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Analysis of chemical composition, structure of *Grifola frondosa* polysaccharides and its effect on skin TNF-α levels, lgG content, T lymphocytes rate and caspase-3 mRNA

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ABSTRACT

The polysaccharide was extracted from *Grifola frondosa* mushroom. The chemical composition of *G. frondosa* polysaccharide was analyzed by the gas chromatography–mass spectroscopy (GC–MS). The *G. frondosa* polysaccharide was found to consist of glucose, mannose and galactose with a molar ratio of 6.5:1:2.6. The structure of *G. frondosa* polysaccharide was characterized by infrared (IR) spectrum, 1D NMR and 2D NMR spectroscopy. The absorption peaks of 1415 cm^{-1}, 1154 cm^{-1} and 851 cm^{-1} suggested the polysaccharide consisted of pyranoside. It was found that *G. frondosa* polysaccharide was a heteropolysaccharide consisting of the repeating disaccharide unit. Comparing control rats, *G. frondosa* polysaccharide treatment markedly enhanced skin TNF-α levels, reduced lgG content, T lymphocytes rate and caspase-3 mRNA expression in group 2 rats. Administration of *G. frondosa* polysaccharide significantly reduced skin levels of TNF-α, increased lgG content, T lymphocytes rate and caspase-3 mRNA expression in groups 3 and 4 rats. In conclusion, *G. frondosa* polysaccharides are beneficial for those with skin diseases.

1. Introduction

The edible mushroom, *Grifola frondosa*, is a basidiomycete fungus belonging to the Polyporaceae family. Fruit bodies and liquid-cultured mycelium from this mushroom are reported to contain useful antitumor polysaccharides (Adachi, Okazaki, Ohno, & Yadomae, 1994; Borchers, Stern, & Hackman, 1999; Nagesh & Shanthamma, 2009), and show some promise as immunomodulatory agents. They may also be beneficial for the treatment of hyperlipidemia, hypertension, and hepatitis (Mayell, 2001). It is reported that D-Fraction, a polysaccharide extracted from the maitake mushroom, *G. frondosa*, activates macrophages, dendritic cells, and T cells, inhibits tumor cell growth (Kodama, Asakawa, Inui, Masuda, & Nanba, 2005), enhances the cytotoxicity of NK cells by inducing the production of IL-12, and improves the symptoms and secondary diseases caused by HIV (Nanba, Kodama, Schar, & Turner, 2000).

The aging process of the skin is a complex biological phenomenon and it can be divided into intrinsic and extrinsic aging. Intrinsic aging, which is largerly genetically determined, affects the skin in a manner similar to most internal organs (Tzaphlidou, 2004). In this study, polysaccharides was extracted from *G. frondosa* mushroom. Chemical composition, structure of the polysaccharides was analyzed using GC–MS, IR and NMR. The effects of *G. frondosa* polysaccharides on the skin TNF-α levels, lgG content, T lymphocytes rate and caspase-3 mRNA expression in aged rats were investigated. This study might shed a light on the potential use of *G. frondosa* polysaccharides for a treatment of skin disease in human.

2. Materials and methods

2.1. Material

G. frondosa mushroom was purchased from a local shop in Taizhou city, Zhejiang province.

2.2. Extraction and quantification of G. frondosa polysaccharides

G. frondosa mushroom (50 g) powder were precisely weighted. The powder was extracted with 200 ml of 80% ethanol solution at 50 °C for 2 h. After vacuum filtration, the residues were extracted with 200 ml of distilled water at 50 °C for 2 h. After centrifuge at 8000 × *g* for 15 min, the supernatant was collected. The supernatant was concentrated by rotary vacuum evaporator (BC-R203, Shanghai Biochemical Equipment Co., Shanghai, China) at 65 °C, and a four-fold volume of ethanol was added to precipitate *G. frondosa* polysaccharides, which was finally obtained by centrifugation.

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Dialysis against distilled water was performed to remove salts from *G. frondosa* polysaccharides. Then they were lyophilized for structural analysis.

The content of polysaccharides was determined by the phenol–sulphuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956; Brizio, Brandsch, Douka, Wait, & Barile, 2008). Protein content was converted from nitrogen content (N × 6.25), which was determined by a PE-2400 series II automatic elemental analyser (PerkinElmer, Waltham, Massachusetts, USA).

