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Full paper

Identification of schisandrin as a vascular endothelium protective component in YiQiFuMai Powder Injection using HUVECs binding and HPLC-DAD-Q-TOF-MS/MS analysis

Fang Li, Yi-Sha Tan, Hong-Lin Chen, Yan Yan, Ke-Feng Zhai, Da-Peng Li, Jun-Ping Kou, Bo-Yang Yu*

Jiangsu Key Laboratory of TCM Evaluation and Translational Research, Department of Complex Prescription of TCM, China Pharmaceutical University, 639 Longmian Road, Nanjing, 211198, PR China

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ABSTRACT

YiQiFuMai Powder Injection (YQFM) is a re-developed preparation based on the well-known traditional Chinese medicine formula Sheng-mai-san. It has been widely used for the treatment of cardiovascular disease with definite clinical efficacy in China, but its bioactive molecules remain obscure. In this study, an effective method has been employed as a tool for screening active components in YQFM, using human umbilical vein endothelial cells (HUVECs) extraction and liquid chromatography coupled with electrospray ionization quadrupole time-of-flight tandem mass spectrometry (Q-TOF MS/MS). Nine compounds, which could interact with HUVECs, were identified as ginsenosides Rb1, Rc, Rb2, Rd, 20(S)-Rg3, 20(R)-Rg3, Rk1/Rg5 and schisandrin by comparing with reference substances or literature. *In vitro* assays showed that schisandrin at concentrations of 10−100 μM protected HUVECs from hypoxia/reoxygenation (H/R) injury, increased cell viability, nitric oxide (NO) content and decreased lactate dehydrogenase (LDH) leakage, malonaldehyde (MDA) content and ROS generation. Moreover, schisandrin pretreatment inhibited cell apoptosis, as evidenced by inhibiting activation of caspase-3 and increasing the Bcl-2/Bax ratio. These data indicate that HUVECs biospecific extraction coupled with HPLC-ESI-Q-TOF-MS/MS analysis is a reliable method for screening potential bioactive components from traditional Chinese medicines. Meanwhile, the vascular endothelium protective property of schisandrin might be beneficial for the treatment of cardiovascular disease.

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1. Introduction

Traditional Chinese medicines (TCMs) are attracting increasing attention, not only due to their reliable curative efficacy and long history of clinical trials, but also because TCMs can serve as an important source of bioactive compounds in the search for new drugs. However, the components of TCMs are usually complex mixtures, containing hundreds or even thousands of different compounds, whereas only a few compounds are responsible for the pharmacological effects through complicated synergistic or integrated actions. In most cases, biologically active compounds in TCMs are not or only partially known. Therefore, it is exceedingly important to investigate the bioactive components and therapeutic mechanisms for the exploration and elucidation of TCMs (1,2).

Nowadays, the interaction of drugs with target cells has been successfully employed as a screen for bioactive components in TCMs (3,4). Meanwhile, HPLC-DAD-Q-TOF-MS/MS, a powerful technique for identification of unknown molecules, has been widely applied to the modern pharmaceutical analysis of TCMs (5). So it becomes practicable to explore the biospecific active components from TCMs by extracting with cells and then identifying with HPLC-DAD-Q-TOF-MS/MS technology.

The YiQiFuMai Powder Injection (YQFM) is a traditional Chinese medicine for the treatment of cardiovascular disease (6,7). It has been re-developed based on the well-known TCMs formula

* Corresponding author. Tel./fax: +86 25 86185158.
E-mail address: boyangyu59@163.com (B.-Y. Yu).
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Sheng-mai-san, which is composed of *Panax ginseng* C.A. MEY., *Ophiopogon japonicus* (Thunb.) KER-GAWL and *Schisandra chinensis* (Turcz.) BAILL (1:3:1.5). Considerable clinical trials have indicated that YQFM can be widely used for the treatment of chronic heart failure with better efficacy and fewer side effects compared with standard medical treatments (8). It has also been suggested that the three herbs of YQFM have beneficial effects in the treatment of the cardiovascular diseases and regulation of blood vessel function (9–12). Although both clinical and experimental studies demonstrate the prophylactic or therapeutic efficacy of YQFM, the actual bioactive molecules have not yet been well identified. Various pharmacological studies showed that some bioactive compounds from Ginseng Radix Et Rhizoma Rubra, such as ginsenoside Rg1, Rb1, and Rg3, have curative effects in coronary artery disease, cardiac hypertrophy, heart failure, cardiac energy metabolism, cardiac contractility and arrhythmia, and could represent the active components of YQFM (13–17). Schisandrin B has been reported to be one of the active chemicals with activities improving cardiac function and attenuating myocardial remodeling after myocardial infarction in mice (18). However, it is not fully known about the molecules responsible for the cardiovascular protective effect of the other two herbs.

