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Protective Effects and Active Ingredients of Yi-Qi-Fu-Mai Sterile Powder Against Myocardial Oxidative Damage in Mice

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Abstract. This study aims to evaluate the protective effects of Yi-Qi-Fu-Mai sterile powder (YQFM) on myocardial oxidative damage and tries to identify the active components responsible for its pharmacological benefits. YQFM and the *n*-butanol extract of YQFM (YQFM-Bu) were administered to ISO-induced myocardial injury mice. Left ventricle weight index and histopathological analyses were conducted. Serum enzymatic activities of lactate dehydrogenase (LDH), creatine kinase (CK) and superoxide dismutase (SOD), myeloperoxidase (MPO), and the levels of malondialdehyde (MDA) were also measured. Our results demonstrated that both YQFM and YQFM-Bu significantly restored the abnormal activities of CK, LDH, MPO, SOD, and the levels of MDA in ISO-induced myocardial injury mice, and these biochemical results were further supported by histopathological data. Our in vitro findings also confirmed that both YQFM and YQFM-Bu exhibit significant radical scavenging activity. Furthermore, the major active fractions of YQFM were identified by UPLC–MS/MS. Twenty-five ginsenosides and three lignans were identified from YQFM-Bu. These findings suggested YQFM-Bu is the major active fraction of YQFM with the ginsenosides and lignans as potential active components responsible for its protective effect against myocardial injury, and YQFM exerted its beneficial effects on myocardial injury mainly through inhibiting oxidative damage and maintaining the functional integrity of myocardial tissue.

Keywords: myocardial oxidative damage, Traditional Chinese Medicine, Yi-Qi-Fu-Mai sterile powder, active ingredient, cardioprotection

Introduction

Cardiovascular diseases and their complications have been regarded as the leading cause of morbidity and mortality around the world, especially in developed countries. Myocardial reperfusion injury, a kind of coronary heart disease, is defined as myocardial oxidative damage characterized by induction of cardiomyocyte death and increasing infarct size (1).

Yi-Qi-Fu-Mai sterile powder (YQFM) is a modified preparation derived from a famous Traditional Chinese Medicine (TCM) formula Sheng-Mai-San (SMS), which is extracted from three kinds of crude drugs: *Panax ginseng* C.A. Mey., *Ophiopogon japonicus* (Thunb.) Ker-Gawl, and *Schisandra chinensis* (Turcz.) Baill (1:3:1.5). According to the theory of TCM, this formulation benefits "qi", nourishes "yin", prevents exhaustion, and replenishes bodily fluids. It has been used for the treatment of "qi-yin" deficiency in cardiovascular diseases for a long time. Modern clinical studies have confirmed the beneficial effect of SMS in patients with coronary heart disease, viral myocarditis, cardiac arrhythmia, and cardiogenic shock (2 – 5).

Although YQFM and SMS are each prepared from the same three herbs, our previous study found that the chemical compositions of YQFM are quite different from SMS possibly due to the different preparation process. Previous studies pay more attention to SMS, but there is little research on YQFM. Whether YQFM can exert protective effects against myocardial injury, the most common complications from heart disease, has not been studied. Moreover, the active components of

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YQFM responsible for the beneficial effect to cardiovascular diseases remain unknown. All of these has become the major obstacle for the clinical application and further pharmaceutical research of YQFM. Therefore, this project aims to investigate protective effects and active components of YQFM on myocardial oxidative damage. YQFM and YQFM-Bu were prepared and evaluated in ISO-induced myocardial injury mouse models induced by isoprenaline (ISO). Our results demonstrated that YQFM-Bu is the main active fraction of YQFM for myocardial injury. Furthermore, a UPLC–MS/MS method was developed to qualitatively investigate the chemical constituents of YQFM-Bu in order to further identify the active components of YQFM.

Materials and Methods

Reagents and materials

Chromatographic grade acetonitrile (Tedia, Fairfield, OH, USA); Analytical grade *n*-butanol (Hanbang Science & Technology, Nanjing, P.R. China); Luminol (Fluka Chemie, Buchs, Switzerland); pyrogallol and EDTA (Shanghai Chemical Reagent Corporation, Shanghai, China); Na_2CO_3 and $NaHCO_3$ (Nanjing Chemical Reagent Corporation, Jiangsu, China); Water for UPLC analysis was purified by a Milli-Q academic water purification system (Milford, MA, USA). Standards of ginsenoside Rg1, Re, Rf, Rb1, schizandrol A, schizandrin A, and schizandrin B were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (NICPBP, Beijing, China). Lactate dehydrogenase (LDH), creatine kinase (CK), superoxide dismutase (SOD), myeloperoxidase (MPO), and malondialdehyde (MDA) assay kits were produced by the Institute of Nanjing Jiancheng Biology Engineering (Nanjing, P.R. China). Isoprenaline was purchased from Sigma Biotechnology (Sigma, St. Louis, MO, USA).

YQFM (Batch No. 20111008) was obtained from Tianshili Pharmaceuticals Company, which is the prescription of *Panax ginseng* C.A. Mey., *Ophiopogon japonicus* (Thunb.) Ker-Gawl, and *Schisandra chinensis* (Turcz.) Baill (Tianjin, P.R. China); the specimen was identified by professor Bo-Yang Yu (Specimen No. 20111008) and deposited in the Herbarium of Department of Traditional Chinese Medicine of China Pharmaceutical University. Propranolol was purchased from Huazhong Pharmaceutical Co., Ltd. (Hubei, P.R. China).

