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Optimisation of extraction procedure for black fungus polysaccharides and effect of the polysaccharides on blood lipid and myocardium antioxidant enzymes activities

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ABSTRACT

Optimal conditions for the extraction of black fungus polysaccharides were 350 W, 5, 35 min and 90 °C, for ultrasonic power, ratio of water to sample, extraction time and extraction temperature, respectively. Gas chromatography (GC) analysis showed that black fungus polysaccharides contained glucose, xylose, mannose and ribose. Their molar percentages were 6.8%, 34.2%, 50.7% and 8.9%, respectively. FT-IR and NMR analysis showed typical chemical structure of black fungus polysaccharides. In animal experiment, high fat diet feeding for 29 days markedly reduced myocardium and blood antioxidant enzyme activities and enhanced lipid peroxidation level. Administration of black fungus polysaccharides had significantly enhanced myocardium and blood antioxidant enzyme activities and reduced lipid peroxidation level in high fat mice. Our results indicated that black fungus polysaccharides could be beneficial for protection against cardiovascular diseases and its complications.

1. Introduction

Cardiovascular diseases (CVDs) are the leading causes of disability and death in industrialized nations and much of the developing world. Over the past three decades it has become clear that the onset and progression of atherosclerosis, the pathological basis of CVD, result from a combination of abnormalities in lipoprotein metabolism, oxidative stress and chronic inflammation (Hansson, 2005). A number of risk factors have been associated with the occurrence of CVD including high blood concentrations of total cholesterol (TC), triglycerides (TG) and homocysteine, low HDL-cholesterol (HDL-C), hypertension, obesity and diabetes (Lusis, 2000). In line with the oxidation hypothesis, dietary antioxidants are increasingly recognized as potentially important factors in the prevention of cardiovascular disease. Epidemiological studies suggest that a high intake of dietary antioxidants such as vitamin E, β-carotene and vitamin C is associated with a reduced risk of cardiovascular disease (Gey, Brubacher, & Stahelin, 1987; Kardinaal et al., 1993; Rimm et al., 1993). Recent observations suggest that potentially beneficial effects may not be limited to these well-known antioxidants. High intake of flavonoids from tea and vegetables was also associated with a reduced risk of coronary heart disease (Hertog, Feskens, Hollman, Katan, & Kromhout, 1993).Due to their interesting biological activities, mushrooms have recently become an attractive source material for the development of pharmaceutical products (VanCott et al., 1996; Adebayo-Tayo et al., 2010; Mavundza et al., 2010). Many polysaccharides have been isolated from mushrooms, fungi, yeast, algae, lichens, and plants in recent years, and screened for biological activity (Murata, Shimamura, Tagami, Takatsuki, & Hamuro, 2002; Markova et al., 2003). Most polysaccharides derived from plants are relatively nontoxic and do not cause significant side effects. These could allow development of an effective natural anticancer with few side effects. The mushroom black fungus, belonging to heterobasidiae of basidiomycetes and also called Jew's ear, wood ear, red ear, black tree fungus or ear fungus, is frequently consumed as a food and a traditional medicine in the far east. Its nutritional value and taste components have been investigated (Blinova et al., 2003; Vattem & Shetty, 2003), and a few studies have reported its biological activity and active substances. Lentinan, a polysaccharide from the Shiitake mushroom (*Lentinula edodes*), has been demonstrated to have strong activity (Djordjevic et al., 2009; Feng et al., 2010; Vattem & Shetty, 2003).

Ultrasonic-assisted extraction (UAE) is an expeditious, inexpensive and efficient alternative to traditional extraction techniques and, in some cases, even to supercritical fluid and microwave-assisted extraction, which has been demonstrated by application to both organic and inorganic analytes in a wide variety of samples (Jalbani et al., 2006). Therefore, ultrasonic treatment is widely used in the fractionation of plant materials (Riera et al.,

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2010) and well established in the processing of plant materials, particularly, for extracting low molecular substances (Banjoo & Nelson, 2005; KaŽys & Svilainis, 1997; Salisova, Toma, & Mason, 1997).