2.3. Purification

The crude polysaccharides was subjected to gel filtration on a column (1.5 cm × 100 cm) of Sephadex G-100, eluted with distilled water and monitored using the phenol–sulfuric acid method (Ge, Duan, Fang, Zhang, & Wang, 2009; Li, Yuan, & Rashid, 2009). The flow rate was 0.2 ml/min.

2.4. GC–MS analysis

GC analyses were performed on an Agilent Technologies 6890 N Network gas chromatograph coupled to an Agilent Technologies 5973 Network quadrupole mass selective spectrometer and provided with a split/splitless injection port. Helium was the carrier gas, at a linear velocity of 38 cm/s. Compounds were separated on a HP-5MS capillary column (Hewlett-Packard, Avondale, PA, USA) and successively on a SPB-1 capillary column (Supelco Ltd., Bellefonte, PA, USA), both 30 m × 0.25 mm ID, 0.25 μm film thickness. Column temperature was held at 40 °C for 5 min and increased to 75 °C at 4 °C/min, then at 8 °C/min to 250 °C holding 10 min. The injector temperature was 250 °C, and samples (1 μl) were injected in the splitless mode.

The temperatures of the ion source and the transfer line were 175 and 280 °C, respectively. Positive ion electron impact mass spectra were recorded at 70 eV ionisation energy, 2 scan/s.

2.5. Fourier-transformed infrared spectroscopy

The major structural groups of purified *G. frondosa* polysaccharides were detected using Fourier-transformed infrared (FT-IR) spectroscopy. Pellets for infrared analysis were obtained by grinding a mixture of 2 mg *G. frondosa* polysaccharides with 200 mg dry KBr, followed by pressing the mixture into a 16-mm-diameter mould. The FT-IR spectra were recorded in the region of 4000–400 cm^{-1} on a Perkin-Elmer spectrum GX FT-IR system (PerkinElmer, USA).

2.6. NMR spectroscopy

NMR spectra were obtained using a DRX-500 spectrometer (Bruker, Germany) for 3–5% solutions of oligo- and polysaccharides in D_2O at 60 °C (acetone as internal standard; δ_H 2.225 ppm, δ_C 31.45 ppm). The samples were lyophilised twice from a D_2O solution before the experiments were carried out. Signals in NMR spectra were assigned on the basis of data from two-dimensional (2D) experiments (COSY, TOCSY, ROESY, and ^1H and ^{13}C HSQC) as described previously (Wu, Duan, Liu, & Cen, 2010). Two-dimensional experiments were carried out using the standard procedures of the Bruker Company. ROESY experiments were carried out with a mixing time of 200 ms. TOCSY experiments were carried out using 60 ms duration of the MLEV 17 spin-lock. The positions of substitutions and the sequences of monosaccharide residues were determined by two-dimensional experiments with the determination of the nuclear Overhauser effect (ROESY).

2.7. Analysis of TNF-α levels, lgG content and T lymphocytes rate

The rats were randomly divided into four groups of eight each as follows. Thirty-two rats were randomly divided into four groups as follows: group 1 (2 months old), comprising eight individuals and serving as normal control; group 2 (13 months old), comprising eight individuals and serving as aged model control; groups 3 and 4 rats (13 months old) were orally received polysaccharides (100 mg/kg and 200 mg/kg B.W.) for 40 days. At the end of the experiment, the animals were deeply anesthetized and killed. Skin samples were immediately removed and weighed after the removal of the surrounding connective tissues carefully, and then the skin was placed in a buffer solution of phosphate buffered saline (PBS, 50 mM Tris, 150 mM NaCl, pH 7.4) at 4 °C for biochemical analysis.

Level of the TNF-α level was measured in plasma and myocardium by ELISA.

LgG content were measured by rat immunoglobulin isotyping cytometric bead array kit (CBA). T lymphocytes rate (%) was measured by flow cytometry analysis.

2.8. Reverse transcription-PCR

The levels of mRNA for caspase-3 were analyzed using a reverse transcription (RT)-PCR approach as previously described (Yakovlev et al., 2001). In brief, total RNA was isolated by acidic phenol extraction, and 10 μg of it was reverse transcribed with Moloney murine leukemia virus reverse transcriptase (Invitrogen) in 30-μL reaction mixture. The resulting cDNA (3 μL) was amplified by PCR. The primers for PCR amplification of caspase-3 were based on the available rat mRNA sequences. The number of cycles and the reaction temperature were estimated to be optimal to provide a linear relationship between the amount of input template and the amount of PCR product generated over a wide concentration range: from 1 to 20 μg of total RNA. The amplified cDNA was analyzed by agarose gel electrophoresis. After staining with ethidium bromide, gel images were captured under the UV light and analyzed on a PC using the public domain NIH Image program. The levels of individual mRNA were expressed in arbitrary units as the proportion of individual PCR product mean optical density (inverted image) to a control product mean optical density obtained from the same RNA sample. The cDNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control.