Preparation and chemical profile of YQFM

YQFM (Batch No. 20111008) was obtained from Tianshili Pharmaceuticals Company (Tianjin, P.R. China). It consists of the ethanol extract (78°C) of *Panax ginseng* C.A. Mey., water extracts (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. In order to ensure the quality control of YQFM used in this study, an UPLC–MS/MS method was applied for determining the chemical profile; the experiment was described in UPLC–MS/MS analysis and the results are shown in Fig. 2 and Table 5.

Preparation of samples

YQFM (10.4 g) was dissolved in water (100 mL) and extracted four times with 300 mL water-saturated *n*-butanol. These *n*-butanol fractions were combined and concentrated by a rotary evaporator and then *n*-butanol totally removed to obtain the dry extract. The dry extract of *n*-butanol fractions was dissolved in water (100 mL) and filtered through a 0.22-μm filter. Two thirds of the filtrate was lyophilized by using Freeze Drying Equipment to obtain the sterile powder for injection of YQFM-Bu; one third of the filtrate was stored at −20°C for further examination. Propranolol was used here as a positive control drug.

In vitro free radical scavenging by YQFM and YQFM-Bu

Superoxide radical scavenging activity was measured by the chemiluminescence method, as previously reported by Qin et al. (6). Carbonate buffers (pH 10.0) were prepared by mixing of appropriate volumes of 0.1 M Na_2CO_3 and 0.1 M $NaHCO_3$. Luminol solution was prepared by mixing Luminol and 0.1 M carbonate buffer (pH 10.0). Pyrogallol solution was prepared by dissolving pyrogallol and EDTA in water. The chemiluminescence emission was detected by the BPCL system (Academia Sinica Biophysics Institute, Beijing, China). Rutin, a well-known radical scavenger, was used as a positive control.

Experimental animals and models

All experiments were performed with ICR mice, weighing 18 – 22 g, obtained from the experimental animal center of Yang-Zhou University. The experimental animals were kept in plastic cages at 22°C ± 2°C with free access to pellet food and water and on a 12-h light/dark cycle. Seventy-two mice were divided into 9 groups randomly: control group, ISO group, propranolol group (0.02 $g \cdot kg^{-1}$/day), three YQFM groups (1.352, 0.676, and 0.338 $g \cdot kg^{-1}$/day), and three YQFM-Bu groups (Equivalent to YQFM 1.352, 0.676, and 0.338 $g \cdot kg^{-1}$/day). All of these doses were converted according to the clinical dose. When the experiment began, YQFM, YQFM-Bu, and propranolol were administered to the animals once a day, while the control group and the ISO

group were administered with saline for 7 consecutive days. From the 5th day, all animals except those in the control group received ISO (0.02 $g \cdot kg^{-1}$/day), i.p. simultaneously once per day for 3 days, while the control group was injected with saline.

All procedures were in accordance with the National Institute of Health's guidelines regarding the principles of animal care and approved by the authors' institutional animal care guidelines.

Determination of the MDA levels and LDH, CK, SOD, MPO activities

At the end of the experiment, blood was drawn from retro orbital veins and centrifuged to separate serum after clotting. The supernatant was taken out and stored at −70°C prior to assay. The activity of LDH, CK, SOD, and MPO were each detected on a microplate reader. The operations of all the assay kits follow the manufacturer's instructions.

Left ventricle weight index and histopathological analysis

Mice were sacrificed and their hearts were removed and dried with filter paper; the pericardium and large vessels were removed, the atria and right ventricle were separated along the atrial/ventricular septal wall, and the left ventricular weight (LVW) was measured. The left ventricular weight/body weight ratio (LVW/BW) was then calculated (7).

Hearts were fixed in 10% formalin. The myocardial tissues samples were separated from the hearts and embedded in paraffin and 5-μm sections were prepared. After fixation, the sections were stained with hematoxylin and eosin (HE). Finally, the sections of the myocardium were observed under a light microscope to examine the architecture of the myocardium. In order to make a quantitative estimation of cardiac damage, pathological scores were determined by an investigator who was kept unaware of the experimental treatment groups. The following morphological criteria were considered: score 0, no damage; score 1 (mild), a few neutrophils infiltrate, local cellular edema; score 2 (moderate), interstitial edema, diffuse myocardial cell swelling, and focal necrosis; score 3 (severe), neutrophil infiltrate, swelling of myocardial fiber, necrosis with the presence of contraction bands; and score 4 (highly severe), wide-spread necrosis and fusion area, swelling of myocardial fiber, a large number of neutrophils infiltrate, and hemorrhage (8).

Statistical analysis

The results were expressed as the mean ± S.D. and analyzed by one-way ANOVA, followed by Student's two-tailed *t*-test for comparison between two groups and Dunnett's test when the data involved three or more groups. A probability (*P*) value of less than 0.05 was considered statistically significant and *P* less than 0.01 being very significant.

UPLC analysis

The YQFM cannot be injected directly into the UPLC for analysis—that would damage the column. Therefore the YQFM-Bu was adopted for analysis to determine the chemical profile of YQFM. After the YQFM-Bu solutions (2 mL) were centrifuged at 12,000 rpm for 10 min, a 5-μL aliquot of supernatant was injected into the UPLC–MS/MS system. Stock solutions of reference compounds including ginsenoside Rg1, Re, Rf, Rb1, schizandrol A, schizandrin A, and schizandrin B were prepared in methanol; all working solutions were prepared by diluting the stock solutions with methanol. All of the solutions were stored at 4°C and used at room temperature.