On the other hand, endothelial dysfunction is a key event in cardiovascular disease. Endothelial cells release substances such as nitric oxide (NO), which are essential for controlling vascular growth, vasomotor reactivity, coagulation and inflammatory responses. During normal physiological conditions, endothelial cells appear able to regulate cardiac contractility, myocardial metabolism and heart rate. Endothelial damage can lead to inflammation, leukocyte and platelet extravasation, vascular damage, resulting myocardial infarction or acute coronary syndromes (19–21). Furthermore, the excessive apoptosis of the endothelial cells induced by hypoxia/reoxygenation (H/R) stress are closely associated with the cardiovascular diseases. Therefore, blockage of pro-apoptotic pathways in the endothelial cells has been considered as an attractive therapeutic strategy to prevent or ameliorate the progression of the cardiovascular diseases (22,23). In the present study, in order to find out the vascular endothelium protective components from YQFM, firstly HUVECs, were co-cultured with YQFM to extract the potential cardiovascular active components. Then HPLC-ESI-MS/MS analysis was employed for the structure elucidation of the components extracted in HUVECs. Eventually, to verify the vascular protective activity, some molecules bounded to HUVECs were investigated by cell viability assay and anti-apoptosis assay.

2. Materials and methods

2.1. Reagents and materials

YQFM was provided by Tasly Pharmaceutical Co., Ltd. (Tianjin, China) with the batch number of 20121210, which is the prescription of *Panax ginseng* C.A. MEY., *Ophiopogon japonicus* (Thunb.) KER-GAWL and *Schisandra chinensis* (Turcz.) BAILL. The specimen was identified by professor Bo-Yang Yu (Specimen No. 20121210) and deposited in the Herbarium of Department of Traditional Chinese Medicine of China Pharmaceutical University. Reference substances of ginsenoside Rb1 and schisandrin were purchased from Nanjing Zelang Bio-Technology Co., Ltd. (Nanjing, China), Rc, Rb2, Rd, 20(R)-Rg3 were kindly given by Dr. Jingrong Wang at Macau University of Science and Technology. The purity of each compound was determined to be higher than 98% by HPLC. HPLC-grade acetonitrile was purchased from Tedia (Fairfield, OH, USA). Deionized water for HPLC-MS analysis was prepared using a Milli-Q system (Milford, MA, USA). Formic acid (Aladdin Chemistry Co. Ltd) was used for all analyses. Methanol for sample extraction was from Hanbon Reagent Company (Jiangsu, China). Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were from GIBCO/BRL (Life Technologies, California, USA). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was from AMRESCO (Cleveland, OH, USA) and phosphate-buffered saline (PBS, pH7.4) was prepared in our laboratory. All other chemicals were of analytical grade. All solvent and samples were filtered through 0.22 μm membranes before use.

2.2. Preparation of YQFM and standard samples

YQFM (Batch No. 20121210) was provided by Tasly Pharmaceutical Co., Ltd. (Tianjin, China). It comprises of the ethanol extract (78 °C) of *Panax ginseng* C.A. MEY., water extract (100 °C) of *Ophiopogon japonicus* (Thunb.) KER-GAWL and *Schisandra chinensis* (Turcz.) BAILL combined in the ratio of 1:3:1.5. The yield of extracts is 23.64%, following by precision processes of multiple filtering, lyophilizing, and aseptic packaging. Then the YQFM was dissolved in 5 mL DMEM medium (pH = 7.4) to make the 100 mg/mL solution and filtered through a 0.22 μm membrane, then the filtrate was used for HUVECs biospecific extraction and HPLC-Q-TOF-MS/MS analysis (sample A5 and B5). For qualitative analysis, a stock solution of reference substances was prepared in methanol and stored at 4 °C until use (sample A3 and B3).

2.3. HUVECs extraction and sample preparation

HUVECs line was purchased from Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). HUVECs were grown at 37 °C and 5% CO_2 cultured in 10% FBS DMEM medium. YQFM prepared as afore mentioned was added when the cell density reached 90%. The drug solution was removed after incubation for 1 h and the 10-cm dish was washed with 8 mL PBS for 5 times to remove unbound components, collected the last 1.5 mL PBS eluate as a control for HPLC-MS analysis (sample A1 and B1). Finally, HUVECs were denatured and extracted with 4 mL of 80% methanol by ultrasonic extraction to liberate components bound to the cells. The desorption eluate was centrifugated and the supernatant was condensed to 100 μL for HPLC-MS analysis (sample A4 and B4). The control sample, in which YQFM was replaced by DMEM, was prepared using the same procedures as described above (sample A2 and B2).

