serum levels of alanine aminotransferase and aspartate aminotransferase, inhibited the formation of malondialdehyde in liver and tumor necrosis factor-alpha (TNF-α) in serum and restored the liver activities of superoxide dismutase, glutathione peroxidase in BCG/LPS-induced immunological liver injury mice. The results suggested that CCPS had a significant protective effect against BCG/LPS-induced immunological liver injury. The hepatoprotective effect of CSPS might be partly due to its immunoregulatory effect by inhibiting TNF-α production and antioxidant activities to protect biological systems against the oxidative stress, which were dependent on the chemical and structural properties of CCPS. Further work on the structure of CCPS is in progress.

1. Introduction

Hepatic damage, both acute and chronic, is a common pathology worldwide (Sun et al., 2008). It can lead to liver fibrosis and end stage cirrhosis, which contribute to life-threatening complications of portal hypertension, liver failure and increased incidence of hepatocellular carcinoma (Zou et al., 2006). The main etiology of liver injury is represented by viral infections (hepatitis B virus, hepatitis C virus and hepatitis D virus), drugs and alcohol abuse (Hoek and Pastorino, 2002). There is now substantial evidence that oxidative stress is involved in the pathogenesis and progression of liver diseases (Campo et al., 2001; Hsieh et al., 2004; Nanji et al., 2001; Vazquez-Gil et al., 2004). Free radicals, formed as a result of oxidative stress, can injure the cell membrane of hepatocytes by lipid peroxidation or other means. They cause extensive damage to DNA, proteins, lipids and carbohydrates, leading to various liver injuries (Sastre et al., 2007; Zhong et al., 2007). Therefore, some natural products with antioxidant and free radical scavenging activities have attracted great attention as potential functional ingredients to protect liver injuries (Wang et al., 2009, 2012). Polysaccharides, important natural compounds widely existed in plants, animals and microorganism, have been demonstrated to possess potent antioxidant activity and to protect liver injury induced by various chemicals.

Cipangopaludina chinensis, one of invertebrates, is a common group of vivipard gastropod and widespread in China and many other countries. This species resides in pools, lakes, streams and other water bodies and lives on organic particles and microbes (Chiu et al., 2002; Li, 2012; Sastre et al., 2007). Its fresh meat contains high amounts of protein, essential amino acids, taurine, calcium, iron and zinc and is traditionally used as food supplement in china (Mulia et al., 2009; Cao and Yao, 2005). It has been processed into many kinds of foods by canning, saucing or cooking. In recent years, it has been used in the preparation of foods such as meat sauce and jerky to improve its commercial value (Liu, 2002; Zhang et al., 2005). In addition, *C. chinensis* have a long history as a traditional medicine for the treatment of liver diseases and alcoholic poisoning in China (Zhao, 2011). Recent studies have demonstrated that extracts of *C. chinensis* meat exert various pharmacological effects, such as anticancer activities and protecting cardiac muscle cells against injuries (Fu et al., 2010; Cui and Zhao, 1989). However, little attention has been devoted to the extraction and biological functions of polysaccharides from *C. chinensis*. Therefore, the objectives of this study were to determine the *in vitro* antioxidant activities of CCPS and to evaluate its

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hepatoprotective effect on immunological liver injury in mice. Furthermore, CCPS was characterized by chemical analysis, gas chromatography (GC) and Fourier transform-infrared spectroscopy (FT-IR).

2. Materials and methods

2.1. Materials

C. chinensis was purchased from Huaian Aquatic Product Market (Huaian, China). Arabinose, fucose, galactose, glucose, mannose, rhamnose, xylose, glucuronic acid and Lipopolysaccharide (LPS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Bacillus Calmette-Guerin (BCG) was obtained from Shanghai Institute of Biological Products (Shanghai, China). Assay kits for protein, alanine aminotransferase (ALT), aspartate aminotransferase (AST), malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). An enzyme-linked immunosorbent assay kit (ELISA) for rat tumor necrosis factor-alpha (TNF-α) was purchased from Shanghai Senxiong Biotech Industry Co., Ltd (China). The male Kunming mice were purchased from the Experimental Animal Center of Guangzhou University of Traditional Chinese Medicine (Guangzhou, China). All other reagents were of analytical grade.