2.9. Western blot analysis

Twenty-microliter sample was mixed with 5 μl of sample buffer, and then boiled for 5 min. Equal amount of proteins (30 μg) were separated by SDS-PAGE, and molecular weight markers (R&D Systems Inc., Minneapolis, MN, USA) were loaded on each gel for protein band identification. The proteins on the gel were subsequently transferred onto a nitrocellulose membrane using a semidry transfer apparatus. Blots were probed with antibody reactive with caspase-3 antibody. Finally, the color reaction was observed by incubation of membrane with 3,3′-diaminobenzidine. The staining results were scanned into computer and the integrated optical densities of the protein bands were analyzed by gel image analyzer (Aalpha Innotech Co., USA).

2.10. Statistical analysis

The results are expressed as means ± SD of eight measurements. To compare several groups analysis of variance (ANOVA) was used. Where it was appropriate, data by two-way ANOVA were tested. Spearman correlation coefficient (R) and P-value were used to show correlations and their significance. P values of <0.05 were adopted as statistically significant.

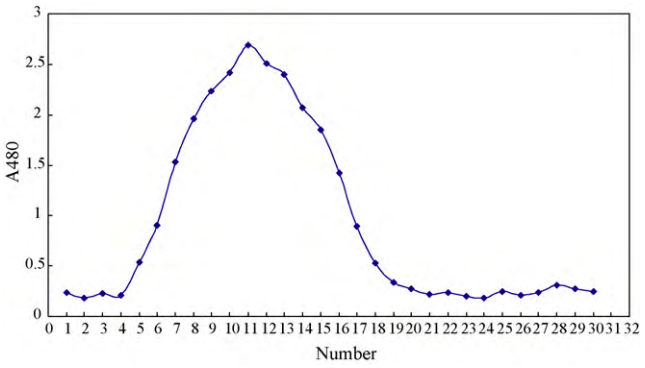

Fig. 1. Elution curve of crude *Grey Polypore* polysaccharides on a column (1.5 cm × 100 cm) of Sephadex G-100.

3. Result

3.1. Purification of G. frondosa polysaccharides

The crude polysaccharide was prepared from the cultured *G. frondosa* by hot-water extraction, EtOH precipitation and dialysis. The purity of the isolated polysaccharides was examined by Sephacryl S-300HR column, and the elution profiles are shown in Fig. 1.

G. frondosa polysaccharides was eluted as a single symmetrical peak corresponding to an average molecular weight of 1.3×10^4 as determined by gel filtration. The absorbance at 280 nm and its positive reaction in the Folin–phenol reaction indicated that this polysaccharide contained a protein moiety. The sugar composition of *G. frondosa* polysaccharides was then determined by GC–MS.

3.2. GC–MS analysis of monosaccharides

By comparing retention times of unknown peaks with reference sugar standards and cochromatography with added standards (rhamnose, arabinose, xylose, mannose, glucose and galactose), three monosaccharides including glucose, mannose and galactose were identified, with the retention times being 5.783, 8.342 and 12.079 min, respectively, and the molar ratio at 6.5:1:2.6. This result clearly demonstrated that both glucose and galactose were the dominant monosaccharides in *G. frondosa* polysaccharides.

3.3. FT-IR spectra analysis of G. frondosa polysaccharides

Fig. 2 showed the FT-IR spectrum of *G. frondosa* polysaccharides. The FT-IR spectra of the *G. frondosa* polysaccharides were recorded in the region 4000–400 cm^{-1}. The *G. frondosa* polysaccharides had IR bands at 1000–1100 cm^{-1}, 1400–1530 cm^{-1}, 2800–2900 cm^{-1}, and 3100–3500 cm^{-1}, which were distinctive absorptions of polysaccharides. *G. frondosa* polysaccharides exhibited a typical absorption of β configuration at 851 cm^{-1}. The absorption peaks of 1415 cm^{-1}, 1154 cm^{-1} and 851 cm^{-1} suggested the polysaccharide consisted of pyranoside (Fig. 2).

