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Biological and physicochemical properties of two polysaccharides from the mycelia of *Grifola umbellate*

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ABSTRACT

In the present study, we firstly reported the antioxidant, antitumor and immunomodulatory effects of two polysaccharides (GUMP-1-1 and GUMP-1-2) isolated from *Grifola umbellata* mycelia. Chemical analysis indicated that two polysaccharide fractions contained different content of neutral sugar, uronic acid and protein, as well as varying monosaccharide compositions and average molecular weight. We found that they could significantly inhibit the growth of H22 implanted tumor and enhance the spleen index and splenocyte proliferation of H22 tumor-bearing mice. In addition, GUMP-1-2 had the stronger free radicals scavenging and ferrous ion chelating abilities than GUMP-1-1 in vitro. These results indicated that antitumor activity of two purified polysaccharides might be achieved by improving immune response and the different chemical composition and average molecular weight could affect their antitumor, antioxidant and immunomodulatory activities.

1. Introduction

Grifola umbellata, a fungus that belongs to Polyporaceae, Basidiomycetes, is widely distributed in China, Japan, Europe and North America. Its fruiting bodies growing above-ground is a delicious edible mushroom and its sclerotium growing under-ground has been used as a traditional Chinese medicine for the treatments of inhibited urination, beriberi, edema and other diseases for centuries (Zhou, Shao, & Chen, 2009). Polysaccharides isolated from *G. umbellata* have been reported to the main bioactive components to enhance human immunity and to treat certain cancers (Han, 1988; Haranaka et al., 1985; Miyazaki & Oikawa, 1973; Miyazaki, Oikawa, Yamada, & Yadomae, 1978; Miyazaki et al., 1979; Ueno, Okamoto, Yamauchi, & Kato, 1982; You, Hau, Chen, & Huang, 1994). Particularly, *G. umbellata* polysaccharide is effective in inhibiting tumor growth. Moreover, many clinical studies show the therapeutic alliance of *G. umbellata* polysaccharides and chemical medicines can enhance the therapeutic efficacy and reduce the side effects of the chemical counterparts (Lv, Liu, & Ning, 2005; Zhang et al., 2007). These reports greatly stimulate the consumption of this wild medicinal materials and the harvest rate is exceedingly rapid than that of rebirth speed by species themselves. Because of a lack of effective protection and strong consumer demand, wild *G. umbellata* resources have been seriously destroyed in China. So, there is an urgent demand for a feasible alternative to obtain the bio-active polysaccharides. Fortunately, this situation has been found and much effort has been made done, mainly focusing on domestic cultivation. Since the solid-state culture could not provide high yields of bio-active polysaccharides, the submerged culture becomes the only approach to produce mycelia and polysaccharides on a larger scale. Recent studies have shown that the main ingredients of the polysaccharides extracted from *G. umbellata* sclerotia and submerged culture mycelia are the same (Xu, Li, Li, Liang, & Jin, 2004). In spite of numerous studies on *G. umbellate* polysaccharide, there are no available reports regarding its anti-hepatoma potential in vivo. In this study, we aimed to investigate whether *G. umbellate* mycelia polysaccharides could inhibit mouse hepatoma H22 engrafted tumor in a rat model, and then to preliminarily investigate its antioxidant and immunopotentiating activities.

2. Materials and methods

2.1. Materials and chemicals

G. umbellata mycelia were from Hubei Chaihong Pharmaceutical Co. Ltd., China. DEAE-52 cellulose and Sephacryl S-400 were

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obtained from GE Healthcare Bio-Sciences Co., Piscataway, NJ, USA. T-series Dextran (MW standards), ethylene diamine tetra-acetic acid (EDTA), ascorbic acid, concanavalin A (Con A) and lipopolysaccharide (LPS) were purchased from Sigma, Chemical Co., Saint Louis, MO, USA. RPMI-1640 medium and fetal calf serum (FCS) were purchased from Gibco, Grand Island, NY, USA. Aqueous solutions were prepared with ultra-pure water from a Milli-Q water purification system (Millipore, Bedford, MA, USA). All the other chemicals used were of analytical grade.