UPLC was performed on a Waters Acquity^{TM} ultra-performance liquid chromatography (UPLC) system (Waters Corporation, Milford, MA, USA), which is equipped with an on-line degasser, binary solvent delivery system, auto-sampler, column oven, and photodiode-array detection (DAD) system. A Zorbax Eclipse plus C_{18} column (50 mm × 2.1 mm, 1.8 μm) was used for all chromatographic separations. The mobile phase was A) water–formic acid (100:0.01, v/v) and B) acetonitrile. The UPLC eluting conditions were as follows: 0 – 3 min, 2% – 20% B; 3 – 9 min, 20% – 30% B; 9 – 12 min, 30% – 38% B; 12 – 14.5 min, 38% – 40.5% B; 14.5 – 17.5 min, 40.5% – 55% B; 17.5 – 19.5 min, 55% – 99% B; 19.5 – 20 min, 99% – 2% B. The flow rate was 0.5 mL/min. The monitoring detection was set at 203 nm, and DAD spectra were recorded from 190 to 400 nm. The column temperature was 25°C, and the sample injection volume was 5 μL.

Mass spectrometry

UPLC–MS/MS analysis was carried out using an Acquity UPLC system connected on-line to a Micromass Quattro Premier XE triple-quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with an electrospray ionization (ESI) source operated in positive and negative ion modes respectively. The cone voltage was set at 30 V and the capillary voltage, at 3.0 kV; the source temperature was 130°C; and the desolvation temperature was 400°C. The desolvation gas was nitrogen and its flow rate was 800 L/h; the flow rate of the cone gas was 50 L/h. An *m/z* range of 200 – 1200 was scanned. MassLynx 4.1 software (Waters) was used for system control and data processing.

Results

The effects on free radical scavenging

The half-maximal inhibitory concentrations (IC_{50}) of YQFM, YQFM-Bu, and rutin were calculated by linear regression of log-dose versus radical yields (Table 1). From our result, YQFM and YQFM-Bu dose-dependently scavenged superoxide radicals. The 50% free radical scavenging concentrations are 8.79 and 10.66 μg/mL for YQFM and YQFM-Bu, respectively. Rutin, a well-known superoxide radical scavenger, was used as a reference compound, which showed an IC_{50} value of 6.88 μg/mL. It was suggested that both YQFM and YQFM-Bu have a good effect on scavenging superoxide free radicals.

Effect on myocardial hypertrophy and histopathological examination of heart tissues

The hypertrophied hearts were evidenced with the left ventricle weight index. ISO-induced model mice showed serious myocardial hypertrophy ($P < 0.01$). Compared with the model group, left ventricle weight index indices were significantly reduced in the YQFM and YQFM-Bu groups ($P < 0.01$). It was confirmed that both YQFM and YQFM-Bu have an inhibiting effect on myocardial hypertrophy (Table 2).

On histopathological examination, the myocardial tissue of control group mice didn't have any obvious pathological lesion (Fig. 1A). Myocardial tissue from

Table 1. The 50% free radical scavenging concentration of YQFM and YQFM-Bu

Radicals	YQFM (μg/mL)	YQFM-Bu (μg/mL)	Rutin (μg/mL)
Superoxide anion	8.79 ± 0.36	10.66 ± 0.36	6.88 ± 0.36

IC_{50} for half-maximal inhibitory concentrations. Data are expressed as the mean of three IC_{50} determinations ± S.D., n = 3.

Table 2. Effect of YQFM and YQFM-Bu on the left ventricular weight index in ISO-induced myocardial injury mice

Treatment	LV weight (mg)	LVW/BW (mg/g)
Normal control	73.0 ± 4.9	2.86 ± 0.14
ISO-control	97.5 ± 9.1$^{\#\#}$	3.86 ± 0.27$^{\#\#}$
Propranolol	82.3 ± 5.8**	3.15 ± 0.18**
YQFM H	80.9 ± 4.3**	3.18 ± 0.22**
YQFM M	79.7 ± 3.4**	3.05 ± 0.18**
YQFM L	81.3 ± 5.6**	3.21 ± 0.18**
YQFM-Bu H	80.0 ± 2.0**	3.11 ± 0.15**
YQFM-Bu M	81.2 ± 5.2**	3.17 ± 0.18**
YQFM-Bu L	83.3 ± 6.2**	3.19 ± 0.10**

LVW/BW for left ventricular weight/body weight, ISO-control for model group, L for low dose, M for middle dose, H for high dose. Data are expressed as means ± S.D., n = 8 in each group. **$P < 0.01$ vs. ISO-control group, $^{\#\#}P < 0.01$ vs. normal control group.