In this work, an ultrasonically assisted extraction technique was utilized for the extraction of polysaccharides from black fungus by a response surface methodology design. The present study was still designed to investigate the efficacy of black fungus polysaccharides as sources of water-soluble antioxidants on myocardium oxidative injury in cholesterol-fed mice. This study will allow us to ascribe antiatherogenic effects to antioxidant properties of the intervention.

2. Materials and methods

2.1. Plant material

Black fungus was purchased from a herb shop, Shanghai city, China. These black fungi originally grew in Shandong province, China. The plant material was identified at the department of pharmacology, Phd Hong where a voucher specimen 20100326 was deposited. The medicine was dried at room temperature and ground in a rotary mill and then sieved (60 mesh).

2.2. Preparation of black fungus polysaccharides

Black fungus polysaccharides (100 g) were ground into fine powder (60 mesh). The extraction was performed using an ultrasonic cleaner (SB-5200DTD, Xinzhi Biotech Co., Ningbo, China, 40 kHz), using selected ultrasonic power and temperature for various durations. 10 g dry sample powders were extracted by immersing in water at a selected ratio, then heating in water at selected temperature for various periods of time. The supernatant was collected for the determination of polysaccharides yield.

2.3. Box–Behnken design

According to the principle of Box–Behnken design, extraction temperature, extraction time, ratio of water to sample and extraction number, which were identified to have strong effects on the response in preliminary one-factor-at-a-time experiments (Martendal, Budziak, & Carasek, 2007), were taken as the variables tested in a 27-run experiment to determine their optimum levels. As shown in Table 1, the four factors chosen for this study were designated as X_1, X_2, X_3, X_4 and prescribed into three levels, coded +1, 0, −1 for high, intermediate and low value, successively. Three test variables were coded according to the following equation (1):

$$x_i = \frac{(X_i - X_0)}{\Delta X} \quad i = 1, 2, 3 \tag{1}$$

where x_i is the coded value of an independent variable; X_i is the actual value of an independent variable; X_0 is the actual value of an independent variable at centre point; ΔX is the step change value of an independent variable.

2.4. Analysis of carbohydrate composition

The polysaccharides sample (2 mg) was hydrolysed in 2 ml of 2 M trifluoroacetic acid (TFA) at 110 °C for 2 h. A small portion of the residue was subjected to thin layer chromatography (TLC) analysis, and the remaining portion was transformed into the corresponding alditol acetates, which was analyzed by GC (Shimadzu, Kyoto, Japan) on a HP-5 chromosorb column and detected by a flame ionization detector (temperature 250 °C). The column temperature was increased from 170 to 215 °C in a rate of 2 °C/min and then 8 °C/min to 250 °C (Dong, Yao, & Fang, 2003).

Table 1
Experimental design and response values.

RUN	X_1	X_2	X_3	X_4	Y_1
1	−1 (350 W)	−1 (4)	0 (35 min)	0 (90 °C)	15
2	−1	1 (6)	0	0	17.2
3	1 (450 W)	−1	0	0	17.5
4	1	1	0	0	18.3
5	0 (400 W)	0 (5)	−1 (30 min)	−1 (80 °C)	16
6	0	0	−1	1 (100 °C)	16.2
7	0	0	1 (40 min)	−1	16.1
8	0	0	1	1	16
9	−1	0	0	−1	16.2
10	−1	0	0	1	16.3
11	1	0	0	−1	18
12	1	0	0	1	18
13	0	−1	−1	0	16.6
14	0	−1	1	0	16.7
15	0	1	−1	0	17.5
16	0	1	1	0	17.6
17	−1	0	−1	0	14.8
18	−1	0	1	0	16.8
19	1	0	−1	0	17.5
20	1	0	1	0	17.8
21	0	−1	0	−1	16.4
22	0	−1	0	1	16.3
23	0	1	0	−1	17.6
24	0	1	0	1	17.8
25	0	0	0	0	19.2
26	0	0	0	0	19.6
27	0	0	0	0	19.5

2.5. FT-IR spectroscopy

FT-IR was analyzed using the KBr disc for detecting functional groups of black fungus polysaccharides.

2.6. NMR spectroscopy

Samples were dissolved in D_2O (99.96% of atom), filtered through a 0.45-μm syringe filter, and freeze–dried to remove exchangeable protons. After exchanging the samples three times by freeze–drying from D_2O, samples were transferred to Shigemi tubes for analyses. One-dimensional (1D) ^1H NMR experiments were performed on a Varian 500 MHz VXR-500 spectrometer equipped with 5-mm triple resonance tunable probe with standard Varian software at 279, 298 and 313 K.

