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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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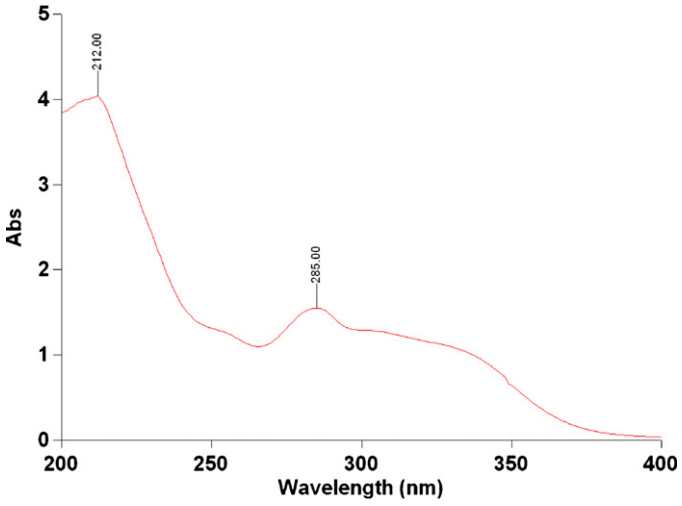

Fig. 1. Ultraviolet analysis of Salvia heteroglycan.

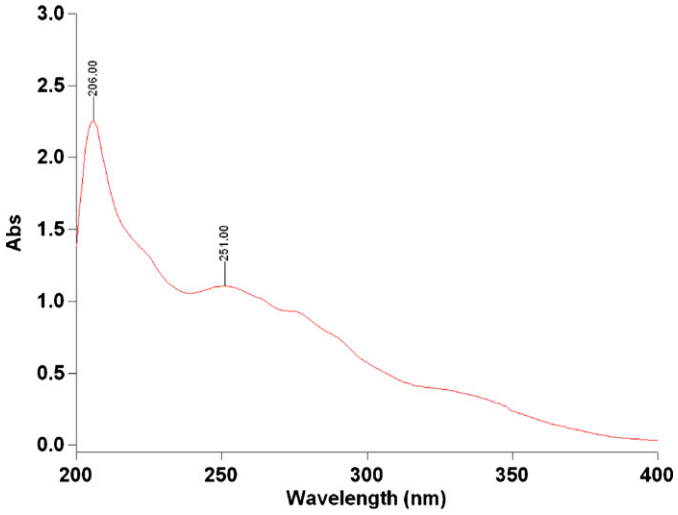

Fig. 2. Ultraviolet analysis of ethanol extract of Salvia.

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

day 5 of culturing, 24 h before the beginning of the hypoxic period. The cardiomyocytes were incubated at 37 °C in an air-tight incubator where normal air was replaced by 95% N_2/5% CO_2 for 24 h to produce hypoxia, and then the air was replaced by 95% O_2/5% CO_2 for 4 h to produce reoxygenation. Corresponding control cells were incubated under the same conditions, but kept in normoxia (ambient atmosphere).

Viability was assessed by trypan blue exclusion and the number of viable cells was determined by counting on a hemocytometer.

2.5. Immunohistochemical examination of Fas

Immunohistochemical staining was performed on the 4 mm thick, 10% formalin-fixed, paraffin-embedded serial sections with a monoclonal antibody (MAb) to the Fas (APO-1; Dako, Japan) using the labelled streptavidin biotin (LSAB) method (Dako). Positive or negative immunoreactivity for Fas was defined as follows: the sections for Fas were judged to be positive when the membrane and cytoplasm of the myocardial cells were stained or when only the membrane of the myocardial cells were stained. They were judged to be negative when neither the membrane nor cytoplasm of the myocardial cells were stained or when only the cytoplasm of the myocardial cells was stained.

2.6. Annexin V staining and caspase assay

For the annexin V assay, cells plated on coverslips or 35 mm dishes were subjected to hypoxia/reoxygenation and stained according to manufacturer's (Roche) protocol. Cells that stained positive for annexin V without nuclear staining (negative for propidium iodide) were considered apoptotic. All fields in the coverslips or dishes were analyzed. Apoptosis index was evaluated according to the manufacturer's (Sigma) protocol.

2.7. Statistical analysis

Data were expressed as means ± SEM for per group. Statistical analysis was carried out by the Student's "*t*" test. The calculations were performed using STATISTICA 6.0 (for Windows, StatSoft Inc. software, Tulsa, OK, USA). A difference of $P < 0.05$ was considered significant between the both groups treated and untreated with extracts.