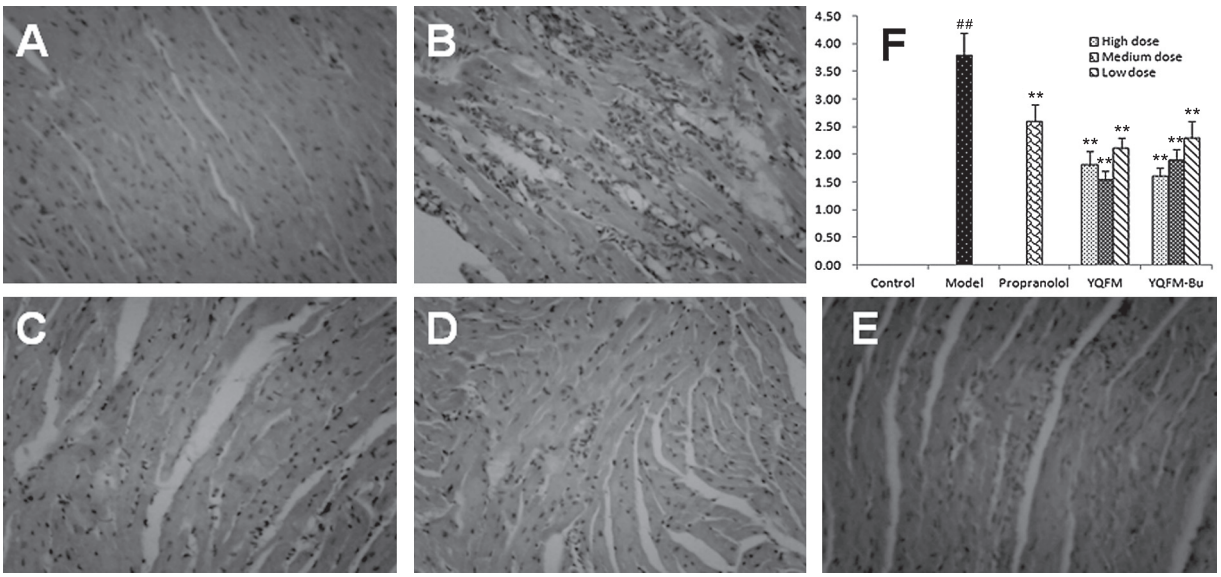

Fig. 1. Effect of YQFM and YQFM-Bu on histopathological changes. A – E) Representative light microscopic appearance of mice myocardial histopathological morphology (hematoxylin staining, original magnification × 200) for normal control (A), isoproterenol-treated group (B), propranolol group (C), YQFM group (D), YQFM-Bu group (E); F) Representative effects of YQFM and YQFM-Bu on histopathological scores. Data were expressed as the mean ± S.D., n = 8 in each group. **$P < 0.01$ vs. ISO model group, $^{\#\#}P < 0.01$ vs. normal control group.

ISO-treated mice showed serious myocardial degeneration, which was demonstrated by swelling of myocardial fiber, increased necrosis and fusion area, and a large number of inflammatory cells infiltrating the myocardial tissue (Fig. 1B). All YQFM treatment groups showed significantly improved myocardial tissue damage compared with the ISO group. The myocardial tissue of YQFM-treated mice showed reduced infiltration of inflammatory cells and no sign of cell edema and necrosis (Fig. 1D). The myocardial tissue of YQFM-Bu–treated mice also showed significantly reduced inflammatory cell infiltration and local cellular edema and no noticeable cell degeneration and necrosis (Fig. 1E). Myocardial tissue of propranolol-treated mice, the positive control group, also showed reduced inflammatory cell infiltration, local cellular edema, and occasional individual cell necrosis as we expected (Fig. 1C). For a quantitative estimation of myocardial tissue damage, pathological scores were used as a parameter to evaluate myocardial oxidative damage severity. The pathological scores of mice treated with YQFM or YQFM-Bu were considerably lower than those of the ISO group (Fig. 1F).

Effect on cardiac marker enzymes

As shown in the Table 3, the activities of CK and LDH were significantly increased in the ISO group compared with the control group ($P < 0.01$). This demonstrated that the ISO group had significant myocardial oxidative damage. Compared with the ISO group, significant fall in serum LDH was seen in the YQFM and YQFM-Bu groups ($P < 0.05$), except in the low-dose group of YQFM-Bu, and the activity of CK in YQFM and YQFM-Bu groups both decreased significantly ($P < 0.05$), except in the high-dose groups of YQFM and YQFM-Bu.

Table 3. Effect of YQFM and YQFM-Bu on the LDH and CK activity in ISO-induced myocardial injury mice

Treatment	LDH (U/L)	CK (U/mL)
Normal control	3043.26 ± 484.95	0.417 ± 0.043
ISO-control	4473.56 ± 322.52$^{\#\#}$	0.950 ± 0.035$^{\#\#}$
Propranolol	3722.57 ± 615.24*	0.570 ± 0.087**
YQFM H	3639.46 ± 479.92**	0.481 ± 0.188
YQFM M	3544.63 ± 379.92**	0.523 ± 0.108*
YQFM L	3753.18 ± 371.28*	0.755 ± 0.180**
YQFM-Bu H	3786.88 ± 443.22**	0.504 ± 0.280
YQFM-Bu M	3825.52 ± 406.70*	0.582 ± 0.066*
YQFM-Bu L	4301.61 ± 967.77	0.770 ± 0.092**

ISO-control for model group, L for low dose, M for middle dose, H for high dose. Data were expressed as means ± S.D., n = 8 in each group. *$P < 0.05$, **$P < 0.01$ vs. ISO-control group, $^{\#\#}P < 0.01$ vs. normal control group.