2.2. Preparation of CCPS

The crude CCPS was prepared according to the reported method with some modifications (Jiang et al., 2011). Briefly, fresh *C. chinensis* was collected and washed carefully with cold water. After removing the shells and impurities, the flesh was crushed by a high speed disintegrator and the homogenate was kept in 90% of ethanol (v/v) for two weeks. Then, the collected flesh was air-dried at 50 °C and extracted with distilled water in a ratio of 1:25 (raw material to water, w/v) for 3 h at 90 °C for three times. After treatment, the mixture was centrifuged at 5000 rpm for 20 min, and the insoluble residue was treated again as mentioned above. The supernatants were collected, concentrated to a proper volume by using a vacuum rotary evaporator, deproteinated by the method of Sevag et al. (1938) and mixed with three times volume of absolute ethanol. The mixture was stirred vigorously and then kept overnight at 4 °C. The precipitate was collected by centrifugation at 5000 rpm for 20 min and air-drying at 50 °C to a constant weight, affording the crude CCPS.

2.3. Preliminary characterization of CCPS

2.3.1. Determination of contents of total sugars, sulfate, protein and uronic acid

The total sugar content of CCPS was determined using the phenol–sulfuric acid method (Dubois et al., 1956). The protein content was determined by the method of Bradford using bovine serum albumin as the standard (Bradford, 1976). The content of uronic acid was determined according to the method of Blumenkrantz and Asboe-Hansen (1973) using D-glucuronic acid as the standard. The content of sulfate radical was determined according to the reported method (Doigson and Price, 1962).

2.3.2. FT-IR spectral analysis

FT-IR spectrum of CCPS was recorded with a Nicolet 6700 FT-IR Spectrometer (Thermo Co., USA) using KBr disks method. Briefly, samples were dried at 35–44 °C in vacuum over P_2O_5 for 48 h, ground with potassium bromide (KBr) powder and then pressed into pellet for FT-IR spectral measurement in the frequency range of 4000–400 cm^{-1}.

2.3.3. Analysis of monosaccharide composition of CCPS

The monosaccharide composition of crude CCPS was determined using the method reported by Gan et al. (2011) with slight modification. Briefly, the polysaccharide sample (5.0 mg) was hydrolyzed with 4 ml trifluoroacetic acid (TFA, 2 M) at 120 °C in an oven for 2 h, and the excess TFA was removed by evaporation at a temperature of 40 °C. Then, the hydrolyzate was repeatedly co-concentrated with methanol to dryness and acetylated by the addition of a mixture of methanol, pyridine and acetic anhydride. The monosaccharide standards including rhamnose, arabinose, fucose, xylose, mannose, glucose and galactose were acetylated in the same way. Finally, the acetylated samples were analyzed by a 7890N GC (Agilent Technologies, Santa Clara, CA, USA) equipped with flame ionization detector and a HP-5 fused silica capillary column (30 m × 0.32 mm × 0.25 mm). The oven temperature was maintained at 120 °C for 3 min, and then increased gradually to 210 °C at a rate of 3 °C/min. The temperatures of detector and injector were set at 280 °C and 250 °C, respectively. The flow rates of N_2, H_2 and air were 25, 30 and 400 ml/min, respectively.

2.4. Determination of antioxidant activity in vitro of CCPS

2.4.1. Assay of Fe^{2+} chelating activity

The Fe^{2+} chelating activity of CCPS was determined according to the reported method (Liu et al., 2007). One milliliter polysaccharide sample at different concentration (0, 0.4, 0.8, 1.6, 3.2 and 4.0 mg/ml) was added to 0.05 ml of ferrous chloride ($FeCl_2$) solution (2 mM), 0.2 ml of ferrozine solution (5 mM) and 2.75 ml of water. The mixture was shaken well and incubated for 10 min at room temperature, and then the absorbance of the mixture was determined at 562 nm. The Fe^{2+} chelating activity was calculated by the following formula:

$$Fe^{2+}\text{chelating activity } (\%) = 100 \times (A_0 - A_1 + A_2)/A_0$$

where A_0 is the absorbance of control sample (water instead of sample), A_1 is the absorbance in the presence of tested sample, and A_2 is the absorbance of the sample only (water instead of $FeCl_2$ solution). Ethylenediaminetetraacetic acid disodium salt (EDTA-2Na) was used as positive control in the present study.