2.2. Extraction, isolation and purification of polysaccharide

Briefly, the dried mycelia (200 g) were preliminarily treated with 1000 mL of 80% ethanol for 24 h to remove the interfering components, such as monosaccharide, disaccharide, oligosaccharide and polyphenol in the samples at 80 °C. After filtration, the residue was extracted twice with hot distilled water at 90 °C for 4 h. The decoction was left to cool at room temperature, filtered and then freeze-dried to obtain crude polysaccharides. The crude polysaccharide was treated by Sevag reagent (Staub, 1965) to remove protein and extensively dialyzed (cut-off Mw 3500 Da). The retentate portion was then concentrated and centrifuged to remove insoluble material. Finally the supernatant was lyophilized to obtain crude polysaccharide, name as GUMP.

The GUMP was purified sequentially by chromatography of DEAE-52 cellulose and Sephacryl S-400 column chromatography. Briefly, 5 mL of GUMP solution (10 mg/mL) was applied to a column of DEAE-52 cellulose, and the column was stepwise eluted with 0, 0.2, 0.4 and 0.6 M sodium chloride solutions at a flow rate of 60 mL/h. Eluate was collected automatically (8 mL/tube), and the carbohydrates were determined by the phenol–sulfuric acid method. As a result, two fractions of polysaccharides (GUMP-1 and GUMP-2) were obtained, concentrated, dialyzed against distilled water and further purified through a column of Sephacryl S-400 (2× 60 cm), affording GUMP-1-1 and GUMP-1-2, respectively. The two purified fractions were collected, concentrated, dialyzed and lyophilized for further study, respectively.

2.3. Molecular weight and monosaccharide composition analysis

The molecular weight of the polysaccharide was determined by high performance gel permeation chromatography (HPGPC). The sample solution was applied to Waters High Performance Liquid Chromatography (HPLC) equipped with a TSK-GEL G3000 SWXL column (7.8 mm × 300 mm), eluted with 0.1 mol/L Na_2SO_4 solution at a flow rate of 0.6 mL/min and detected by a Waters 2414 Refractive Index Detector. The columns were calibrated with Dextran T-series standard of known molecular weight (200,000, 70,000, 40,000, 10,000, 5000 Da). The molecular weight of GUMP-1-1 and GUMP-1-2 was estimated by reference to the calibration curve made above.

The polysaccharide was hydrolyzed by 2 M TFA at 120 °C for 3 h into monosaccharides under airtight condition, and the monosaccharides were conventionally converted into the alditol acetates as described (Xin et al., 2012) and were analyzed by gas chromatography (GC, Agilent 6890, USA) equipped with a HP-5 column (30 m × 0.25 mm × 0.25 μm) and flame-ionization detector (FID). The operation was performed using the following conditions: 160 °C for 2 min, then to 200 °C at 6 °C/min, then to 215 °C at 0.2 °C/min, then to 240 °C at 6 °C/min for 30 min. Nitrogen was used as the carrier gas at 1 mL/min; injection temperature was 250 °C; detector temperature was 300 °C. Monosaccharides identification was done by comparison with reference monosaccharides. The relative molar proportions were calculated by the area normalization method.

Total neutral sugar content was determined by the reaction with phenol in the presence of sulfuric acid at 486 nm (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) using glucose as standard. Protein was determined by photometric assay (Bradford, 1976) using bovine serum albumin as the standard. Uronic acid was determined by measuring the absorbance at 525 nm using the m-hydroxybiphenyl photometric procedure, with D-glucuronic acid as the standard (Blumenkrantz & Asboe-Hansen, 1973).

2.4. Assay for antioxidant activity

2.4.1. Hydroxyl radical scavenging activity

The hydroxyl radical scavenging capacity was examined using the method described previously (Chen, Ju, Li, & Yu, 2012). For the control, sample was substituted by ascorbic acid. All values were determined in three replicates. The percentage of hydroxyl radical scavenging capacity was calculated according to the following equation:

$$\text{Scavenging effect}\,(\%) = \frac{A_{532(\text{blank})} - A_{532(\text{sample})}}{A_{532(\text{blank})}} \times 100$$

where $A_{532(\text{blank})}$ was the absorbance of the control (deionized water, instead of sample) and $A_{532(\text{sample})}$ was the absorbance of the test sample mixed with reaction solution.