2.7. Animals and dietary treatment

Thirty kunming mice weighing 16 ± 1 g were housed in stainless steel cages in a room with controlled lighting (12-h light:dark cycle), constant temperature (24 °C) and relative humidity (60%). The animals were randomly divided into four groups of 10 each and fed a different diet for 4 weeks, as follows: one group fed a diet containing 1% cholesterol and 0.5% cholic acid, i.e. high cholesterol diet (HCD) and the other group fed the same diet supplemented with black fungus polysaccharides (0.6% and 1.2%). Another mice fed with basic diet and served as control. Diets and tap water were freely available. The animals were weighed weekly. We followed the general guidelines on the use of living animals in scientific investigations (Council of European Communities, 1986).

2.8. Antioxidant enzyme measurements

On day 29, the mice were fasted overnight, killed and blood and heart samples were collected. Then, it was centrifuged at 3000 × *g* for 15 min at 4 °C to obtain the serum for the measurement of TG, TC, HDL-c and LDL-c levels, according to the commercial instructions

for the automatic biochemical analyser (Biochemical analytic Center of Maigaoqiao Hospital, Nanjing, China).

Lipid peroxidation was estimated by measuring thiobarbituric acid-reactive substances (TBARS) and expressed in terms of malondialdehyde (MDA) content, according to the method of Draper and Hadley (1990). Reduced glutathione levels (GSH) were determined by Ellman method (1959) modified by Jollow, Mitchell, Zamppaglione, and Gillette (1974).

Superoxide dismutase activity was measured at 412 nm by the NADH oxidation procedure (Elstner, Youngman & Obwald, 1983). Glutathione peroxidase was determined by the method of Paglia and Valentine (1967) using cumene hydroperoxide as substrate. Catalase activity was determined by the method of Aebi (1974) by measuring the rate of decomposition of H_2O_2 at 240 nm.

2.9. Statistical analysis

Results are expressed as means ± standard deviations (SD). Significant differences among the groups were determined by one-way ANOVA with Duncan's multiple range test. Differences were considered significant if $P < 0.05$.

3. Results and discussion

3.1. Effect of different extraction parameters on extraction yield of the polysaccharides

As shown in Fig. 1A, ultrasonic power of 400 W is favourable for the extraction of the polysaccharides. As shown in Fig. 1B, the extraction time of 35 min was enough to obtain maximum extraction yield of the polysaccharides. As shown in Fig. 1C, 5 times volume of water was proper for extraction of this polysaccharides. As shown in Fig. 1D, extraction yield did not markedly increased when temperature was between 90 °C and 100 °C. Therefore, high extraction yield can be achieved with the increase of extraction temperature.

Table 2
Analysis of variances in the regression model for optimisation of polysaccharide extraction from black fungus .

	Master model	Predictive model
Mean	17.12963	17.12963
R-Square	93.55%	89.01%
Adj. *R*-square	86.03%	85.71%
RMSE	0.454377	0.459619
CV	2.652578	2.683184

3.2. Optimisation of extraction process

After the RSREG procedure, the regression equation was given as follows:

$$Y_1 = 19.43333 + 0.9 \times X_1 + 0.625 \times X_2 - 1.133333 \times X_1 \times X_1 - 0.995833 \times X_2 \times X_2 - 1.608333 \times X_3 \times X_3 - 1.445833 \times X_4 \times X_4 \quad (2)$$

The significance of each coefficient in Eq. (2) was determined using the Student's *t*-test and *p* value as shown in Table 2. It was evident that the linear coefficients (ultrasonic power, ratio of water to sample), and four quadratic coefficients (ultrasonic power, ratio of water to sample, extraction temperature and extraction time) were significant ($p < 0.05$), while all the cross product coefficients were insignificant ($p > 0.5$). These results suggest that ultrasonic power and ratio of water to sample were the most important factors because it affected the polysaccharides extraction the most ($p < 0.01$).