2.4.2. Assay of lipid peroxidation inhibition effect

The lipid peroxidation inhibition effect of CCPS was determined by thiobarbituric acid-reactive-substances (TBARS) assay using mouse liver homogenate as the lipid rich media with some modification (Yen and Hsieh, 1998). Briefly, 1.0 ml of 1% (w/v) mouse liver homogenate was mixed with 1.0 ml sample solution with different concentrations (0, 0.4, 0.8, 1.6, 3.2 and 4.0 mg/ml), then 0.05 ml of $FeCl_2$ (0.5 mM) and H_2O_2 (0.5 mM) were added to initiate lipid peroxidation, which was carried out in a 37 °C water bath for 30 min. Reaction was terminated by adding 1.5 ml of trichloroacetic acid (TCA, 20%, w/v) and 1.5 ml of thiobarbituric acid solution (0.8%, w/v). The resulting mixture was vortexed and heated in a boiling water bath for 10 min. After centrifugation at 4000 rpm for 10 min, the TCA-TBA phase was removed and the absorbance of the upper layer was recorded at 532 nm. Butylated hydroxytoluene (BHT) was used as positive control. The inhibition effect on lipid peroxidation was calculated according to the formula below:

$$\text{Lipid peroxidation inhibition effect } (\%) = 100 \times (A_0 - A_1 + A_2)/A_0$$

where A_0 is the absorbance of control sample (water instead of sample), A_1 is the absorbance in the presence of tested sample, and A_2 is the absorbance of the sample only (water instead of liver homogenate).

2.4.3. Assay of hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity was measured by the method of Liu et al. (2009) with some modifications. The hydroxyl radical was generated in the mixture of 1 ml of 0.75 mM 1,10-phenanthroline, 1.5 ml of 0.15 M sodium phosphate buffer (pH 7.4), 1 ml of 0.75 mM $FeSO_4$ and 1 ml of H_2O_2 (0.01%, v/v). After addition of 1 ml sample, the mixture was incubated at 37 °C for 30 min. The absorbance of the mixture was measured at 536 nm. The hydroxyl radical scavenging activity was calculated by the following formula:

$$\text{Hydroxyl radical scavenging activity } (\%) = 100 \times (A_1 - A_0)/(A_2 - A_0)$$

where A_0 is the absorbance of control sample (water instead of sample), A_1 is the absorbance in the presence of tested samples, and A_2 is the absorbance of water instead of H_2O_2 and sample in the assay system. Ascorbic acid was used as positive control.

2.5. Evaluation of hepatoprotective effects of CCPS

2.5.1. Animal grouping and experimental design

The hepatoprotective effects of CCPS on immunological liver injury in mice were evaluated according to the reported method (Guo et al., 2009) with some modifications. Briefly, male Kunming mice (8-weeks-old, 20 ± 2 g) were used in the present study. All animals were housed under standard environmental conditions (22 ± 0.5 °C, 55 ± 5% humidity and a 12 h light/12 h dark cycle) and maintained with free access to standard laboratory pellet diet and water. All procedures involving animals throughout the experiments were conducted in strict accordance with the Chinese legislation on the use and care of laboratory animals. After a 7-day acclimation period, these mice were randomly divided into six groups (10 for each) including normal control group, model control group, silymarin group (positive control) and CCPS group. On the first day of experiment, 2.5 mg of BCG in 0.2 ml saline per mouse was injected via lateral tail vein, with the exception of the normal control group, which received saline alone. Mice in CCPS groups were fed with CCPS in three different doses (200, 400 and 600 mg/kg BW per day, respectively) by gastric gavage. Mice in positive control group were given silymarin at the dose of 50 mg/kg BW per day for 15 days prior to the LPS treatment. Normal and model groups simultaneously received the same amount of physiological saline.

2.5.2. Biochemical assay

Eight hours after the last CCPS or silymarin treatment, the mice from the BCG/LPS model, silymarin-treated and CCPS-treated groups were treated with 7.5 μg of LPS in 0.2 ml saline per mouse (normal mice received saline alone) via lateral tail

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