2.4. Chromatographic and mass spectrometric conditions

HPLC-DAD-Q-TOF-MS/MS analysis was carried out using an Agilent series 1290 Infinity HPLC instrument (Agilent, Waldbronn, Germany) coupled with an Agilent 6530 Q-TOF mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) equipped with an electrospray (ESI) interface. The mobile phase consisted of acetonitrile (A) and water-0.1% acetic acid (B), and the gradient elution conditions were: 0–30 min, 8–18% A; 30–80 min, 18–50% A; 80–115 min, 50–100% A and then returned to the initial condition. The flow rate was 1.0 mL/min. Chromatographic separation was carried out at 30 °C on an Alltima C_{18} column (4.6 mm × 250 mm, I.D., 5 μm, Serial No. 213040116, GRACE-Alltech, USA). The DAD detector scanned from 190 to 400 nm, and the samples were detected at 203, 254 and 296 nm. The MS detection parameters were optimized as follows: drying gas temperature, 350 °C; drying gas (N_2) flow rate, 9.0 L/min; fragmentor voltage, 120 V; nebulizer, 45 psig and capillary, 3000 V; skimmer, 65 V; Oct RFV, 750 V. The sample collision energy was set at 20–40 V. All the acquisition and analysis of data were controlled by MassHunter software (Agilent Technologies). Mass spectra were recorded in the range of *m/z*

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100–2000 with accurate mass measurement of all mass peaks. Each sample was analyzed in both positive (Fig. 1A) and negative (Fig. 1B) modes to give abundant information for structural identification.

2.5. HUVECs damage induced by H/R

The H/R model was established according to the method with slight modifications (24). Briefly, HUVECs were incubated under hypoxia using a 94% N_2, 5% CO_2 and 1% O_2 for 4 h in a humidified N_2/CO_2 incubator and then in a standard incubator with 5% CO_2 in normal atmosphere at 37 °C for 2 h. Cells were pretreated with the compound at 0.1–100 μM for 12 h before subjecting to H/R. The control groups were treated without the compound and H/R treatment.

2.6. Cell viability assay and measurement of LDH activity

HUVECs growing exponentially were trypsinized and then approximately 8000 cells/well were seeded into 96-well plates. After the experimental treatment, HUVECs were incubated with MTT at a final concentration of 0.5 mg/mL for 3 h at 37 °C. Then, the medium was discarded and 150 μL DMSO was added to dissolve the formazan crystals. The absorbance was read at 570 nm with a reference wavelength of 650 nm and cell viability was expressed as percentage of absorbance to control values. To further measure the extent of cell injury, the LDH activity was also tested. At the end of the incubation period, the culture supernatants were collected. The activity of LDH was detected at 490 nm according to the LDH assay kit (Beyotime Institute of Biotechnology, Shanghai, China).

2.7. Measurement of nitric oxide (NO), the malondialdehyde (MDA) and intracellular ROS

The content of NO and MDA were determined with assay kits (Jiancheng Bioengineering Research Institute, Nanjing, China) according to the manufacturer's instructions. In brief, cells were harvested by trypsinization and cellular extracts were lysed with RIPA lysis buffer. Then lysed cells were centrifuged and the supernatant was subjected to the measurement of MDA, NO levels and the protein contents. MDA and NO levels were then normalized to milligram protein. ROS levels were determined using 2′, 7′-dichlorofluorescein diacetate (DCFH-DA, Jiancheng Bioengineering Research Institute, Nanjing, China). Cells were incubated for 30 min with DCFH-DA (10 μmol/L), then the cells were washed with PBS and visualized by fluorescence microscopy.

2.8. Western blot analysis

As reported (25), after washing with the ice-cold PBS, HUVECs were lysed with RIPA lysis buffer (Vazyme Biotech, Nanjing, China) supplemented with protease inhibitor (Vazyme Biotech, Nanjing, China). Equal amount of proteins were separated on 12.5% SDS-PAGE and transferred to PVDF membrane (Millipore Corporation, Billerica, MA, USA). The membranes were blocked with 3% BSA in TBS/T and stained with primary antibodies against caspase-3, Bax, Bcl-2 and β-actin (dilution 1:1000, 1:1000, 1:1000, 1:4000, respectively; Cell Signaling Technology, Boston, MA, USA) overnight at 4 °C. Membranes were then probed with peroxidase conjugated secondary antibody at a 1: 8000 dilution (Bioworld Technology, Nanjing, China). The antigen–antibody complexes were then detected with chemiluminescene (ECL) reagent (Vazyme Biotech, Nanjing, China). The immunoreactive bands were visualized by ChemiDoc™ MP System (Bio-Rad) and analyzed using Image Lab™ Software (version 4.1, Bio-Rad).