It is evident that the model was highly significant, as was evident from the model *F*-value and a very low probability value (*P* model, $F < 0.0001$). The goodness of the model could be checked by the determination coefficient R^2 (0.9355) and the multiple correlation coefficient *R* (0.8603). The closer the values of *R* (multiple correlation coefficient) to 1, the better the correlation between the experimental and predicted values (Lin, Yang, Hsu, Hsu, & Chang, 2006; Liu, Miao, Wen, & Sun, 2009). Here, the value of *R* (0.9355) indicated good agreement between the experimental and predicted values of extraction yield of polysaccharides.

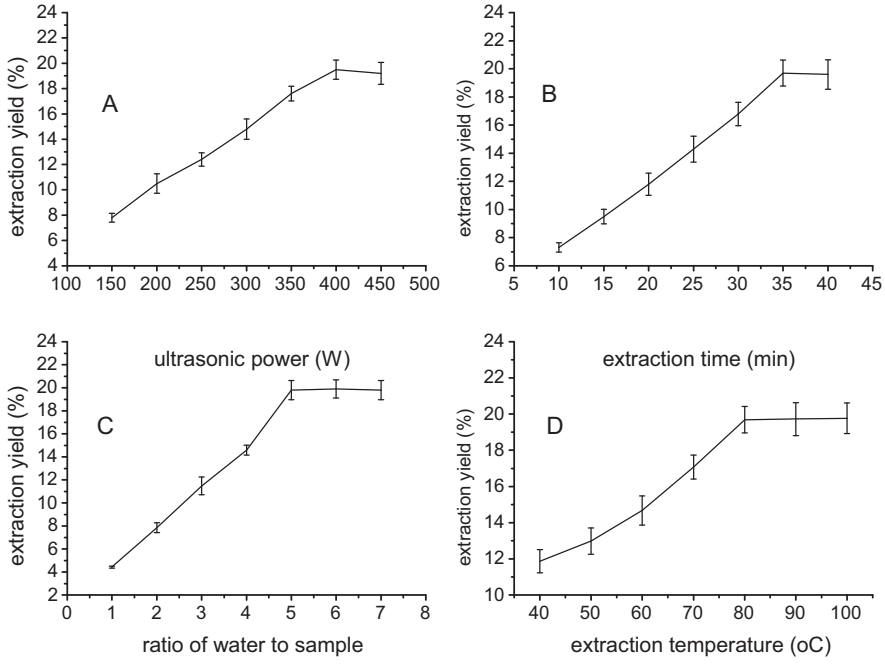

Fig. 1. Effect of different extraction parameters on extraction yield of the polysaccharides.