Effect on enzymic antioxidants and lipid peroxidation

As shown in the Table 4, after ISO injection, MPO activity and MDA level significantly increased while SOD activity decreased in the ISO group ($P < 0.05$). This means that animals of the ISO group had significant oxidative damage in their myocardial tissue. Both YQFM and YQFM-Bu substantially attenuated the increase of MDA production compared with the ISO group ($P < 0.01$). YQFM and YQFM-Bu treatment substantially reversed the increase of MPO activity compared with the ISO group ($P < 0.05$), except in the low-dose

Table 4. Effects of YQFM and YQFM-Bu on the MDA levels, MPO and SOD activities in ISO-induced myocardial injury mice

Treatment	MDA (nmol/mL)	MPO activity (U/L)	SOD activity (U/mL)
Normal control	7.06 ± 0.38	40.60 ± 12.89	85.29 ± 6.71
ISO-control	12.66 ± 1.89$^{\#\#}$	90.44 ± 12.19$^{\#\#}$	54.18 ± 6.80$^{\#\#}$
Propranolol	8.94 ± 1.75**	63.08 ± 8.81**	78.75 ± 11.57**
YQFM H	8.10 ± 1.59**	43.26 ± 8.16**	80.80 ± 17.46*
YQFM M	8.68 ± 1.63**	48.18 ± 6.11**	80.62 ± 26.58*
YQFM L	7.04 ± 1.43**	50.64 ± 7.07**	82.80 ± 15.75*
YQFM-Bu H	8.54 ± 2.50**	45.98 ± 9.27**	85.56 ± 26.49*
YQFM-Bu M	9.96 ± 0.87**	48.77 ± 5.96*	80.60 ± 12.14*
YQFM-Bu L	7.93 ± 2.22**	52.44 ± 22.89	83.63 ± 20.08**

ISO-control for model group, L for low dose, M for middle dose, H for high dose. Data were expressed as means ± S.D., n = 8 in each group. Data were analyzed by one-way ANOVA, followed by Student's two-tailed *t*-test for comparison between two groups and Dunnett's test when the data involved three or more groups. *$P < 0.05$, **$P < 0.01$ vs. ISO-control group, $^{\#\#}P < 0.01$ vs. normal control group.

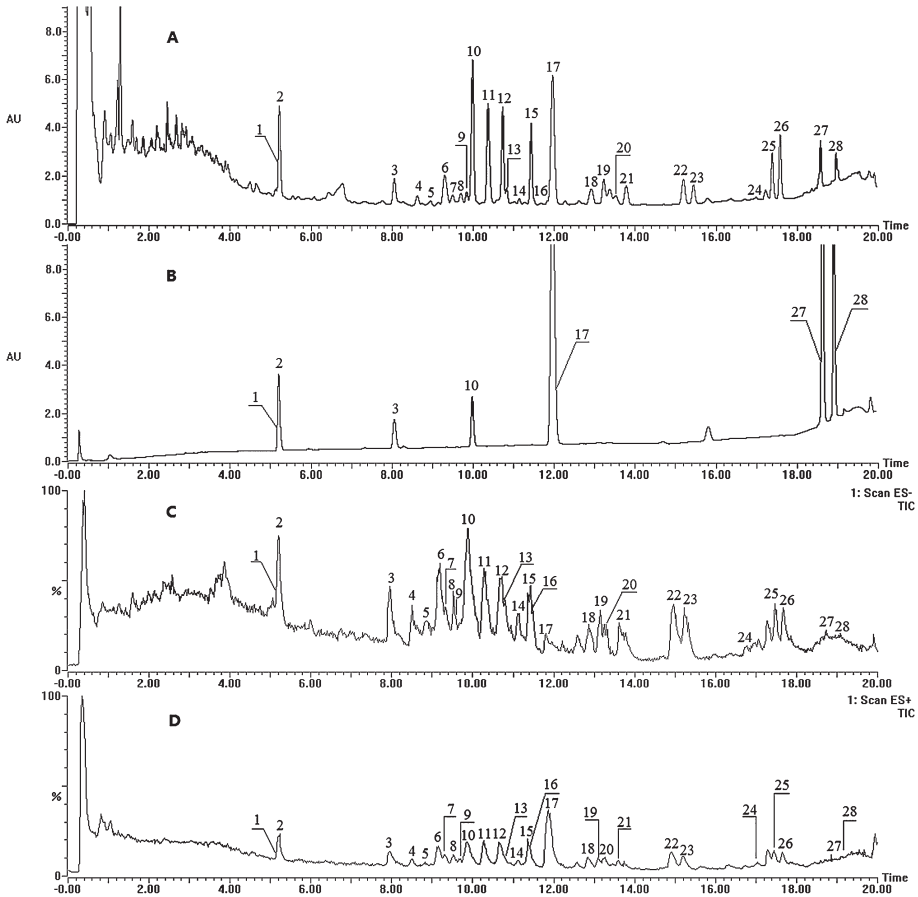

Fig. 2. UPLC–MS/MS analysis of YQFM-Bu. A) UPLC chromatogram at 203 nm, B) UPLC chromatogram of standard mixture at 203 nm, C) Negative ion mode MS spectra, D) Positive ion mode MS spectra.

group of YQFM-Bu. All of the YQFM and YQFM-Bu groups exhibited a significant increase in the serum SOD activity compared to the ISO group ($P < 0.05$).

Identification of main components in YQFM-Bu by UPLC-MS/MS

UPLC–MS/MS was developed to identify potential bioactive compounds from YQFM-Bu. The main chemical constituents were identified through comparing their retention time (t_R) and MS spectra with reference standards and the data in related studies. The UV chromatograms at 203 nm and MS TIC chromatograms of YQFM-Bu are presented in Fig. 2. The MS data and identification results are presented in Table 5. Peaks 1, 2, 3, 10, 17, 27, and 28 were each unequivocally identified by comparison with the respective authentic compound. The other 21 peaks were identified tentatively by comparing their molecular weight and structural information from MS/MS spectra with data in related studies (9 – 12).