2.4.2. Superoxide anion scavenging activity

The superoxide anion scavenging capacity was examined using the method described previously (Chen et al., 2012). For the control, sample was substituted by ascorbic acid. All values were determined in three replicates. The percentage of superoxide anion scavenging capacity was calculated according to the following equation:

$$\text{Scavenging effect}\,(\%) = \frac{A_{560(\text{blank})} - A_{560(\text{sample})}}{A_{560(\text{blank})}} \times 100$$

where $A_{562(\text{blank})}$ was the absorbance of the control (deionized water, instead of sample) and $A_{562(\text{sample})}$ was the absorbance of the test sample mixed with reaction solution.

2.4.3. Metal chelating assay

The chelating activity of sample on Fe^{2+} was measured as reported (Wang, Yang, & Wei, 2012) by measuring the formation of ferrous iron–ferrozine complex. For the control, sample was substituted with EDTA. The chelating activity was calculated as:

$$\text{Chelating ability}\,(\%) = \frac{A_{562(\text{blank})} - A_{562(\text{sample})}}{A_{562(\text{blank})}} \times 100$$

where $A_{562(\text{blank})}$ was the absorbance of the control (deionized water, instead of sample) and $A_{562(\text{sample})}$ was the absorbance of the test sample mixed with reaction solution.

2.5. In vivo antitumor test

BALB/c mice (half male and half female, 18–22 g) were purchased from Animal Experimental Center of the Fourth Military Medical University. The animals were maintained on a 12-h-dark/12-h-light cycle at 25 ± 1 °C, humidity of 50 ± 10% and allowed free access to standard laboratory pellet diet and water during the experiments. Animal experiments were conducted under principles in good laboratory animal care, and approved by ethical committee for Laboratory Animals Care and Use of the Fourth Military Medical University.

Under sterile condition, 0.2 mL of ascites H22 tumor cell suspension (1×10^7 cells/mL) was subcutaneously inoculated into the

right armpit of BALB/c mice at day 0. Twenty-four hours after inoculation, mice were divided randomly into six groups (20 mice in each group): model control group, positive control group and four treatment groups. The mice inoculated with H22 cells were orally administrated with two polysaccharides (100 and 200 mg/kg body weight) or 5-FU (20 mg/kg) once daily for 10 days. The mice in model control group received an equal volume of normal saline (0.2 mL/10 g body weight). Tumor volume was measured with a digital caliper and calculated using the formula $0.52 \times a \times b^2$, where a and b are the largest and smallest diameters as described previously (Cai et al., 2008). Twenty-four hours after last tested administration, all the tumor-bearing mice were sacrificed by cervical dislocation on day 11, and then tumor and spleen were collected and weight. The rest 10 mice in each group were kept to evaluate survival time. The inhibitory rate against the growth of tumor, the spleen index and life prolongation rate were calculated according to the following formula: the inhibitory rate (%) = [(the average volume of the model control group − the average volume of treated groups)/the average volume of the model control group] × 100; spleen index (mg/10 g) = (weight of spleen/body weight); life prolongation rate (%) = [(the survival time of the treated groups − the survival time of the model control group)/the survival time of the model control group] × 100. Body weights were measured at the start and at the last day of treatment.

2.6. Splenocyte proliferation assay

Spleens collected from sacrificed mice under aseptic condition were chopped into small pieces and passed through a fine steel mesh to obtain a homogeneous cell suspension. Recovered spleen cells were resuspended in lysis buffer (0.15 M NH_4Cl, pH 7.4) for 5 min to remove erythrocytes. Then spleen cells were harvested and resuspended in RPMI 1640 complete medium. Cell numbers and viability (over 95%) were assessed microscopically using trypan blue dye exclusion technique. The splenic cells (100 μl) were seeded in 96-well plates (1×10^6 cells/well) together with Con A (2.5 μg/ml) or LPS (5.0 μg/ml). After the cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2 for 48 h, 20 μl MTT (5 mg/ml) were added to each well and incubated for another 4 h. After the incubation, the cell suspensions were centrifuged at 800 rpm for 10 min and the supernatants were removed, then 150 μl of dimethyl sulfoxide (DMSO) working solution was added. The absorbance was measured in an automatic ELISA plate reader at 570 nm with a 630 nm reference after 30 min. The stimulation index (SI) was calculated based on the following formula: SI = the absorbance value for mitogen-cultures divided by the absorbance value for non-stimulated cultures.