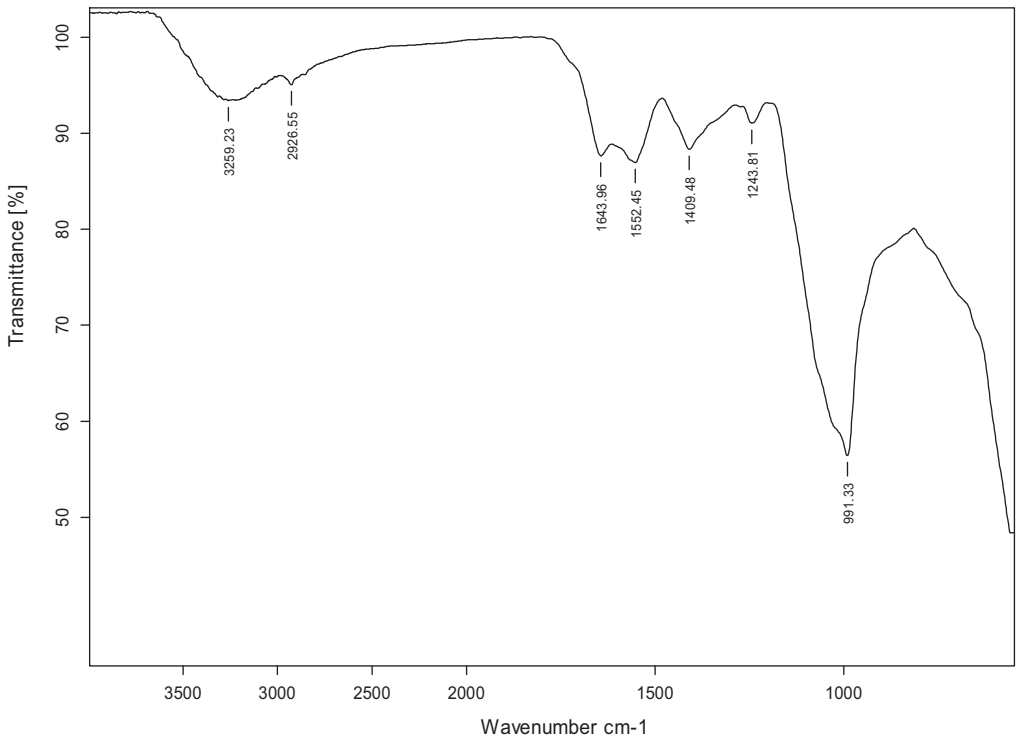

Fig. 2. FT-IR spectroscopy of black fungus polysaccharides.

3.3. Chemical composition and structure of black fungus polysaccharides

The purified black fungus polysaccharides were hydrolysed by TFA into individual monosaccharides that were further trimethylsilylated for gas chromatography analysis. The results showed that four monosaccharides, including glucose, xylose, mannose and ribose, were identified after comparison with the monosaccharide standards. Their molar percentages were 6.8%, 34.2%, 50.7% and 8.9%, respectively.

Fig. 2 shows that the most important wavenumbers related to the variability of black fungus polysaccharides were the bands located at 3259, 2926, 1643, 1552, 1409, 1243, and 991 cm^{-1}. The range (1243–1409 cm^{-1}) is O–H-group vibrations. The frequencies (1243–991 cm^{-1}) were polysaccharides with mannose, glucose and xylose, constituents. The band at 2926 cm^{-1} is associated with the vibrations of C–H bond. The band at 991 cm^{-1} is associated with the presence of β-pyran ring, which indicated the presence of β glucosidic bond in black fungus polysaccharides.

The signals of ^{1}H NMR were 5.05 (α-C-1), 4.73 ppm (β-C-1), 3.64 ppm (C-5), 3.52 ppm (C-4), 3.56 ppm (C-3), and 3.43 ppm (C-2) and are shown in Fig. 3A. On the basis of these results, the polysaccharides has been determined to be a novel biomolecule combined by α- and β-linkages.

The signals identified at 95, 75, 73 and 63 ppm in the ^{13}C spectra of black fungus polysaccharides could be assigned to C-1, C-4 and C-6 of α-D-mannose (Fig. 3B). The signals identified at 74, 57 and 56 ppm could be assigned to C-1, C-5 and C-6 of β-D-glucose. Based on the data available in the literature, it was possible to identify that the resonances in the region of 75–95 ppm were attributed to the anomeric carbon atoms of glucopyranose (Glcp) and xylopyranose (Xylp), respectively.

3.4. Inhibition of black fungus polysaccharides against oxidative injury in high fat mice

Medicinal mushroom extracts have been considered as important remedies for the prevention and treatment of many diseases for thousands of years especially in the Orient (Israilides & Philippoussis, 2003; Kidd, 2000; Wasser & Weis, 1999; Matsuo et al., 1996; Djordjevic et al., 2009). A plethora of medicinal effects has been demonstrated for many traditionally used mushrooms including antibacterial, antiviral, antifungal, antitumour and immuno-potentiating activities (Hobbs, 2003; Ooio & Liu, 1999). Among the various bioactive components which have been demonstrated to be most effective as antitumour and immunomodulatory agents are polysaccharides and polysaccharopeptides. A lot of *Auricularia polytricha* were consumed every year in the East. Furthermore, these edible fungi are also well known for its multiple pharmacological effects. It has been reported that *A. polytricha* could suppress platelet aggregating (Hokama & Hokama, 1981), modulate immune function (Sheu, Chien, & Chien, 2004; Hu et al., 2009; Shuai et al., 2010), exhibit antinociceptive (Koyama, Akiba, & Imaizumi, 2002) and antioxidative effect (Mau, Chao, & Wu, 2001). In addition, previous study showed that black fungus polysaccharides treatment can reduced blood lipid level (Han & Xu, 2007; Oyedemi et al., 2009).