2.9. Statistical analysis

All experiments were performed in triplicate and data were expressed as the mean ± standard deviation. Statistical analysis was carried out using Student's two-tailed-t-test for comparison between two groups and one-way analysis of variance (ANOVA),

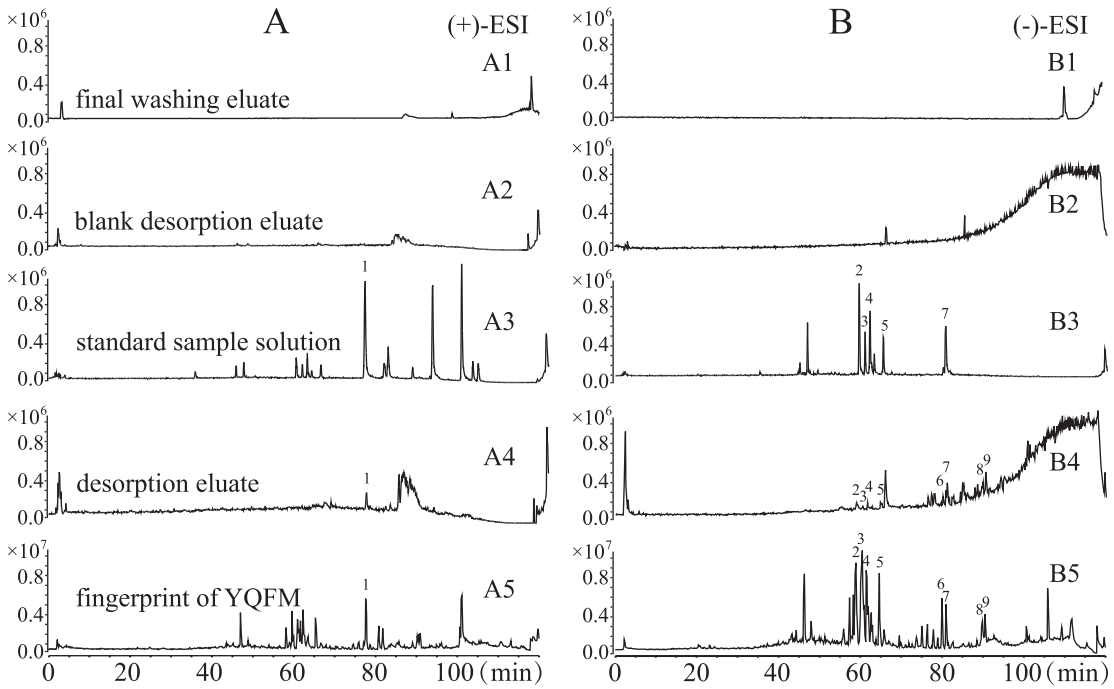

Fig. 1. Detection of YQFM HUVECs-binding molecules by HPLC-DAD-Q-TOF-MS/MS. (A) total ion current (TIC) in positive ion mode: A1 the final washing eluate, A2 the blank desorption elute, A3 standard sample solution, A4 the desorption elute, A5 YQFM, **1** schisandrin. (B) total ion current (TIC) in negative ion mode: B1 the final washing eluate, B2 the blank desorption elute, B3 standard sample solution, B4 the desorption elute, B5 YQFM, **2** ginsenoside Rb1, **3** ginsenoside Rc, **4** ginsenoside Rb2, **5** ginsenoside Rd, **6** 20(S)-ginsenoside Rg3, **7** 20(R)-ginsenoside Rg3, **8**/**9** ginsenoside Rk1/Rg5.

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followed by Dunnett's test when the data involved three or more groups. $P < 0.05$ was defined as significant.

3. Results

3.1. HUVECs-binding components of YQFM

It is critical to completely wash out the compounds which not specially binding with HUVECs, after the components have combined with the receptors on HUVECs, generally washed with PBS for several times to scour off the possible non-selectively combining components. The eluates were discarded except for the last one, which was collected as contrast for HPLC-MS analysis. As shown in Fig. 1, nine principal peaks were identified in the extraction of HUVECs (A4, B4) by comparing standards (A3, B3) fragmentation behavior and the retention time with YQFM (A5, B5). No comparable peaks were detected in the final washing eluate (A1, B1) and blank desorption eluate (A2, B2). Therefore, it was assumed that these nine components were extracted by some receptors or other targets of HUVECs in analogical physiological condition, which indicated that these binding molecules as potential vascular endothelium protective compounds of YQFM.

3.2. HPLC-ESI-MS/MS analysis for HUVECs-binding components of YQFM

Compounds **1**–**7** could easily be identified as schisandrin, ginsenoside Rb1, Rc, Rb2, Rd and 20(R)-Rg3 by comparing retention time and the fragmentation behaviors with the standard sample solution and the fingerprint of YQFM, respectively. Among these compounds, 20(S)-Rg3 was identified by comparing with literature (26) and showed similar MS behaviors to 20(R)-Rg3 (Fig. 2A). In addition, compound **8** and **9** were tentatively identified as a group of geometric isomer. They showed the same $[M-H]^-$ ions at *m/z* 765.4914, in the positive ion MS/MS spectra, they afforded ions at *m/z* 587.4301, 443.3884, 425.3778, 407.3673 (Fig. 2B). Therefore, they were tentatively identified as ginsenoside Rk1 and Rg5 according to publication (26).