2.7. Statistical analysis

All results were presented as mean ± SD. Data were analyzed by one-way ANOVA using SPSS and Dunnett's test. p values less than 0.05 were considered significant.

3. Results and discussion

3.1. Isolation, purification and composition of two polysaccharides fractions

The crude polysaccharide (GUMP) was isolated from the hot water extract of *G. umbellata* mycelia, followed by ethanol precipitation, deproteinization and lyophilization. After being fractioned on DEAE-Sepharose CL-6B column, GUMP-1 (6.34%) and GUMP-2 (5.45%) were obtained from the distilled water and NaCl eluent, respectively (Fig. 1A). GUMP-1 and GUMP-2 were further purified by Sephadex G-100 gel permeation chromatography, affording

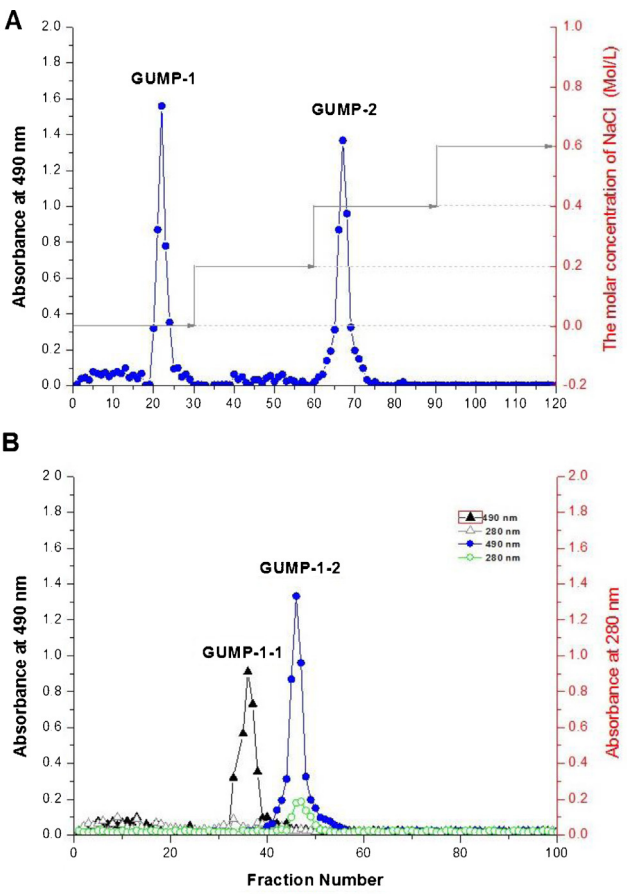

Fig. 1. (A) Elution curve of crude polysaccharide extracted from *Grifola umbellata* mycelia on a DEAE-52 cellulose anion-exchange column. (B) Purification of polysaccharide fractions GUMP-1 and GUMP-2 on a Sephacryl S-400 column.

two fractions of GUMP-1-1 and GUMP-1-2 as detected by the phenol–sulfuric acid assay (Fig. 1B). The physicochemical properties of two polysaccharides were summarized in Table 1. GUMP-1-1 had a negative response to the Bradford method, and no absorption was detected by the UV spectrum at 280 and 260 nm, indicating the absence of protein and nucleic acid. However GUMP-1-2 contained a large amount of uronic acid (12.5%) and protein (13.6%), indicating GUMP-1-2 is an acid protein–polysaccharide. Accordingly, the total sugar content evaluated in GUMP-1-1 and GUMP-1-2 were 92.3% and 75.1%, respectively.

High-performance gel permeation chromatography (HPGPC) was used to determine the purity and molecular weight of the polysaccharide. Each polysaccharide showed only one single symmetrical peak on HPGPC, suggesting that they were homogenous

Table 1
Components of monosaccharide and properties of two polysaccharide fractions from *Grifola umbellata* mycelia.