MDA and GSH levels of BFP-treated and untreated high fat mice are presented in Figs. 4 and 5. Compared with normal control, MDA level and increased GSH level in myocardium and blood were markedly increased and decreased 29 days after high fat diet was fed. Black fungus polysaccharides treatment significantly decreased MDA level and increased GSH level in myocardium and blood.

Results are given in Fig. 6. As seen from the table, blood TC, TG, LDL-c levels in untreated model control mice were significantly

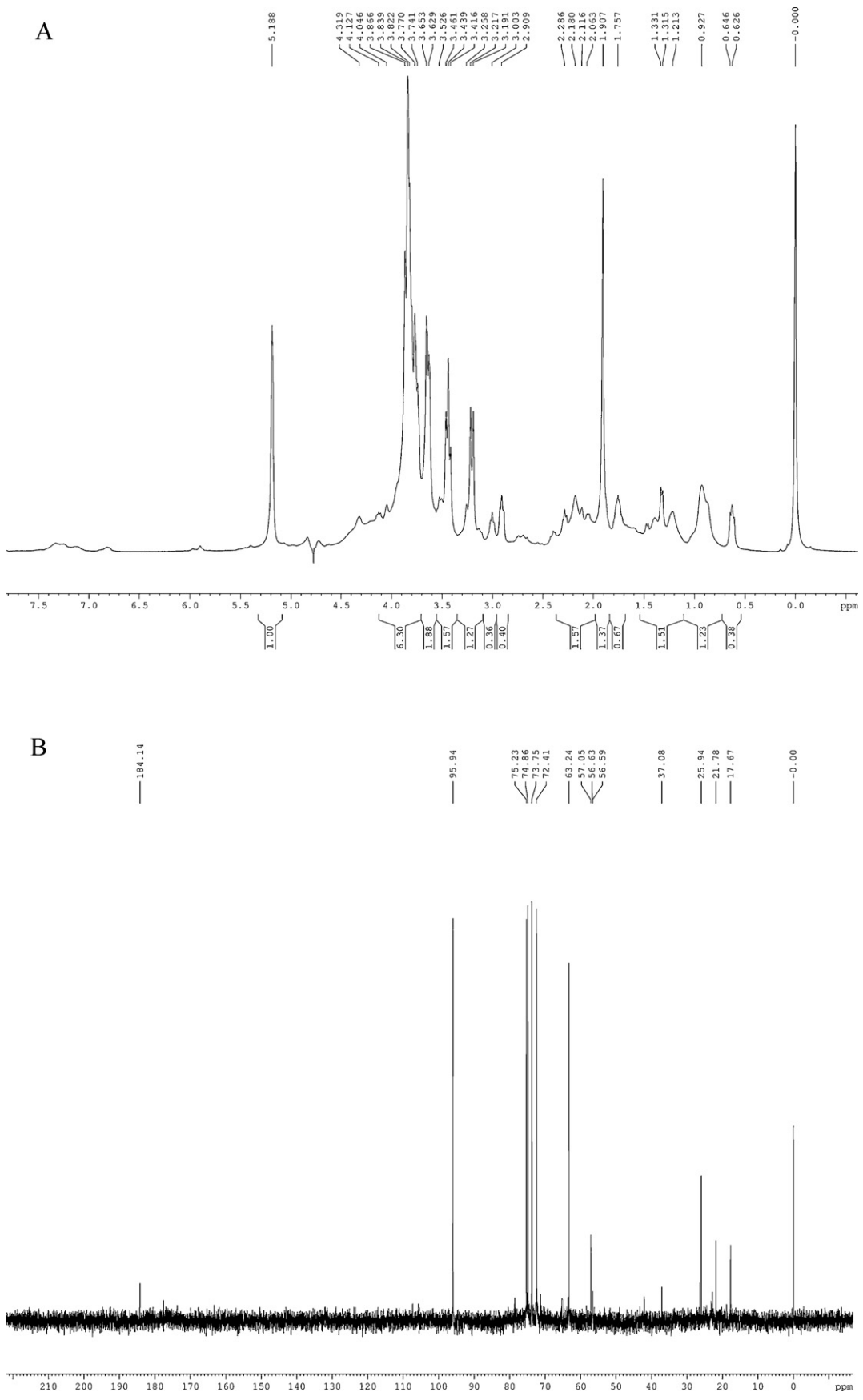

Fig. 3. NMR spectroscopy of black fungus polysaccharides.

