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Protective effects of fractions from *Pseudostellaria heterophylla* against cobalt chloride-induced hypoxic injury in H9c2 cell

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ABSTRACT

Ethnopharmacological relevance: Except for as a well-known tonic Chinese herbal medicine for the treatment of splenic asthenia, anorexia, lassitude and weakness, the roots of *Pseudostellaria heterophylla* was also used in Chinese medicines for the treatment of palpitation.

Aim of the study: The study was designed to determine whether fractions from *Pseudostellaria heterophylla* could provide cardioprotection on hypoxic cardiomyocytes, what structural types of compounds were responsible for the observed effects, and which is the possible mechanism of action.

Materials and Methods: The roots of *Pseudostellaria heterophylla* were extracted successively with 70% aqueous ethanol and water to give a 70% ethanol extract and a water extract. The latter was first precipitated by 80% ethanol and then protein-removed by the Sevag method to give a fraction enriched in polysaccarides (PHP). The former was separated by column chromatography into a fraction enriched in small-molecule sugars and amino acids (PHSSAC), saponins (PHS), cyclopeptides (PHCP), and sapogenins (PHSG). UV spectral or chemical methods were used to confirm the five fractions. The cardioprotective effects of the fractions were evaluated by measuring the viability and the leakage of lactate dehydrogenase (LDH) of the fraction-pretreated cardiomyocyte H9c2 after exposure to $CoCl_2$-induced hypoxia. The mechanism of action was studied by investigating the nature of cell death inhibition (by Annexin V/PI flow cytometric analysis) and their effects on the levels of malonaldehyde (MDA), superoxide dismutase (SOD) and intracellular reactive oxygen species (ROS).

Results: Fractions PHS and PHP could attenuate $CoCl_2$-induced hypoxic damage to an extent higher than or comparable to the effect of the positive control *N*-acetyl-L-cysteine (NAC). Pretreatment of the cells with 800 μg/mL of PHS or 10 mg/mL of PHP markedly decreased the level of MDA, reduced intracellular ROS, increased the activity of SOD, and reduced leakage of LDH to the levels close to or better than that with 326 μg/mL of NAC. Reduction of apoptosis was also observed for both fractions.

Conclusions: The overall results suggested that the traditional use of this plant for the treatment of palpitation may be attributed to the presence of cardioprotective agents in *Pseudostellaria heterophylla*. PHP and PHS were the two active fractions responsible for its cardioprotective effect. The mechanism might involve protections of the cell membrane from hypoxic damage and of the cells from oxidative injury *via* preventing increased oxidative stress. Protection of the cells *via* inhibition of cellular apoptosis may also be involved.

1. Introduction

Ischemic heart disease (IHD), which often presents as acute myocardial infarction, remains the leading cause of death and heart failure worldwide (Luo et al., 2012). Failure to meet oxygen demand in the heart is usually one of the direct causes of IHD (Al-Nimer, 2012). Anti-ischemic therapy, which uses antianginal drugs to relieve or prevent acute ischemic episodes by increasing myocardial oxygen supply and/or decreasing myocardial oxygen demand, has been proved to be a very important treatment of IHD (Al-Nimer, 2012). Cardiomyocyte hypoxia is one of the most important aspects of ischaemia in IHD (Wu et al., 2011) and plays a key role in cardiomyocyte death (Sharov et al., 2005) that may eventually lead to morbidity and mortality. Thus, to protect the cardiomyocyte from hypoxic injury (and hence the heart from myocardial hypoxic damage) has been one of the reasonable therapeutic strategies to reduce the risk of IHD.

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Cobalt chloride ($CoCl_2$), a well-known hypoxia mimetic agent, was reported to be able to mimic the hypoxic response in many aspects (*e.g.*, to decrease cell viability, dissipate mitochondrial membrane potential, activate caspase-3, and induce apoptosis) (Lan et al., 2011). Except for as a tonic Chinese herbal medicine (CHM) in Traditional Chinese Medicine (TCM) and ethnic medicines for the treatment of splenic asthenia, anorexia, lassitude and weakness, the roots of *Pseudostellaria heterophylla* was also used for the treatment of palpitation (Editorial Committee of Chinese Materia Medica, 1999), which suggested that the cardioprotective effect of *Pseudostellaria heterophylla* deserved evaluation. Previous pharmacological investigations (Lin et al., 2010) showed that, except for immunostimulating, anti-lipid peroxidative, hypoglycemic, antibacterial and anti-inflammatory activities, extracts from *Pseudostellaria heterophylla* possessed cardioprotective effects either in rat myocardial infarction (Shen et al., 2007, 2008) or in norepinephrine-induced cardiac myocyte injury (Xiao et al., 2012). It is reported (Lin et al., 2010) that saccharides, amino acids, steroid saponins, cylcopeptides and essential oils were its major constituents, but no direct evidence showed that which types of compounds were responsible for the cardioprotective effects. The purpose of the present study was to determine whether the fractions from *Pseudostellaria heterophylla* could provide cardioprotection on hypoxic cardiomyocytes and what structural types of compounds were responsible for the observed effects. Efforts were also made to investigate the underlying mechanism of action.

2. Materials and methods

2.1. Plant material

The roots of *Pseudostellaria heterophylla* were collected in August 2010 from the Shibing County, Guizhou Province and was authenticated by one of the authors, Professor Qing-De Long of Guiyang Medical College. A voucher specimen (accession No. PH110827) was deposited in Guiyang Medical College. Plant material was sun-dried and ground.