3.3. Effects of schisandrin on H/R-induced injury in HUVECs

HUVECs were incubated with schisandrin (Fig. 3A) at concentrations of 0.1–100 μM and YQFM at 400 μg/mL for 12 h and exposed to hypoxia of 4 h followed by 2 h reoxygenation. As shown in Fig. 3B, the viability of HUVECs subjected to H/R was only 52.9% compared to the untreated cells ($P < 0.01$). However, H/R induced HUVECs injury was significantly attenuated by pretreatment with schisandrin in a concentration-dependent manner and YQFM. As shown in Fig. 3C, the percentage of LDH, an indicator for cell damage, was 12.7% in the control, which significantly increased to 39.2% after H/R ($P < 0.01$). While pretreatment with schisandrin at concentrations of 10–100 μM and YQFM significantly inhibited the increase in LDH release. These results indicated that schisandrin had an obvious protective effect against H/R-mediated damage in HUVECs.

3.4. Effects of schisandrin on MDA, NO content and intracellular ROS of HUVECs subjected to H/R

MDA was one of the most frequently used indicators of lipid peroxidation, which was indirectly involved in the degree of damage to cells. When HUVECs were subjected to H/R injury, the content of MDA increased remarkably ($P < 0.01$). Whereas, the concentration of MDA significantly decreased after pre-treated with schisandrin (Fig. 4A). Meanwhile, compared to the control group, NO secretion was markedly decreased in the H/R group ($P < 0.05$). Following pre-treatment with schisandrin, the decrease in H/R-induced NO production was inhibited in a dose-dependent manner (Fig. 4B). Moreover, cells pretreated with schisandrin showed a substantial reduction in ROS levels (Fig. 4C).

3.5. Effects of schisandrin on H/R induced cell apoptosis

To evaluate whether the protective effect of schisandrin correlated to the anti-apoptotic mechanism, the expression of anti-apoptotic and pro-apoptotic proteins were determined by western blot. There was a tendency of increase in expression of cleaved

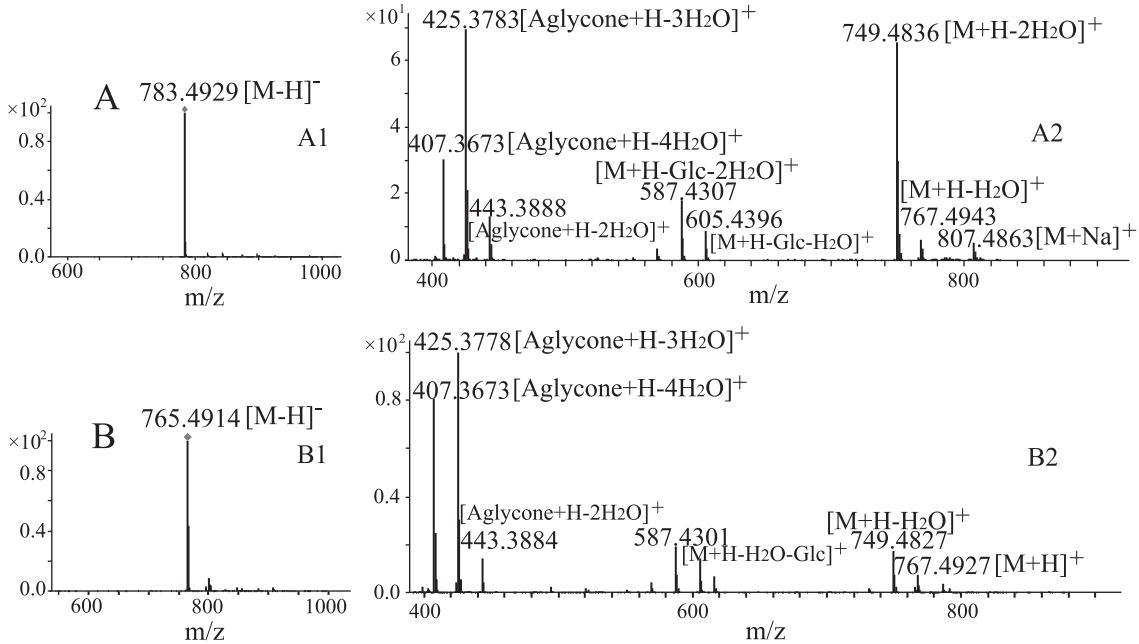

Fig. 2. ESI-MS ionization spectrum of compound **6** and compounds **8**/**9**. (A) Full-scan product ion spectra of $[M-H]^-$ ions (A1) and fragmentation pathways for compound **6** (A2). (B) Full-scan product ion spectra of $[M-H]^-$ ions (B1) and fragmentation pathways for compounds **8**/**9** (B2).