3.4. Structure analysis of G. frondosa polysaccharides

The 1H NMR spectrum of *G. frondosa* polysaccharides was assigned using 2D COSY and relayed COSY experiments (Fig. 3a). Based on the vicinal coupling constant values, seven sugar spin systems were assigned to α-Glup, β-Manp, and β-Galp. Actually, the two anomeric signals labeled as Iα and Iβ originate from the terminal reducing Gal unit. The influence of anomerization on the chemical shift of α-GluII H-1 (δ 3.89 and 3.87), β-ManIII H-1 (δ 3.80 and 3.75) and β-GalII H-1 (δ 3.75 and 3.69) is apparent. The ^{13}C NMR spectrum of *G. frondosa* polysaccharides (Fig. 3b) was assigned using H-detected 1H, ^{13}C HMQC experiments (Fig. 3a and b). Significant downfield displacements of the signals for C-2, C-4 of GalI, and C-4 of β-ManIII, as compared with their position in the spectra of corresponding nonsubstituted monomers, confirmed the linkage positions. These data confirmed the substitution pattern and revealed the sequence of residues in the polysaccharide.

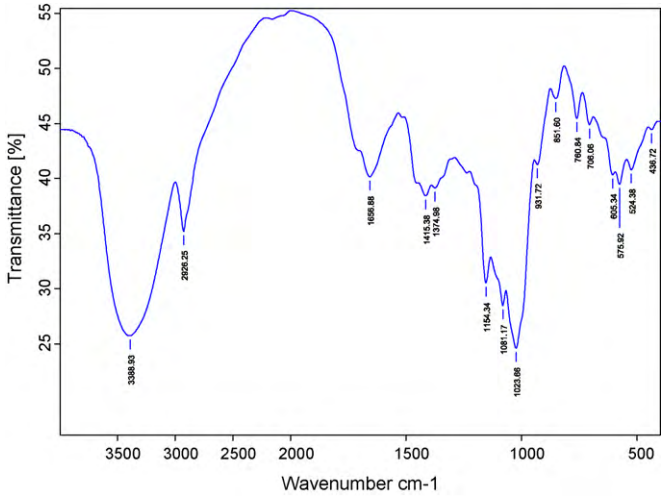

Fig. 2. FT-IR spectroscopy of *Grey Polypore* polysaccharides.

3.5. Effect of G. frondosa polysaccharides on skin TNF-α levels, lgG content and T lymphocytes rate in different groups

To analyze the possible role of *G. frondosa* polysaccharides in treating skin disease, skin TNF-α levels, lgG content and T lymphocytes rate in aged rats were investigated. As shown in Table 1, significantly increased skin TNF-α levels were observed in aged rats (group 2). The increased levels of skin TNF-α were significantly suppressed by *G. frondosa* polysaccharides treatment in polysaccharides-treatment rat (groups 3 and 4). In addition, significantly decreased skin lgG content and T lymphocytes rate were observed in aged rats (group 2). The decreased levels of skin lgG content and T lymphocytes rate were significantly enhanced by *G. frondosa* polysaccharides treatment in polysaccharides-treatment rat (groups 3 and 4). Recent evidence indicates that aging does not appear to alter lipid raft (membrane microenvironment) formation in which the TCR resides in activated T lymphocytes (Xu, George, & Jolly, 2004). However, the same group showed that aging resulted in the inability of T lymphocytes to properly assemble the necessary intracellular signaling molecules for appropriate T-lymphocyte proliferation (Switzer, Fan, Wang, McMurray, & Chapkin, 2004). Administration of *G. frondosa* polysaccharides dose-dependently enhanced skin lgG content and T lymphocytes rate, reduced TNF-α

Table 1
Effect of *Grifola frondosa* polysaccharides on skin TNF-α levels, lgG content and T lymphocytes rate in different groups.

Group	TNF-α (average grey scale)	lgG (mg/ml)	T lymphocytes rate (%)
1	107.6 ± 8.8	9.32 ± 0.24	87.42 ± 2.31
2	160.3 ± 11.1[a]	6.21 ± 0.32[a]	65.04 ± 1.83[a]
3	142.2 ± 10.5[b]	7.88 ± 0.17[b]	79.37 ± 2.03[b]
4	121.9 ± 13.1[b]	9.06 ± 0.24[b]	89.02 ± 2.55[b]

[a] $P < 0.05$, compared with group 1.
[b] $P < 0.05$, compared with group 2.