Fractions	GUMP-1-1	GUMP-1-2
Total sugar (%)	92.3	75.1
Uronic acid (%)	–	12.5
Protein (%)	–	13.6
Average molecular weights (10^5 Da)	2.5	0.5
Sugar components (mol%)		
Glucose	17.4	11.4
Mannose	1.4	1.2
Galactose	1.2	1.1
Fructose	0.4	0.2
Glucuronic acid	–	2.1

polysaccharides. The average molecular weights of GUMP-1-1 and GUMP-1-2 were calculated by the calibration curve with standard T-series Dextrans to be 2.5 and 0.5×10^5 Da, respectively.

According to GC analysis, GUMP-1-1 was composed of glucose, mannose, galactose and fructose in the molar ratios of 17.4:1.4:1.2:0.4. GUMP-1-2 contained glucose, mannose, galactose, fructose and GlcA in the molar ratios of 11.4:1.2:1.1:0.2:2.1. These data revealed glucose was the main neutral monosaccharide incorporated in two biopolymers, followed by mannose, galactose and fructose.

3.2. The antioxidant activity of two polysaccharides fractions

3.2.1. Scavenging ability on hydroxyl radicals

Hydroxyl radical is the most reactive oxygen radical known in chemistry, which can severely damage almost any biological molecule in living cells and induce severe damage to the adjacent biomolecules. It is considered to be involved in aging, and the development of cancer and several other diseases (Valentão et al., 2003). Thus, removal of hydrogen peroxide is very important for protection of cells from oxidative damage. The scavenging abilities of GUMP-1-1 and GUMP-1-2 on hydroxyl free radical are shown in Fig. 2A. The scavenging rates of the GUMP-1-2 and ascorbic acid against hydroxyl radicals were demonstrated in a dose-dependent manner (Fig. 3A). Moreover, the neutral polysaccharide GUMP-1-1 exhibited weaker scavenging effect than the acidic polysaccharide GUMP-1-2 at every concentration point. All these confirmed that the uronic acid content and average molecular weight have significant effect on scavenging hydroxyl radical (Chen et al., 2012; Hu, Liu, Chen, Wu, & Wang, 2010; Yu et al., 2003).

3.2.2. Scavenging ability on superoxide radicals

Superoxide anion, which is a relatively weak oxidant formed in viable cells during several biochemical reactions, can be magnified by its effect on cell damage because it can generate dangerous species of free radicals and oxidizing agent (Chen et al., 2012). As shown in Fig. 2B, a significant increase of the scavenging activity on superoxide radicals was observed at the concentration range (0–8 mg/mL) of GUMP-1-2. Especially at the dose of 8 mg/mL, the scavenging potency of GUMP-1-2 on superoxide radicals was close to that of ascorbic acid. However GUMP-1-1 containing no uronic acid showed a little or no scavenging activity on superoxide radicals compared with GUMP-1-2 and ascorbic acid. This demonstrated that the uronic acid content and average molecular weight affected their antioxidant activities, which was in accordance with the fact that higher uronic acid content and small molecular weight showed greater scavenging effect on superoxide radical (Chen et al., 2012; Hu et al., 2010; Yu et al., 2003).

3.2.3. Chelating effect on ferrous ions

Transition metals, such as ferrous iron (Fe^{2+}), can facilitate the generation of ROS. Thus the chelating capacity of polysaccharides against Fe^{2+} can be an important approach for retarding metal-catalyzed oxidation (Hu et al., 2010). As shown in Fig. 2C, the ferrous ion chelating ability climbed following a rise in polysaccharide concentration, with a peak level being attained for GUMP-1-2 (96.5%) at 8 mg/mL. Conversely, a poor ferrous ion chelating activity was found for GUMP-1-1 at all tested concentrations. It is worth pointing out that the compounds with structures containing two or more of the following functional groups: —OH, —SH, —COOH, $—PO_3H_2$, C, O, $—NR_2$, —S— and —O— in a favorable structure–function configuration can show metal chelating activity (Yuan, Bone, & Carrington, 2005). Similarly, a lack of carboxyl group may lead to a lower metal ion chelating capacity of GUMP-1-1 at every concentration point compared to acidic polysaccharide GUMP-1-2.