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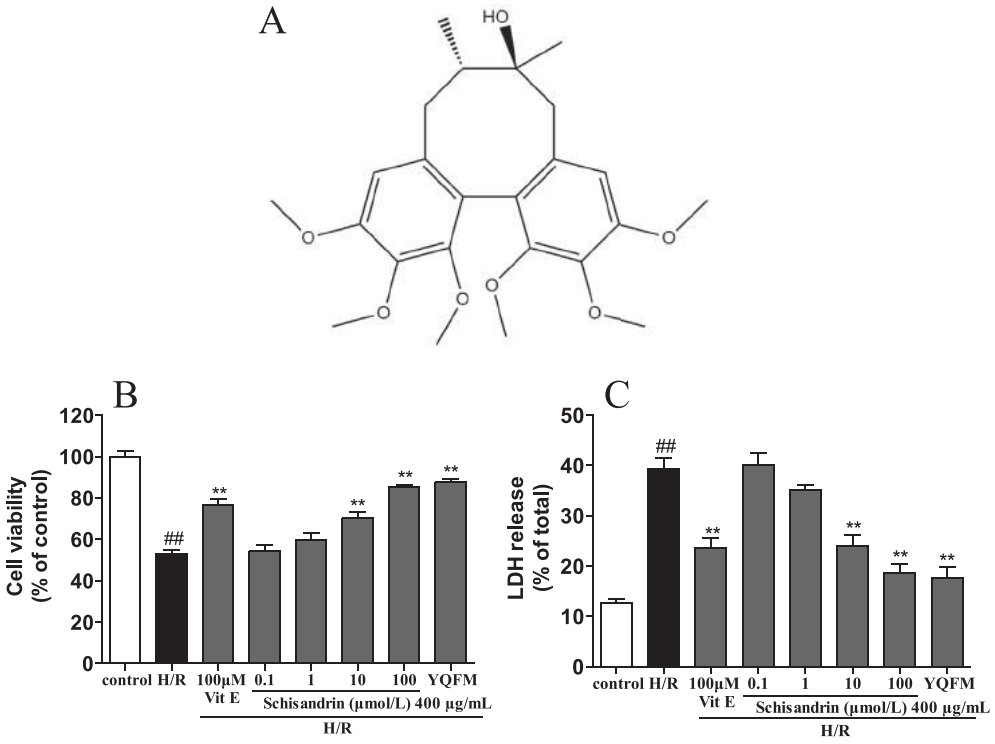

Fig. 3. Chemical structure of schisandrin (A) and protective effects of schisandrin and YQFM on cell viability (B) and LDH activity (C) in HUVECs subjected to H/R injury. HUVECs were pretreated with schisandrin at the concentration of 0.1–100 μmol/L and 400 μg/mL YQFM for 12 h and then exposed to hypoxia of 4 h followed by 2 h reoxygenation. Vit E was used as a positive control drug. Cell viability was measured by the MTT assay described in method. The LDH was detected using LDH cytotoxicity assay kit. Results were obtained from three independent experiments and were presented as mean ± SD. $^{\#\#}P < 0.01$ vs. Control group without H/R, $^{**}P < 0.01$ vs. group treated with H/R alone.