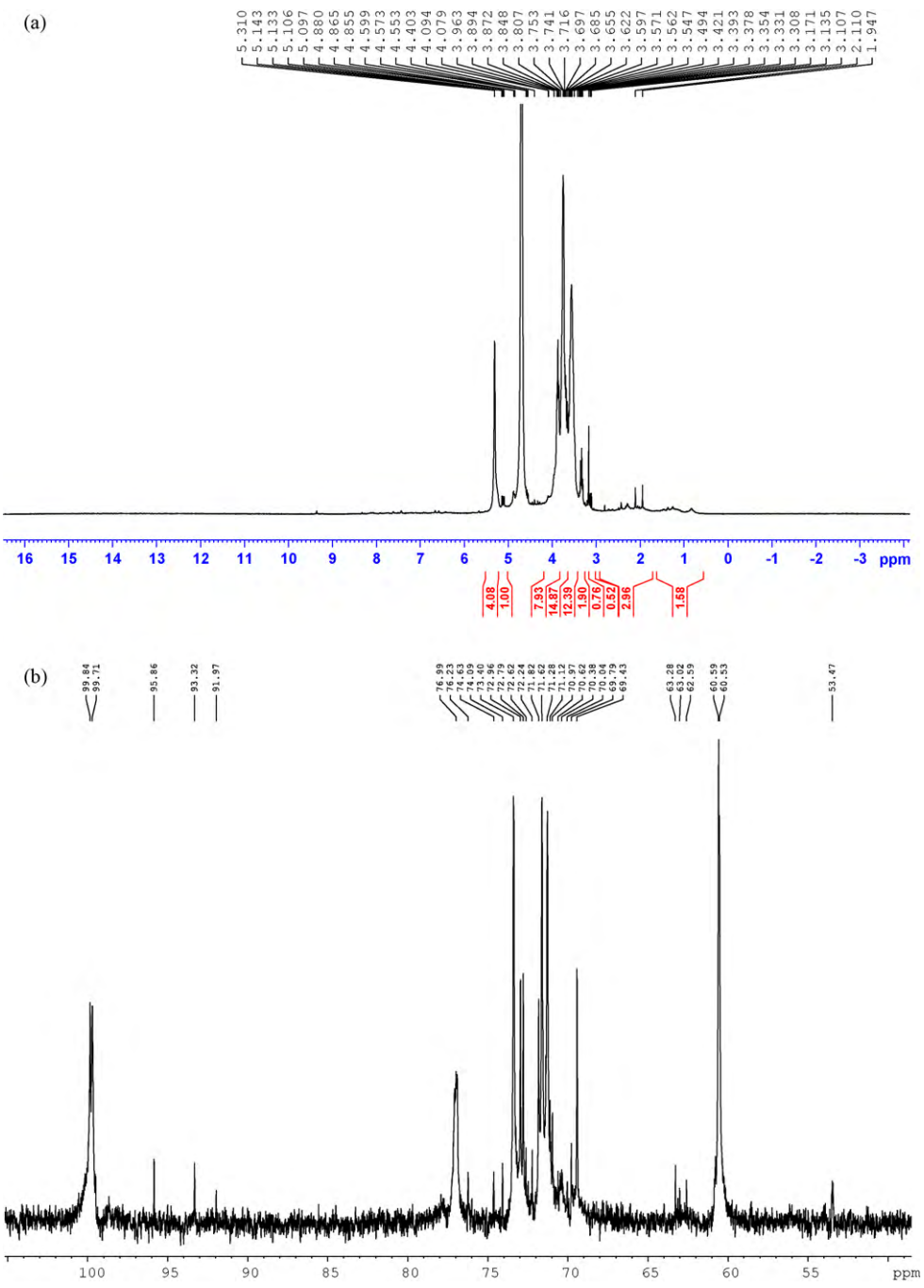

Fig. 3. (a) ^{1}H NMR analysis of *Grey Polypore* polysaccharides; (b) ^{13}C NMR analysis of *Grey Polypore* polysaccharides.

levels, suggesting that *G. frondosa* polysaccharides may enhanced immunity function of experimental rats.

