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Composition identification of Salvia extracts and testing of its inhibiting myocytes cell death caused by hypoxia/reoxygenation

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ABSTRACT

A four-factor, three-level factorial design was used to evaluate the effects of the following factors on the extraction efficiency: extraction temperature (*A*), extraction time (*B*), particle size (*C*) and ratio of water to solid (*D*). The optimal conditions for extraction of Salvia heteroglycan were determined, using the ridge analysis, as extracting 2.5 h at 80 °C for three times. HPLC analyses showed the presence of rhamnose, glucose and galactose as constituents of Salvia heteroglycan. A water and ethanol extract of Salvia could enhance cell viability, Fas protein expression and myocardial apoptosis index in a dose-dependent manner. These indicated that water and ethanol extract of Salvia may reduce hypoxia/reoxygenation injury of myocytes cells.

1. Introduction

For several decades, *Salvia miltiorrhiza* root (Labiatae, Laminaceae) has been widely used in clinics in China, Korea, Japan and other Asian countries for the treatment of various microcirculatory disturbance-related diseases, such as cardiovascular disease, cerebrovascular disease, liver dysfunction, renal deficiency and diabetic vascular complication.

Danshen is frequently used for the treatment of cardiovascular diseases in clinic, including coronary heart disease, hypertension, diabetes, atherosclerosis and chronic heart failure (Wang, Wang, Xiong, Mao, & Li, 2006; Yang, Han, Sheng, He, & Liang, 2006; Zhang, Liu, & Huang, 2006). Chemical compounds from *S. miltiorrhiza* can be classified into two major categories: hydrophilic compounds and lipophilic diterpenoid quinines (LDQ). Both hydrophilic and lipophilic compounds of Danshen have multiple pharmacological activities, such as improving the microcirculatory disturbance, protecting against cardiotoxicity induced by doxorubicin, inhibiting the proliferation of vascular smooth muscle cells, anti-inflammatory, anti-platelet, anti-oxidant and vasorelaxation (Han et al., 2007; Jiang et al., 2009; Seon-Il et al., 2003; Wang, Gao, & Zhang, 2005; Wu et al., 2009).

Ischemia and reperfusion (I/R) occurs in a wide range of situations, including trauma, vascular reflow after contraction, percutaneous transluminal coronary angioplasty, thrombolysis treatment, organ transplantation, and hypovolemic shock with resuscitation. I/R exerts multiple insults in microcirculation, frequently accompanied by endothelial cell injury, enhanced adhesion of leukocytes, macromolecular efflux, production of oxygen free radicals and mast cell degranulation (Han et al., 2001).

Cardiac myocytes, the cellular components of the heart, play important roles in heart health and disease. During the development and progression of heart failure, changes occur in both the structure and function of these cells, resulting in a wide range of abnormalities which affect cell growth, death, and physiological function. This study, using in vitro cell culture techniques, examined the effect of *S. miltiorrhiza* extracts on these cardiac cells with respect to the cell viability and apoptosis.

2. Materials and methods

2.1. Orthogonal array design

Salvia was collected in Xian city in May 2010. The plant material was stored at room temperature in a dry place prior to use.

Orthogonal array design (OAD) is a type of experimental design in which an orthogonal array is used to assign factors to a series of experimental combinations and results can be analyzed using a mathematical procedure (Kolaiti & Koukouvinos, 2006; Lan, Wong, Chen, & Sin, 1995; Lee, Yi, Park, & Park, 2003). Effects of extraction temperature, time, particle size and ratio of liquid to solid were investigated on the yield of Salvia heteroglycan. An orthog-

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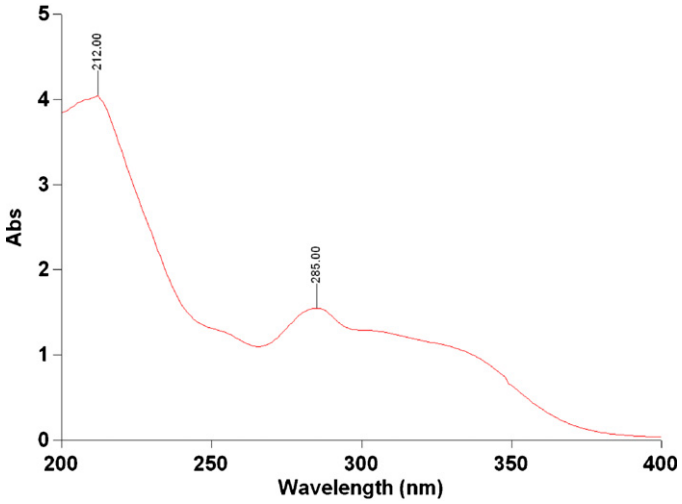

Fig. 1. Ultraviolet analysis of Salvia heteroglycan.