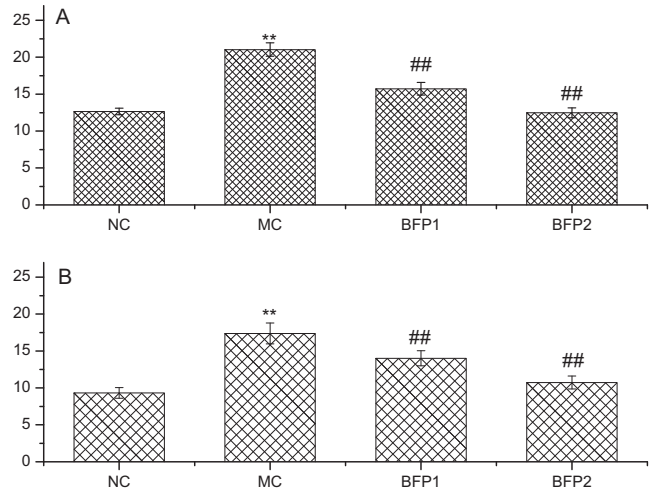

Fig. 4. Black fungus polysaccharides affecting myocardium (A) and blood (B) MDA level. $^{**}P<0.01$, NC group vs MC group; $^{\#\#}P<0.01$, BFP1, BFP2 groups vs MC group.

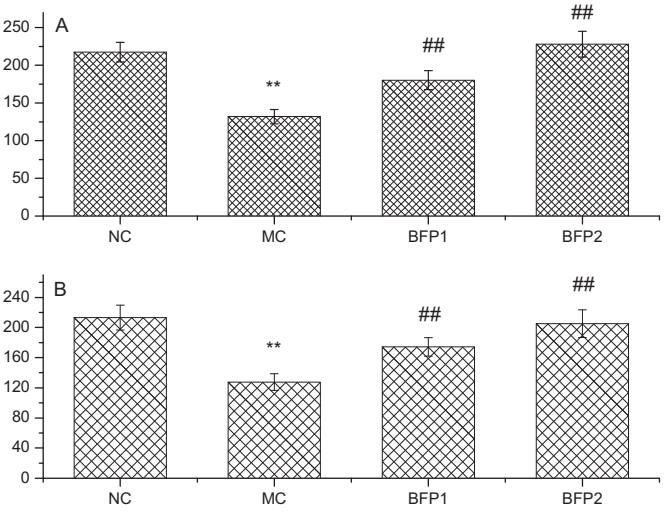

Fig. 5. Black fungus polysaccharides affecting myocardium (A) and blood (B) GSH level. $^{**}P<0.01$, NC group vs MC group; $^{\#\#}P<0.01$, BFP1, BFP2 groups vs MC group.

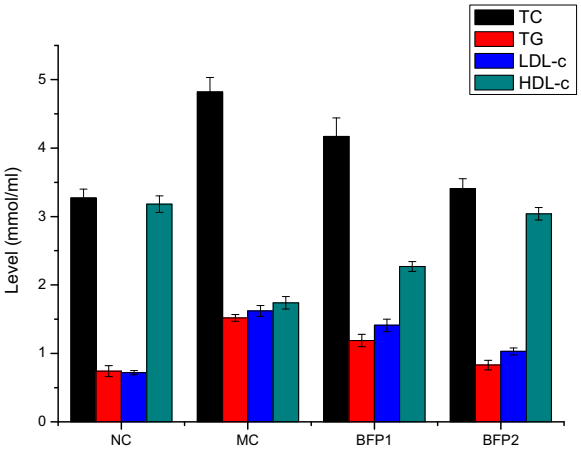

Fig. 6. Black fungus polysaccharides affecting blood TC, TG, LDL-c and HDL-c levels. $^{**}P<0.01$, NC group vs MC group; $^{\#\#}P<0.01$, BFP1, BFP2 groups vs MC group.