3.6. *Effect of G. frondosa polysaccharides on skin caspase-3 mRNA expression and protein level in different groups*

In skin mRNAs of caspase-3 were detected by RT-PCR analysis (Fig. 4). The expression levels of caspase-3 were compared between young-adult and aged rats. The levels of caspase-3 mRNAs were significantly up-regulated in aged rats ($P < 0.05$). After *G. frondosa* polysaccharides administration, mRNAs of caspase-3 of aged rats were significantly ($P < 0.05$) reduced compared to those of young-adult rats. Level of skin caspase-3 protein were also detected by Western blot analysis (Fig. 5). Consistent with RT-PCR observations, level of skin caspase-3 protein was significantly enhanced in aged rats ($P < 0.05$). Compared with aged rats (group 2), administration of *G. frondosa* polysaccharides dose-dependently significantly reduced levels of caspase-3 protein in skin of aged rats (groups 3 and 4).

Classical caspase-dependent apoptosis is a short-lasting process, bringing cell to death in few hours up to 1 or 2 days. Cell death in aging process is instead a slow process (Deligezer, Erten, Akisik, & Dalay, 2006). It has been proposed that some forms of caspase-independent programmed cell death may account for tissue cell elimination in aging. While not directly involved in cell execution, caspase-3 activity has been found to be increased in aging (Lacelle, Xu, & Wang, 2002). In this framework, it is not surprising to have found a significant higher level of caspase-3 mRNA in the aged

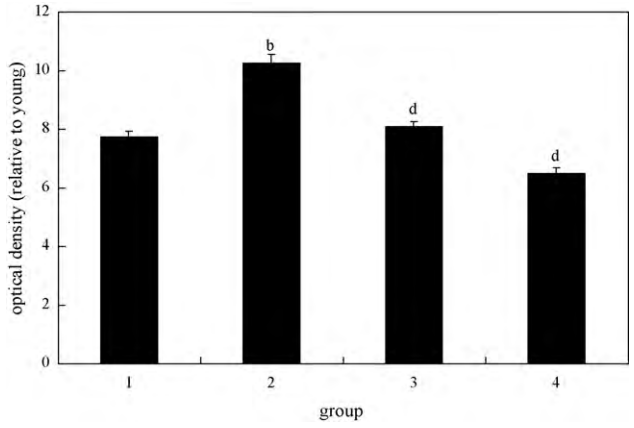

Fig. 4. Effect of *Grey Polypore* polysaccharides on skin caspase-3 mRNA expression. $^{a}P < 0.05$, compared with group 1; $^{b}P < 0.05$, compared with group 2.

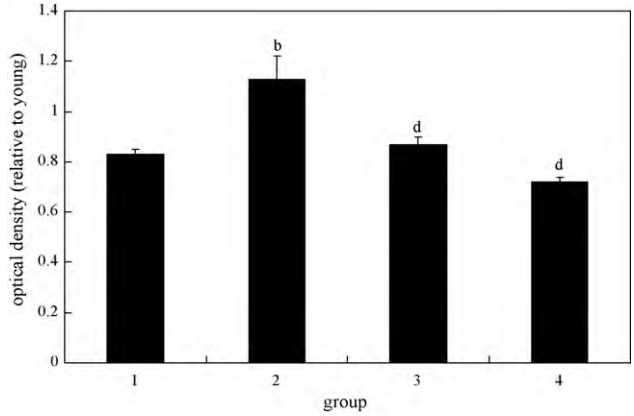

Fig. 5. Effect of *Grey Polypore* polysaccharides on skin caspase-3 protein. $^{a}P < 0.05$, compared with group 1; $^{b}P < 0.05$, compared with group 2.

skins, where cell death has also been shown to be increased. The anti-skin-aging effect of administration of *G. frondosa* polysaccharides by the decreased caspase-3 mRNA expression may be one of the factors promoting the longer life span. These results suggest that *G. frondosa* polysaccharides are beneficial for those with skin diseases.

4. Conclusion

In conclusion, the most appropriate extraction and purification condition for determination of polysaccharide in *G. frondosa* was as follows: a volume ratio of *G. frondosa* polysaccharides sample to deionized water at 1:10, followed by shaking at 100 °C for 30 min, concentrating to a small volume under vacuum, adding fivefold volume of 95% ethanol for precipitation for 8 h at −20 °C, hydrolysing protein with 2.5 U/mL of proteinase at pH 8 and 60 °C for 4 h and separating polysaccharide into three fractions. A maximum bleaching (68.39%) of crude polysaccharide was attained by active carbon at 9 mg/mL. Polysaccharide was shown to be composed of mannose, glucose and galactose. Pharmacological experiment suggested that *G. frondosa* polysaccharides was beneficial for those with skin diseases.

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