3. Results and discussion

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*), ratio of water to solid (*C*) and particle size (*D*). In order to estimate the best condition for the extraction of Salvia heteroglycan, 9 experiments were performed. The factors and their respected levels are reported in Table 1. In this study, the focus was on the main effects of the four most important factors. The results of the extraction experiments, based on the percentage of the Salvia heteroglycan are given in Table 1. The analysis of extreme difference revealed that the influential order of the four factors on the extraction yields of Salvia heteroglycan. According to variance analysis, the contributions of extraction time (*A*) and extraction temperature (*B*) were significant for the extraction yield of Salvia heteroglycan ($P < 0.05$).

A four-factor, three-level factorial design was used to evaluate the effects of the following factors on the extraction efficiency: extraction time (*A*), extraction temperature (*B*), ratio of water to solid (*C*) and particle size (*D*). The analysis of extreme difference revealed that the influential order of the four factors on the ethanol extraction yields of Salvia. According to variance analysis, the contributions of extraction time (*A*) and extraction temperature (*B*) were significant for the ethanol extraction yield of Salvia ($P < 0.05$) (Table 2).

Table 1
Orthogonal array design of Salvia heteroglycan.

No	*A*	*B*	*C*	*D*	*Y*
1	1 (2.5 h)	1 (80 °C)	1 (8:1)	1 (40 mesh)	2.512
2	1	2 (90 °C)	2 (9:1)	2 (50 mesh)	2.454
3	1	3 (100 °C)	3 (10:1)	3 (60 mesh)	2.255
4	2 (3.0 h)	1	2	3	2.305
5	2	2	3	1	2.111
6	2	3	1	2	1.717
7	3 (3.5 h)	1	3	2	2.093
8	3	2	1	3	1.943
9	3	3	2	1	1.873
K1	7.221	6.91	6.172	6.496	
K2	6.133	6.508	6.632	6.264	
K3	5.879	5.845	6.459	6.503	
R	1.342	1.065	0.46	0.239	

Table 2
Orthogonal array design of ethanol extract of Salvia.

No	*A*	*B*	*C*	*D*	*Y*
1	1 (60 min)	1 (80 °C)	1 (5:1)	1 (40 mesh)	1.65
2	1	2 (85 °C)	2 (6:1)	2 (50 mesh)	1.51
3	1	3 (90 °C)	3 (7:1)	3 (60 mesh)	1.29
4	2 (70 min)	1	2	3	1.54
5	2	2	3	1	1.26
6	2	3	1	2	1.43
7	3 (80 min)	1	3	2	1.18
8	3	2	1	3	0.89
9	3	3	2	1	0.82
K1	4.45	4.37	3.97	3.73	
K2	4.23	3.66	3.87	4.12	
K3	2.89	3.54	3.73	3.72	
R	1.56	0.83	0.24	0.4	

The carbohydrate was methanolized and the resulting methyl glycosides were trimethysilylated and analyzed by HPLC. These analyses showed the presence of rhamnose, glucose and galactose as constituents of Salvia heteroglycan. Thus, from these data, it was concluded that the polysaccharide was composed of rhamnose, glucose and galactose in the ratio 1.0:1.8:3.3. The molecular weight (Mw) of Salvia heteroglycan calculated using GPC software, were 1,418 (Fig. 3).

Identification of phenolic compounds and purine alkaloids was carried out by comparing HPLC retention time, UV absorption, *m*/*z* of their quasi-molecular ions, and MS2 fragmentation patterns with those of the standards and the literature data. Peaks were identified as, danshensu, salvianolic acid B, cryptotanshinone and tanshinone IIA, respectively, by comparing the retention time and mass spectra with those of reference compounds.

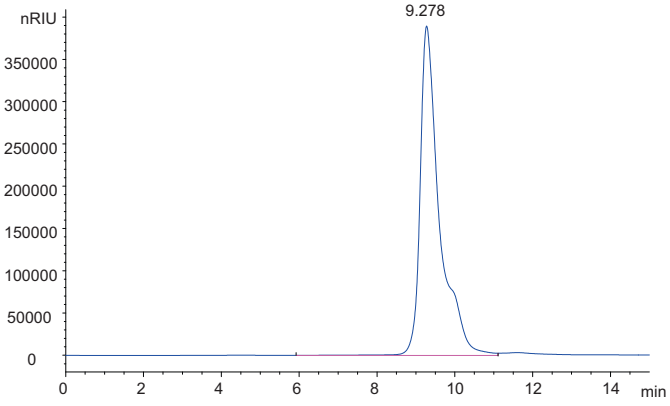

Fig. 3. HPLC analysis of Salvia heteroglycan.