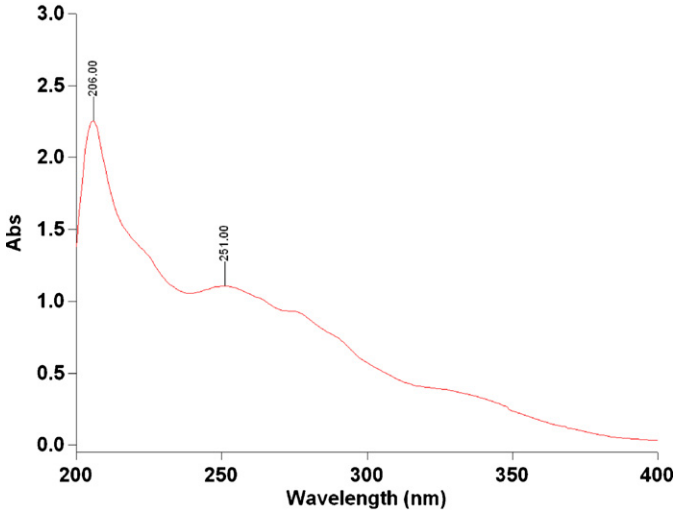

Fig. 2. Ultraviolet analysis of ethanol extract of Salvia.

onal matrix with three factors, each factor containing three levels, was selected to arrange the experiments. Extraction temperatures were 80, 90 and 100 °C; extraction times were 3, 3.5 and 4 h; particle sizes were 40, 50 and 60 meshs; and ratios of water to solid were 5, 6 and 7. The levels for each process variables were selected from a series of preliminary trials without using particular experimental designs.

Effects of extraction temperature, time, particle size and ratio of liquid to solid were investigated on the yield of ethanol extract of Salvia. An orthogonal matrix with three factors, each factor containing three levels, was selected to arrange the experiments. Extraction temperatures were 60, 70 and 80 °C; extraction times were 3, 3.5 and 4 h; particle sizes were 40, 50 and 60 meshs; and ratios of ethanol to solid were 5, 6 and 7. The levels for each process variables were selected from a series of preliminary trials without using particular experimental designs.

2.2. Sugar analysis

Salvia heteroglycan was monitored using UV detector at 280 nm for protein. Result indicated that protein was not present in polysaccharides (Fig. 1). Ethanol extract of Salvia was monitored using UV detector. Results indicated that two absorption peaks could be detected at 251 nm and 206 nm (Fig. 2). This ultraviolet spectrum was similar to spectrum of polyphenol compounds, suggesting that ethanol extract of Salvia was rich in polyphenol compounds.

Sugar compositions were measured by the HPLC post-label fluorescent detection method using the LC-20A system (Shimazu). Briefly, 100 μl of 2 N trifluoroacetic acid was added to 1.2 mg of Salvia heteroglycan, and hydrolysis was carried out at 100 °C for 6 h. After drying in a vacuum, the residue was dissolved in 500 μl distilled water and the solution was filtered through a 0.22-lm membrane filter. In the analysis of sugar composition, the filtrate was injected into a TSKgel Sugar AXG column (15 cm 9 4.6-mm i.d., Shimazu) heated at 70 °C, equilibrated with 0.5 M potassium borate buffer (pH 8.7), and flow rate was 0.4 ml/min. The eluate from the column was combined with 1% (w/v) L-arginine dissolved in 3% (w/v) boric acid solution, flow rate was 0.5 ml/min, and heated at 150 °C within the reaction loop.

2.3. HPLC–DAD–ESI-MS/MS analysis

The HPLC–MS system consisted of a Surveyor MS pump, an autosampler, a diode array detector, and a triple–quadrupole TSQ Quantum mass spectrometer (Thermo Finnigan, San José, CA, USA), with Xcalibur software for data acquisition and analysis. Separations were carried out using a Gemini C18 reverse phase column (250 mm × 4.6 mm i.d., 5 μm, Phenomenex, Torrance, CA, USA), protected with a security guard cartridge (Gemini C18, 4 mm × 2.0 mm i.d., Phenomenex). The samples were analyzed according to Del Rio et al.'s method (Wang, Lu, Miao, Xie, & Yang, 2008) with minor modification. The elution consisted of a linear gradient program from 4% to 25% acetonitrile in 1% formic acid aqueous solution over 60 min. The flow rate was 1 mL/min and 10 μL of samples was injected. A 15 min re-equilibration time was used between HPLC runs. The DAD acquisition wavelength was set in the range of 200–700 nm. After passing through the flow cell of the DAD, the column eluate was split and 0.3 mL/min was directed to a triple–quadrupole tandem mass spectrometer with an electrospray interface (ESI), operating in full scan MS mode from *m*/*z* 50 to 1500. Mass spectra were acquired in both negative and positive modes with ion spray voltage at 3.5 kV, capillary temperature at 350 °C, capillary voltage at 35 V, sheath gas pressure at 35 Arb, auxiliary gas pressure at 11 Arb.

For quantitative purpose, standards and samples were analyzed by a Waters 600E HPLC system, equipped with a Waters 717 plus autosampler and a Waters 2996 photodiode array detector. Chromatographic conditions were the same as described above.

2.4. Hypoxia-reoxygenation

Neonatal hearts were collected from rats between 2 and 4 days of age as previously described (Bordoni et al., 2002). Cells were seeded in Petri dishes in Ham F10 nutrient mixture supplemented with 10% (v/v) FCS and 10% (v/v) HS, and grown at 37 °C, 5% CO_2 and 95% humidity (normoxic condition) until complete confluence. The cardiomyocytes were then divided into five groups: normal control, model control and low, high dose of Salvia extract treatment groups. In Salvia heteroglycan treatment groups, Salvia heteroglycan were added to 10% (v/v) FCS for a final concentration 50 or 100 μg/ml the culture medium at day 5 of culturing, 24 h before the beginning of the hypoxic period. In ethanol extract of Salvia treatment group, ethanol extract of Salvia were added to 10% (v/v) FCS for a final concentration 30 or 60 μg/ml the culture medium at