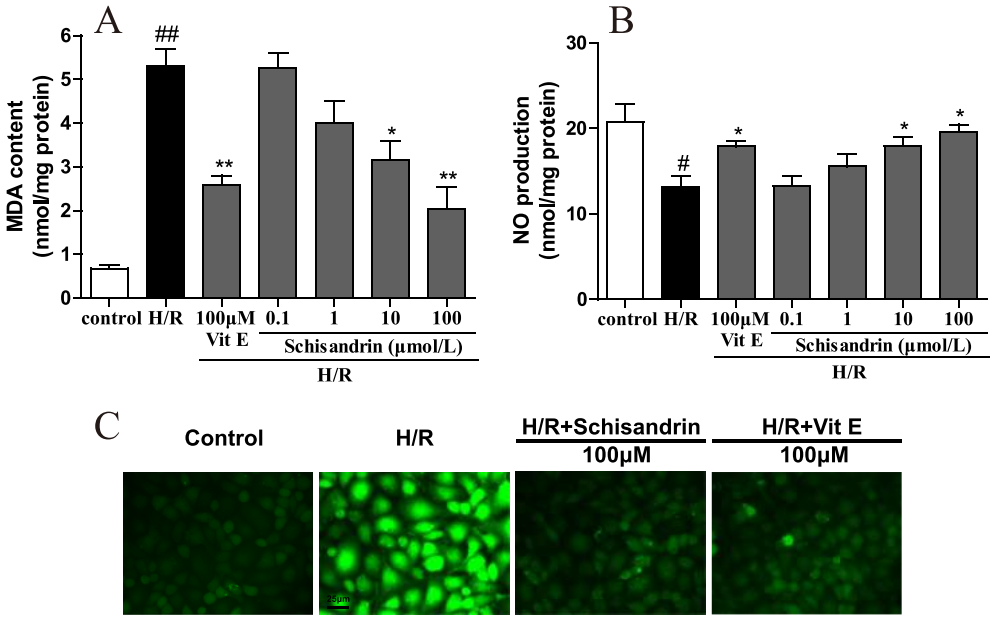

Fig. 4. Effects of schisandrin on MDA content (A), NO production (B) and intracellular ROS (C) in HUVECs subjected to H/R injury. HUVECs were pretreated with schisandrin at the concentration of 0.1–100 μmol/L for 12 h and then exposed to hypoxia of 4 h followed by 2 h reoxygenation. MDA content and NO production were measured by assay kit. ROS levels were determined using 2′, 7′-dichlorofluorescein diacetate (DCFH-DA, Molecular Probes), then the cells were loaded with DCFH-DA for 30 min and visualized by fluorescence microscopy. Vit E was used as a positive control drug. Results were obtained from three independent experiments and were presented as mean ± SD. $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ vs. Control group without H/R, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. group treated with H/R alone.

caspase-3 under the H/R condition (Fig. 5A). In contrast, expressions of Bcl-2/Bax was significantly decreased (Fig. 5B). Whereas, HUVECs treated with schisandrin could increase Bcl-2/Bax expression and conversely decrease the expression of caspase-3 under H/R conditions. The results implied that schisandrin had protective effects against H/R-induced cellular injury by decreasing the apoptosis rate, which offered some new information for the role of schisandrin in the cardiovascular system.

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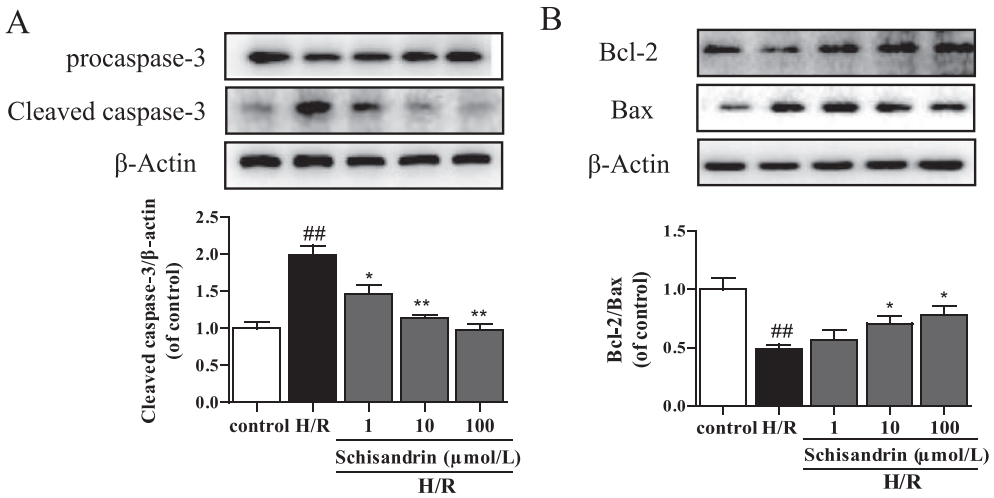

Fig. 5. Effects of schisandrin on the up-regulation of caspase-3 (A) and down-regulation of the ratio of Bcl-2/Bax (B) induced by H/R on HUVECs. HUVECs were pretreated with schisandrin at the concentration of 1–100 μmol/L for 12 h and then exposed to hypoxia of 4 h followed by 2 h reoxygenation. Protein expressions were tested by western blot. Results were obtained from three independent experiments and were expressed as mean ± SD. $^{\#\#}P < 0.01$ vs. Control group without H/R, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. group treated with H/R alone.

4. Discussion

Given the key role of endothelial cells in the cardiovascular diseases (19,20), we chose HUVECs binding as an enrichment method to explore the bioactive components present in YQFM. Nine compounds containing eight ginsenosides were identified. Recently, the clinical utilization of ginseng to treat cardiovascular diseases has increased dramatically. Both *in vitro* and *in vivo* results indicated that ginsenosides had potentially positive effects on cardiovascular disease by means of their various properties including antioxidation, vasomotor regulation, influencing various ion channels and so on. Studies showed that myocardial infarction after ischemia and reperfusion was preconditionally protected by ginsenoside Rb1 (14). Dihydroginsenoside Rg3 could increase the production of NO by increasing phosphorylation and expression of eNOS (27). Moreover, evidence suggested ginsenoside Rd mediated cardioprotective effect against MI/R-induced apoptosis via a mitochondrial-dependent apoptotic pathway (28). These findings indicated that HUVECs-HPLC-MS/MS was a reliable method for screening potential bioactive compounds from complicated systems of TCMs. Due to the substantial evidence in the literature for the use of ginsenosides in the treatment of cardiovascular disorders (9), whereas little is reported about schisandrin, we further investigated the protective effect of schisandrin.