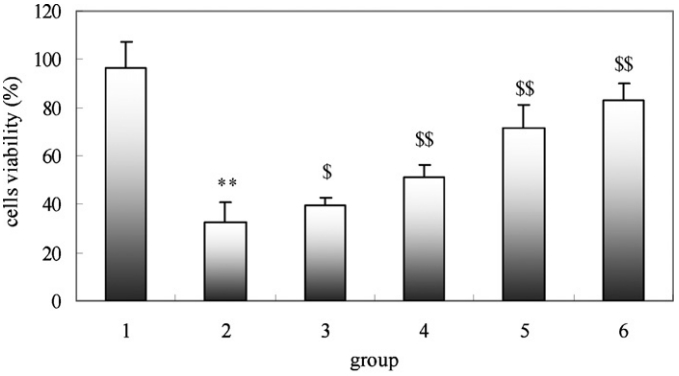

Fig. 4. Effect of Salvia heteroglycan and ethanol extract on myocardial cells viability. Note: 1, normal group; 2, model group; 3, 4, salvia heteroglycan tretment groups; 5, 6, salvia ethanol extract treatment groups.

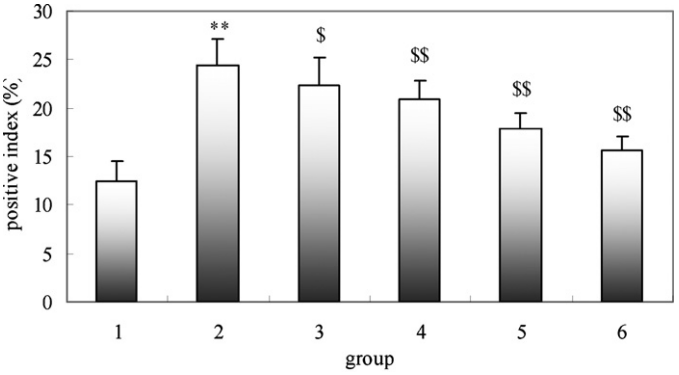

Fig. 5. Effect of Salvia heteroglycan and ethanol extract on Fas protein expression.

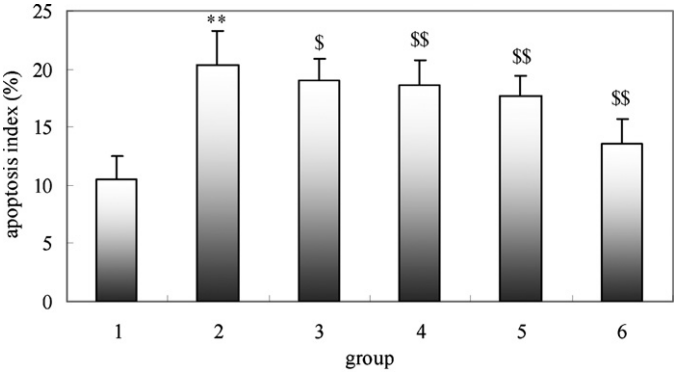

Fig. 6. Effect of Salvia heteroglycan and ethanol extract on myocardial apoptosis index.

To verify that ethanol extract of Salvia and Salvia heteroglycan enhanced the cell proliferation of cardiomyocytes, we first examined the effects of treatment with different concentrations of ethanol extract of Salvia and Salvia heteroglycan on cardiomyocytes. After treatment, survival was correlated with extract concentration (Fig. 4). These data indicate that the ethanol extract of Salvia and Salvia heteroglycan could increase cell viability in a dose-dependent manner (Fig. 4).

Expression of Fas was seen in the cardiomyocytes of the groups (Fig. 5). Expression of Fas in the cardiomyocytes of the model control group was markedly higher than that in normal control group. In the medicine-treated group, the expression of Fas in the cardiomyocytes was significantly decreased compared to model control group.

We characterized the level of apoptosis and the protective role of ethanol extract of Salvia and Salvia heteroglycan under these conditions. Annexin V staining showed that 50% of the cardiomyocytes subjected to hypoxia/reoxygenation were apoptotic. Cells pretreated with ethanol extract of Salvia became more resistant, and demonstrated an average of 24% apoptosis, which was not different from the levels found in cells incubated under normoxia (Fig. 6).

4. Conclusion

The development of optimized extraction parameters is important for industrial commercialization in the view of the economic aspects. A four-factor, three-level factorial design was used to evaluate the effects of the extraction factors on the extraction efficiency. According to variance analysis, the contributions of extraction time (A) and extraction temperature (B) were significant for the extraction yield of Salvia heteroglycan. The Salvia heteroglycan was composed of rhamnose, glucose and galactose. The molecular weight (Mw) of Salvia heteroglycan calculated using GPC software, were 1,418. Pharmacological analysis showed that Salvia heteroglycan and its ethanol extract could increase myocardial cells viability, reduce Fas protein expression and myocardial apoptosis index.

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