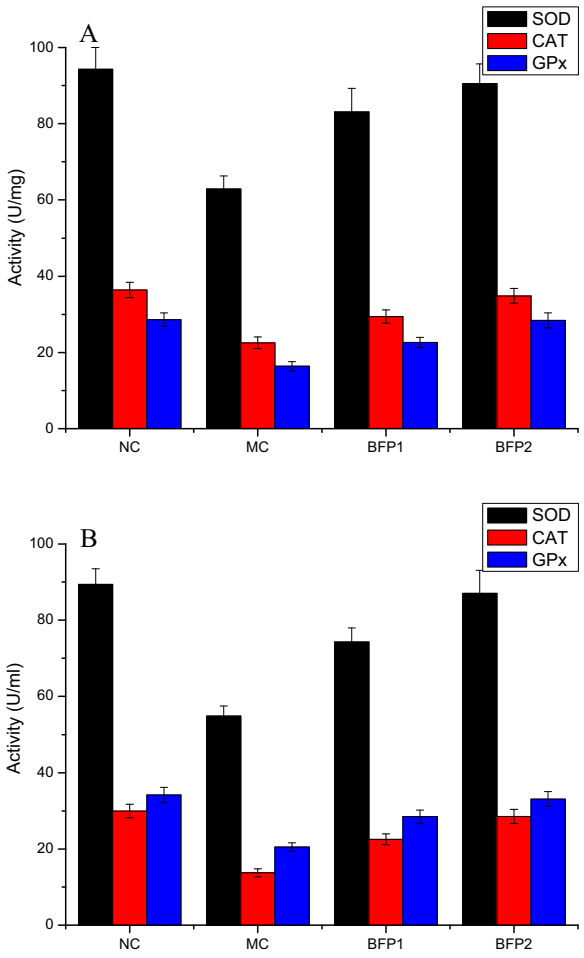

Fig. 7. Black fungus polysaccharides affecting myocardium (A) and blood (B) SOD, CAT and GPx activities. $^{**}P<0.01$, NC group vs MC group; $^{\#\#}P<0.01$, BFP1, BFP2 groups vs MC group.

higher whereas blood HDL-c level was significantly lower than those in the normal control mice. However, blood TC, TG, LDL-c levels were significantly found to be lower in the BFP-treated groups relative to untreated model group. In addition, it has been found that black fungus polysaccharides supplementation significantly enhanced HDL-c level in high fat mice.

The changes in the antioxidant enzyme activities are summarized in Fig. 7. Compared with normal, high fat diet feeding for 29 days significantly reduced myocardium and blood SOD, CAT and GPx activities in untreated model control mice. The myocardium and blood SOD, CAT and GPx activities were significantly increased in the BFP-treated mice compared to the untreated model control group.

Hyperlipidemia is a known risk factor for the development of cardiovascular disease including atherosclerosis. The major risk factors for the development of atherosclerosis are hypercholesterolemia and elevated levels of low-density lipoprotein-cholesterol (LDL-C) (Raza, Babb, & Movahed, 2004). Furthermore, free-radical-mediated peroxidative modification of polyunsaturated fatty acids of LDL and very-low-density lipoprotein (VLDL) is thought to contribute to the progression of atherosclerotic lesions. High fat diet feeding can increase risk of cardiovascular disease (Orsó, Ahrens, Dzenan, & Schmitz, 2009; Aboaba, 2009). Clinical trials have shown that treatment of older high-risk subjects with lipid-lowering drugs can reduce cardiovascular morbidity and mortality (Aronow, 2008). The search for new agents capable of

reducing serum lipid levels has therefore become an important research focus. Our present results had confirmed that black fungus polysaccharides treatment could reduce high-fat-diet-induced oxidative injury in heart tissue. This indicated that black fungus polysaccharides were beneficial for therapy of some cardiovascular diseases.

4. Conclusions

GC analysis showed that black fungus polysaccharides contained glucose, xylose, mannose and ribose. Their molar percentages were 6.8%, 34.2%, 50.7% and 8.9%, respectively. FT-IR and NMR analysis showed typical chemical structure of black fungus polysaccharides. In high fat mice, myocardium antioxidant enzyme activities and lipid peroxidation level were significantly decreased and increased. Black fungus polysaccharides feeding for 29 days significantly enhanced myocardium antioxidant enzyme activities and decreased lipid peroxidation level. The evidence suggests that black fungus polysaccharides could be beneficial for protection against cardiovascular diseases and its complications.

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