Discussion

Oxidative stress has been implicated in the pathogenesis of many cardiovascular diseases. Many studies have confirmed that ISO-induced myocardial injury is mainly caused by oxygen stress and numerous drugs exerted their beneficial effects on myocardial injury mainly through anti-oxidative damage (13 – 16). Free radicals play an important role in myocardial oxidative damage. During reperfusion or reoxygenation of the ischemic tissue, free radicals are formed, which can cause cytomembrane damage, protein oxidation, and DNA injury. Ultimately, this will lead to myocardial cell necrosis and tissue injury. In cellular oxidation reactions, superoxide radical is normally formed first. It can generate other types of free radicals and cause cell-damaging. Therefore scavenging superoxide free radicals might be beneficial in the treatment of ISO-induced myocardial oxidative damage (17 – 21). In our experiment, superoxide anion was generated by pyrogallol and oxygen and then measured by detection of chemiluminescence. Our result shows that both YQFM and YQFM-Bu have a good effect on scavenging free radicals.

Table 5. Identification of main components in YQFM-Bu by UPLC–MS/MS

Peak No.	Retention time (min)	M	Negative ions (m/z)	Positive ions (m/z)	Identification
1	5.215	800	799[M-H]$^-$ 845[M+HCOO]$^-$	801[M+H]$^+$ 621[M+H-Glc-H_2O]$^+$ 603[M+H-Glc-2H_2O]$^+$ 441[Aglycone+H-3H_2O]$^+$ 423[Aglycone+H-4H_2O]$^+$ 405[Aglycone+H-5H_2O]$^+$	Ginsenoside Rg1*
2	5.215	946	945[M-H]$^-$ 991[M+HCOO]$^-$	911[M+H-2H_2O]$^+$ 767[M+H-Glc-H_2O]$^+$ 749[M+H-Glc-2H_2O]$^+$ 441[Aglycone+H-3H_2O]$^+$ 423[Aglycone+H-4H_2O]$^+$ 405[Aglycone+H-5H_2O]$^+$	Ginsenoside Re*
3	8.055	800	799[M-H]$^-$ 845[M+HCOO]$^-$	823[M+Na]$^+$ 603[M+H-Glc-2H_2O]$^+$ 585[M+H-Glc-3H_2O]$^+$ 441[Aglycone+H-3H_2O]$^+$ 423[Aglycone+H-4H_2O]$^+$ 405[Aglycone+H-5H_2O]$^+$	Ginsenoside Rf*
4	8.623	770	769[M-H]$^-$ 815[M+HCOO]$^-$ 805[M+Cl]$^-$	735[M+H-2H_2O]$^+$ 441[Aglycone+H-3H_2O]$^+$ 423[Aglycone+H-4H_2O]$^+$ 405[Aglycone+H-5H_2O]$^+$	Notoginsenoside R2
5	8.948	1241	619[M-2H]$^{2-}$ 766[M-H-Xyl-2Glc-H_2O]$^-$		Ra3/Notoginsenoside R4
6	9.301	784	783[M-H]$^-$ 829[M+HCOO]$^-$	749[M+H-2H_2O]$^+$ 441[Aglycone+H-3H_2O]$^+$ 423[Aglycone+H-4H_2O]$^+$ 405[Aglycone+H-5H_2O]$^+$	20(S)-Ginsenoside Rg2
7	9.495	638	637[M-H]$^-$ 683[M+HCOO]$^-$	621[M+H-H_2O]$^+$ 603[M+H-2H_2O]$^+$ 441[Aglycone+H-3H_2O]$^+$ 423[Aglycone+H-4H_2O]$^+$ 405[Aglycone+H-5H_2O]$^+$	20(S)-Ginsenoside Rh1
8	9.702	784	783[M-H]$^-$ 829[M+HCOO]$^-$	749[M+H-2H_2O]$^+$ 441[Aglycone+H-3H_2O]$^+$ 423[Aglycone+H-4H_2O]$^+$ 405[Aglycone+H-5H_2O]$^+$	20(R)-Ginsenoside Rg2
9	9.838	638	683[M+HCOO]$^-$	621[M+H-H_2O]$^+$ 603[M+H-2H_2O]$^+$ 441[Aglycone+H-3H_2O]$^+$ 423[Aglycone+H-4H_2O]$^+$ 405[Aglycone+H-5H_2O]$^+$	20(R)-Ginsenoside Rh1
10	9.986	1108	1107[M-H]$^-$ 1153 [M+HCOO]$^-$ 783[M-H-2Glc]$^-$ 621[M-H-3Glc]$^-$	767[M+H-2Glc-H_2O]$^+$ 443[Aglycone+H-2H_2O]$^+$ 425[Aglycone+H-3H_2O]$^+$ 407[Aglycone+H-4H_2O]$^+$	Ginsenoside Rb1*
11	10.367	1078	1077[M-H]$^-$ 1123 [M+HCOO]$^-$ 946[M-H-Xyl]$^-$ 538[M-2H]$^{2-}$	1179[M+H]$^+$ 443[Aglycone+H-2H_2O]$^+$ 425[Aglycone+H-3H_2O]$^+$ 407[Aglycone+H-4H_2O]$^+$	Ginsenoside Rc
12	10. 732	1078	1077[M-H]$^-$ 1123[M+HCOO]$^-$ 1141[M-H-2H_2O]$^-$	1079[M+H]$^+$ 443[Aglycone+H-2H_2O]$^+$ 425[Aglycone+H-3H_2O]$^+$ 407[Aglycone+H-4H_2O]$^+$	Ginsenoside Rb2
13	10.828	1078	1077[M-H]$^-$ 1123[M+HCOO]$^-$ 538[M-2H]$^{2-}$	1079[M+H]$^+$ 443[Aglycone+H-2H_2O]$^+$ 425[Aglycone+H-3H_2O]$^+$ 407[Aglycone+H-4H_2O]$^+$	Ginsenoside Rb3
14	11.137	1194	1149[M-H-CO_2]$^-$	443[Aglycone+H-2H_2O]$^+$ 425[Aglycone+H-3H_2O]$^+$ 407[Aglycone+H-4H_2O]$^+$	Malony-Ginsenoside Rb1/isomer