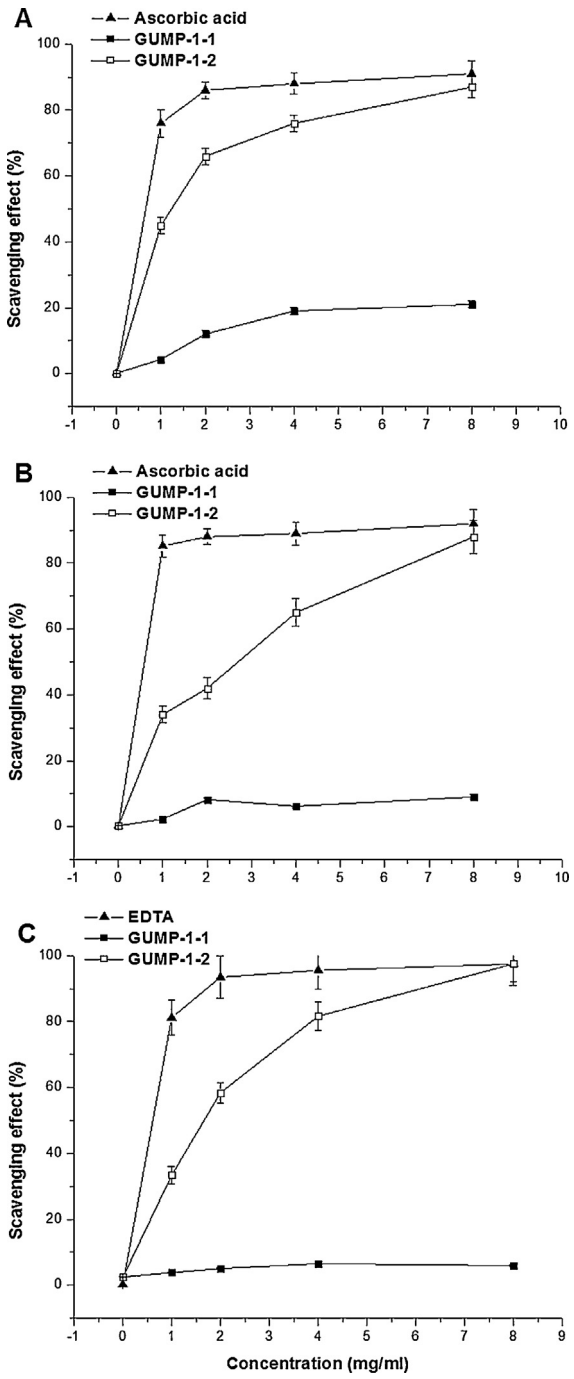

Fig. 2. The antioxidant activity of two polysaccharides fractions from *Grifola umbellata* mycelia in vitro. (A) Hydroxyl radicals scavenging abilities of GUMP-1-1 and GUMP-1-2; (B) superoxide radicals scavenging abilities of GUMP-1-1 and GUMP-1-2; (C) chelating abilities of GUMP-1-1 and GUMP-1-2 to ferrous ions. The values are presented as mean ± S.D. ($n = 3$).

3.3. Effect of two polysaccharides fractions on tumor growth and survival time in H22-bearing mice

To investigate the anti-tumor activity of two polysaccharides fractions in vivo, the mouse hepatoma H22 transplanted model was created by subcutaneous injection of H22 cells into BALB/c mice. The inhibitory effects of GUMP-1-1 and GUMP-1-2 are shown in Fig. 3A. GUMP-1-1 and GUMP-1-2 obviously reduced the tumor volume of H22 tumor-bearing mice, exhibiting the inhibition rate of 45.2% and 63.2%, respectively at the dose of 200 mg/kg, which