2.2. Chemical

Acetonitrile and formic acid were of HPLC grade (Tedia, Tedia Company INC, USA). Distilled water was obtained from Watsons Group Co. Ltd (Hong Kong, PRC). All other chemicals were of analytical grade.

2.3. Preparation of plant fractions

Dried and ground roots (1.2 kg) were extracted with 70% ethanol (12 L, 2 h) two times under reflux Fig. 1). Evaporation of the solvent under reduced pressure provided the 70% ethanol extract (70EE) (465 g; 61%), 420 g of which was then subjected to D101 macroporous resin column chromatography (CC). Successive elution with water, 70% (10 column volume (CV)), 75% (5 CV) and 95% (3 CV) ethanol afforded fractions A, B, C, and D, respectively. Fr. A enriched in small-molecule sugars and amino acids was then named Fr. PHSSAC (370 g; 53.4%). Fraction B was transferred to MCI CC eluted successively with 30% ethanol (10 CV) and 80% ethanol (5 CV) to afford, respectively, fractions B_1 and B_2. Fr. B_2 was further separated over a Sephadex LH-20 column eluted with absolute ethanol to afford fractions B_{2A} and B_{2B}. Similar separation of Fr. C by Sephadex LH-20 CC (absolute ethanol) yielded fractions C_{2A} and C_{2B}. Combination of Fr. B_1 and B_{2A} afforded a fraction enriched in saponins (Fr. PHS) (12 g; 1.7%), while that of Fr. B_{2B} and C_{2A} gave a fraction enriched in cyclopeptides (Fr. PHCP) (180 mg; 0.026%), and that of Fr. D and C_{2B} yielded a fraction enriched in sapogenins (Fr. PHSG) (1.8 g; 0.26%). After extraction with 70% ethanol, the root dregs were sun-dried and then extracted with water (12 L, 2 h) two times to give a water extract (297 g, 39%), precipitation of which by 80% ethanol followed by removal of free proteins by the Sevag method afforded a fraction enriched in polysaccarides (Fr. PHP) (260 g; 34%). Preparations of all the fractions were monitored by UPLC-PDA-MS as well as Liebermann–Burchard, Ninhydrin, and Molish tests. All the samples were concentrated and dried at room temperature and kept at 4 °C until required.

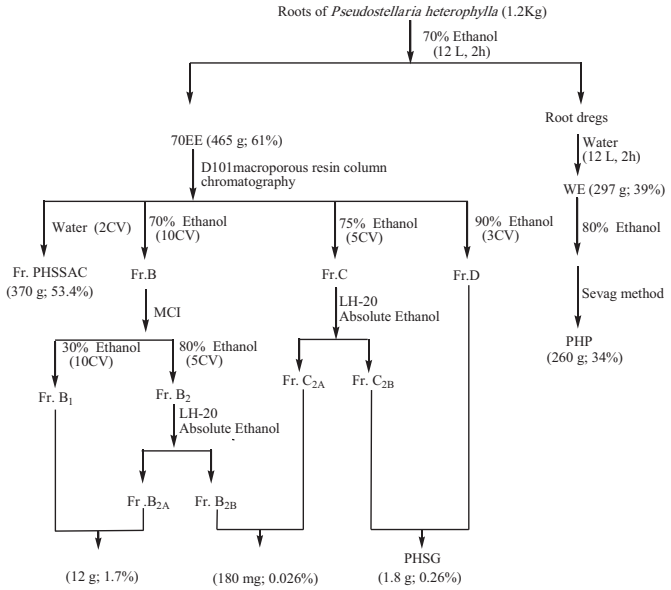

Fig. 1. Flow chart of the extraction procedure.

2.4. Cell culture and treatment

H9c2 cells (Cell Bank of the Chinese Academy of Sciences, Shanghai, China) were grown in the high-glucose Dulbecco's modified Eagle's medium (Gibco, USA) supplemented with 10% (v/v) fetal bovine serum (Hangzhou Sijiqing Biological Engineering Materials Co., Ltd.), 1% (v/v) L-glutamine, penicillin G (100 U/mL), and streptomycin (100 mg/mL) in a humidified 5% CO_2 atmosphere at 37 °C. Cells at exponential growth were dissociated with 0.25% trypsin—0.02% EDTA (Solarbio, Beijing, China) and were seeded in a 96-well plate at a seeding density of $3–4 \times 10^4$/well before being incubated for 24 h. Cells were then treated with either PHSSAC, PHS, PHSG, PHCP, or PHP to figure out their safe concentrations to be used in the bioassay. In order to explore the effects of fractions on $CoCl_2$-induced cell injury, H9c2 cells seeded in the 96-well plate at a density of $1.5–1.8 \times 10^4$/well or in the 6-well plates at a density of $8–10 \times 10^5$/well were preincubated with various concentrations of fractions, *N*-acetyl-L-cysteine (NAC, Sigma), or vehicle at 37 °C for 24 h, the supernatant was then removed, and the washed cells were exposed to 1200 μmol/L $CoCl_2$ in the culture medium for 24 h to induce cell hypoxia. The cells in the 96-well plate were then used for microculture tetrazolium (MTT) and lactate dehydrogenase (LDH) assays, while those in the 6-well plates were subjected to malondialdehyde (MDA) and superoxide dismutase (SOD) tests.

2.5. Cytotoxicity assay

Cytotoxicity was evaluated by measuring the cell viability after treatment with fractions, NAC or vehicle by the MTT method (Zhu et al., 2011). H9c2 cells at exponential growth were seeded at