Peak No.	Retention time (min)	M	Negative ions (m/z)	Positive ions (m/z)	Identification
15	11.214	946	945[M-H]$^-$ 991[M+HCOO]$^-$	947[M+H]$^+$ 443[Aglycone+H-2H_2O]$^+$ 425[Aglycone+H-3H_2O]$^+$ 407[Aglycone+H-4H_2O]$^+$	Ginsenoside Rd
16	11.433	1164	1119[M-H-CO_2]$^-$	443[Aglycone+H-2H_2O]$^+$ 425[Aglycone+H-3H_2O]$^+$ 407[Aglycone+H-4H_2O]$^+$	Malony-Ginsenoside Rb2/Rb3/Rc
17	11.959	432		433[M+H]$^+$ 415[M+H-H_2O]$^+$ 384[M+H-H_2O-CH_3O]$^+$	Schizandrol A*
18	12.916	766	765[M-H]$^-$ 811[M+HCOO]$^-$	767[M+H]$^+$ 603 [M+H-Rha-H_2O]$^+$ 441[Aglycone+H-3H_2O]$^+$ 423[Aglycone+H-4H_2O]$^+$	Ginsenoside Rg6
19	13.223	766	765[M-H]$^-$ 811[M+HCOO]$^-$	603 [M+H-Rha-H_2O]$^+$ 441[Aglycone+H-3H_2O]$^+$ 423[Aglycone+H-4H_2O]$^+$ 405[Aglycone+H-5H_2O]$^+$	Ginsenoside F4
20	13.372	620	619[M-H]$^-$ 665[M+HCOO]$^-$	621[M+H]$^+$ 441[Aglycone+H-3H_2O]$^+$ 423[Aglycone+H-4H_2O]$^+$	Ginsenoside Rk3/Rh4
21	13.779	620	619[M-H]$^-$ 665[M+HCOO]$^-$	621[M+H]$^+$ 441[Aglycone+H-3H_2O]$^+$ 423[Aglycone+H-4H_2O]$^+$	Ginsenoside Rh4/Rk3
22	15.193	784	783[M-H]$^-$ 829[M+HCOO]$^-$	785[M+H]$^+$ 767[M+H-H_2O]$^+$ 749[M+H-2H_2O]$^+$ 731[M+H-3H_2O]$^+$ 587[M+H-Glc-2H_2O]$^+$ 569[M+H-Glc-3H_2O]$^+$ 443[Aglycone+H-2H_2O]$^+$ 425[Aglycone+H-3H_2O]$^+$ 407[Aglycone+H-4H_2O]$^+$	20(S)-Ginsenoside Rg3
23	15.441	784	783[M-H]$^-$ 829[M+HCOO]$^-$	785[M+H]$^+$ 767[M+H-H_2O]$^+$ 749[M+H-2H_2O]$^+$ 605[M+H-Glc-H_2O]$^+$ 587[M+H-Glc-2H_2O]$^+$ 443[Aglycone+H-2H_2O]$^+$ 425[Aglycone+H-3H_2O]$^+$ 407[Aglycone+H-4H_2O]$^+$	20(R)-Ginsenoside Rg3
24	16.987	826	825[M-H]$^-$ 871[M+HCOO]$^-$	791[M+H-2H_2O]$^+$ 443[Aglycone+H-2H_2O]$^+$ 425[Aglycone+H-3H_2O]$^+$ 407[Aglycone+H-4H_2O]$^+$	Ginsenoside Rs3/Isomer
25	17.386	766	765[M-H]$^-$ 811[M+HCOO]$^-$	767[M+H]$^+$ 749[M+H-H_2O]$^+$ 731[M+H-2H_2O]$^+$ 587[M+H-Glc-H_2O]$^+$ 443[Aglycone+H-2H_2O]$^+$ 425[Aglycone+H-3H_2O]$^+$ 407[Aglycone+H-4H_2O]$^+$	Ginsenoside Rk1/ Rg5
26	17.582	766	765[M-H]$^-$ 811[M+HCOO]$^-$	767[M+H]$^+$ 749[M+H-H_2O]$^+$ 731[M+H-2H_2O]$^+$ 587[M+H-Glc-H_2O]$^+$ 443[Aglycone+H-2H_2O]$^+$ 425[Aglycone+H-3H_2O]$^+$ 407[Aglycone+H-4H_2O]$^+$	Ginsenoside Rk1/Rg5
27	18.574	416		417[M+H]$^+$	Schizandrin A*
28	19.141	400		401[M+H]$^+$	Schizandrin B*

Glc for Glucose, Xyl for Xylose, Rha for Rhamnose, M for molecular mass. *Compared with authentic compounds.

LDH and CK play important roles in energy metabolism of myocardial tissue and they are usually assayed in the blood as a biomarker for myocardial oxidative damage. LDH is an enzyme playing an important role in glycolysis, which can catalyze the mutual conversion of lactate and pyruvate. CK is a key enzyme for aerobic metabolism of cells, which can catalyze the conversion of creatine to phosphocreatine. When the myocardium is injured due to ISO-induced oxidative stress, these enzymes can be released to the serum from cells. The activities of these enzymes in serum can be used as surrogate marker of myocardium injury (22 – 24). In our experiment, both YQFM and YQFM-Bu can decrease the activity of LDH and CK compared with the ISO group, which suggested that both of them can protect myocardial tissue from damage caused by ISO.