Schisandrin, the most abundant lignan present in *Schisandra chinensis* (Turcz.) Baill, was previously reported to have the anti-inflammatory activity by upregulation of heme oxygenase-1 via PI3K/Akt and Nrf-2 signaling pathways (29). Other studies reported its neuroprotective effect on the glutamate-induced neuronal excitotoxicity and suggested that it was a potential cognitive enhancer against Alzheimer's disease through anti-oxidative action (30,31). Although some of these activities could reflect by schisandrin, no previous studies have addressed its specific effects on H/R-induced injury in HUVECs. H/R injuries are common causes of mortality and play important roles in human pathophysiology because they occur in clinical conditions such as myocardial ischemia and stroke (32,33). During hypoxia and reperfusion vascular endothelium is a primary site of ROS generation and target of injury. Therefore, cellular models of H/R have been widely used to evaluate the effects of drugs or active compounds (34,35).

Endothelial cell injury caused by H/R plays a critical role in the pathogenesis of cardiovascular diseases. The indicators of oxidative stress-related cell injury were measured in the present study (36). NO was known to be an important effector molecule that had gained recognition as a crucial modulator of vascular disease (37). It was reported that NO had protective effects against apoptosis in a variety of cell types including endothelial cells. In normal physiological conditions, ROS generated from metabolic processes are scavenged by antioxidants that are part of cellular defense mechanisms. However, in disease states such as H/R, overproduction of ROS results in oxidative cellular damage (33). ROS cause oxidation of proteins, affect singnal transduction pathways and finally result in alterations of cardiac function, cellular injury and death (38). Our findings first demonstrated that schisandrin could protect against H/R-induced HUVECs injury by increasing cell viability, NO content and decreasing LDH leakage, MDA content and ROS generation, which was beneficial for the prevention and treatment of cardiovascular diseases. Vitamin E, which is a chain-breaking antioxidant, was used as a positive control drug. Pretreatment with vitamin E has been demonstrated to attenuate hypoxia/reoxygenation injury in cardiomyocytes (39,40).

In addition, we further explored its possible mechanism of schisandrin on cell injury induced by H/R. A balance between cell proliferation and death is critical for tissue homeostasis in multicellular organisms. Many diseases are more or less associated with apoptosis, including cardiovascular diseases. Endothelium is a critical site for the control of apoptosis and the suppression of endothelial cell apoptosis is required for the maintenance of blood vessel integrity (22). Caspase-3 is the key downstream enzyme and the final step of apoptotic pathway, and it can cause DNA degradation and apoptosis when it is activated. The Bcl-2 family, comprised of both anti-apoptotic proteins, such as Bcl-2, and pro-apoptotic members, such as Bax, constitutes a critical intracellular checkpoint for apoptosis within a common cell death pathway. The balance between these proteins is essential for cell survival against H/R injuries (41). Our results showed that schisandrin could increase Bcl-2/Bax expression and conversely decrease the expression of caspase-3 under H/R conditions, which indicated that schisandrin had protective effects against H/R-induced cellular injury by decreasing the apoptosis rate. It is noteworthy that the findings of the present study may shed light on the pharmacological basis for

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the clinical application of traditional Chinese medicine in treatment of cardiovascular diseases relevant to the endothelial cell damage. Further studies on the understanding of schisandrin targets and its molecular mechanism are needed.

Additionally, we investigated the effects of identified nine compounds against H/R-mediated injury in HUVECs. As seen in Supplemental Fig. 1, pretreatment with schisandrin, ginsenoside Rb1, Rc, Rb2, Rd, (R)-Rg3 at the concentration of 10 μM significantly increased the cell viability, while pretreatment with ginsenosides (S)-Rg3 and Rk1/Rg5 at 10 μM did not exert obvious protective effect against H/R-mediated damage in HUVECs. We then determined the content of schisandrin in YQFM as about 0.09047 ± 0.0009 mg/g. The maximum concentration of schisandrin at 100 μM was equivalent to 43.25 μg/mL in our experiment. Moreover, the content of schisandrin in YQFM at concentration of 400 μg/mL was far lower than 43.25 μg/mL. Yet, YQFM presented an excellent protective effect, due to complicated synergistic or integrated actions of multiple active compounds.

In conclusion, this combined specific binding and HPLC-ESI-MS/MS analysis method was applied to screen for potential bioactive components in YQFM via vascular endothelial cell receptors or other targets. Schisandrin, a compound identified by this method, was first demonstrated to exert protective effects against H/R-induced vascular endothelial injury by inhibiting apoptosis pathway. These findings argue that the cardiovascular protective effect of YQFM is at least partially mediated by schisandrin. In addition, some ginsenosides with definite cardiovascular protective effect were also detected, which indicated that HUVECs extract and HPLC-ESI-MS/MS analysis would be very efficient and rapid for screening and identification of bioactive components in YQFM. These results present the prospective application of this method to other complicated systems of TCMs. More research data are urged to be accumulated in the further investigation.

Conflicts of interest

The authors have no conflict of interest to declare.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jphs.2015.02.003.

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