SOD is a very important antioxidant enzyme, which can clear away superoxide anion radicals, prevent cellular damage, and maintain the oxidation and antioxidant balance. MDA is a product of lipid peroxidation. It has the function of interfering with cell proliferation and protein expression through reacting with the free amino groups of proteins and nucleic acids to cross-link biological macromolecules (25, 26). MPO is a basic heme protein which is expressed abundantly in neutrophils and monocytes. It can utilize chloride and H_2O_2 to generate reactive oxidants and free radicals that oxidatively modify lipids and proteins (27 – 29). The damage of an organism introduced by myocardial oxidative damage can cause elevation of MPO activity, decrease of SOD activity, and increased levels of MDA. In our study, both YQFM and YQFM-Bu substantially attenuated the increase in MDA production, MPO activity, and reversed the decrease of SOD activity.

These results suggested that both YQFM and YQFM-Bu have beneficial effects against myocardial oxidative damage by attenuating oxidative stress.

Myocardial hypertrophy was induced using chronic adrenoceptor stimulation in mice, which involves many similarities with human heart failure. ISO is known to induce cardiac hypertrophy in mice which is an easy and standardized model to study the effects of many drugs on cardiac function (30). From the present study, both YQFM and YQFM-Bu have the cardioprotective potential against ISO-induced myocardial hypertrophy.

The myocardial tissue damage resulting in ISO-induced myocardial oxidative damage can be visually detected by observing pathological slices. The pathological score is often used as a quantitative analysis of a pathological section; myocardial pathological scores can reflect the severity of myocardial oxidative damage. From the histopathological examination results, we found that both YQFM and YQFM-Bu could significantly restore myocardial tissue pathological damage.

From the UPLC-MS/MS experiment results, we identified 25 ginsenosides and 3 lignans in YQFM-Bu through comparing their retention time (t_R) and MS spectra with reference standards and the data in related studies.

Many pharmacological investigations have revealed that some of ginsenosides and lignans can exert preventative effects on myocardial oxidative damage. Ginsenosides can protect against myocardial damage by decreasing lipid peroxidation (31). Ginseng can shorten the action potential duration (APD) by inhibiting L-type Ca^{2+} current ($I_{Ca\text{-}L}$) and enhancing the slowly activating delayed rectifier K^+ current (I_{Ks}) for cardiovascular protection against ischemia–reperfusion injury (32). Ginsenoside Re appears to be one of the main electrophysiological actions of ginseng in the heart. It can inhibit Ca^{2+} accumulation in mitochondria during cardiac ischemia/reperfusion, which is attributable to NO-induced Ca^{2+} channel inhibition and K^+-channel activation in cardiac myocytes (33). It also can activate endothelial NO synthase (eNOS) to release NO, resulting in activation of I_{Ks} (34). Some papers had reported that ginsenoside Re can protect cardiomyocytes from oxidant injury induced by both exogenous and endogenous oxidants through scavenging H_2O_2 and hydroxyl radicals (35). Ginsenoside Rb1 has been shown to protect against myocardial–reperfusion injury by enhancing the expression of eNOS and increasing the content of NO as well as inhibiting oxidative stress and protecting the endothelial cells (36, 37). Ginsenoside Rg1 has been reported to promote endogenous NO production, prolong ventricular refractoriness and repolarization, and inhibit left ventricular (LV) hypertrophy in rats (38). Ginsenoside Rb3 has a protective effect against ISO-induced myocardial injury and heart function impairment (39). Ginsenoside Rg2 has the ability to protect myocardial cells against anoxia by inhibiting cell apoptosis, increasing the energy of antioxidase, and eliminating free radicals (40). It was also found that lignan compounds exerted beneficial effects on the cardiovascular system (41). Schizandrol A was an effective scavenger for oxygen radicals such as superoxide anion and hydroxyl radical (42). Schisandrin B can enhance antioxidant capacity of heart tissue by significantly increasing the activity of glutathione peroxidase, glutathione reductase, glutathione transferase, and SOD (43). In our present study, all of these ginsenosides and lignans have been detected in YQFM-Bu. Based on previous publications and our experiment results, we propose that the ginsenosides and lignans in YQFM-Bu can potentially serve as the main active chemicals of YQFM responsible for the protective effect of myocardial oxidative damage.

All these investigations and results also suggested that YQFM may exhibit anti-oxidant effects for myocardial injury by enhancing the expression of eNOS, increasing the content of NO, and scavenging free radicals.

In conclusion, the present study was performed to investigate the protective effects and potential active components of YQFM for the treatment of myocardial oxidative damage. Our results indicated that both YQFM and YQFM-Bu significantly restored myocardial damage induced by ISO in mice. YQFM exerted its beneficial effects on myocardial injury mainly through inhibiting oxidative damage caused by stresses and by maintaining the functional integrity of myocardial tissue; Our result suggested YQFM-Bu is the major active fraction of YQFM with the ginsenosides and lignans as potential active components responsible for its protective effect against myocardial injury. For future studies, we will continue to investigate the biological activity of each chemical identified in YQFM-Bu for the treatment of myocardial oxidative damage and elucidate mechanisms, other than anti-oxidant effects, which may contribute to the protective effect of YQFM on myocardial injury.

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