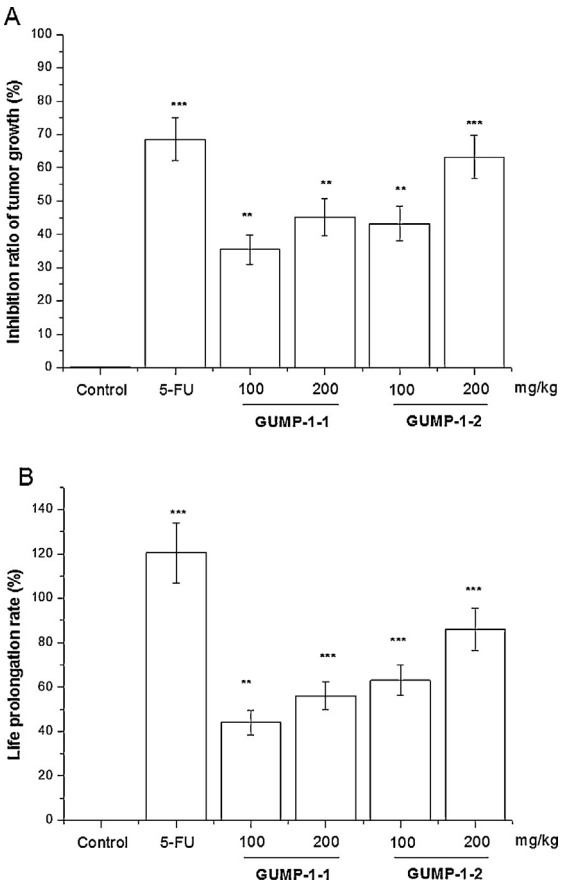

Fig. 3. Effect of two polysaccharides fractions from *Grifola umbellata* mycelia on the tumor volume and the survival time of H22-bearing mice. (A) Inhibition rate of tumor growth; (B) life prolonged rate. The values are presented as mean ± S.D. ($n = 10$). Significant differences compared to negative control group are designated as $^{**}p < 0.01$ and $^{***}p < 0.001$.

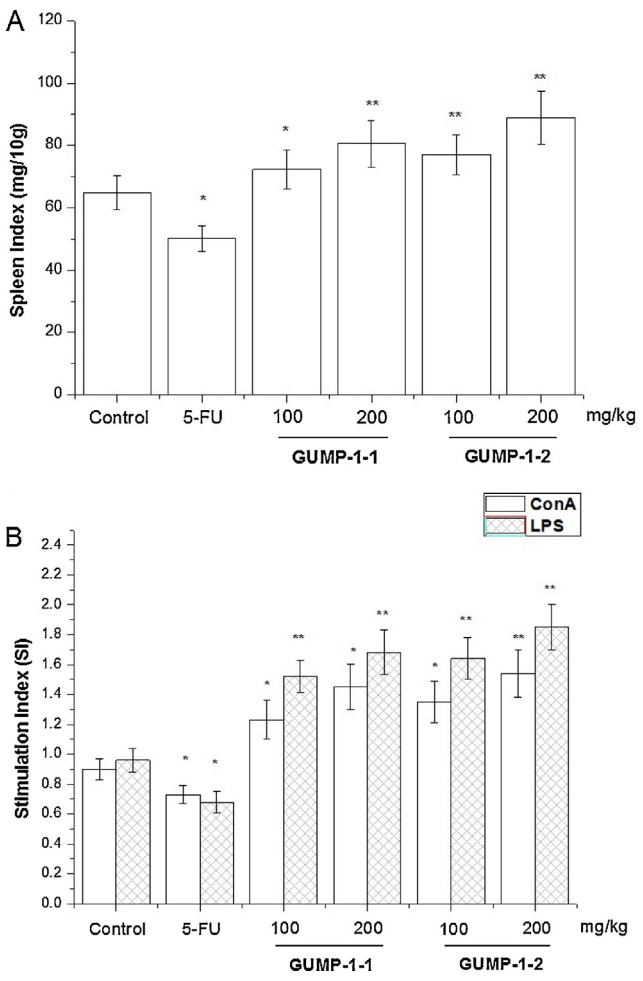

Fig. 4. Effect of two polysaccharides fractions from *Grifola umbellata* mycelia on spleen index and concanavalin A (Con A)- and lipopolysaccharide (LPS)-stimulated splenocyte proliferation of H22-bearing mice. (A) Spleen index; (B) splenocyte proliferation index (SI). The values are presented as mean ± S.D. ($n = 10$). Significant differences compared to negative control group are designated as $^{*}p < 0.05$ and $^{**}p < 0.01$.

indicated that GUMP-1-1 and GUMP-1-2 had significant antitumor activity in vivo against H22 tumor. More importantly, GUMP-1-1 and GUMP-1-2 significantly prolonged the survival period of H22-bearing mice, showing the life prolonged ratios of 56.3% and 85.8%, respectively at the dose of 200 mg/kg (Fig. 3B). As expected, 5-FU significantly prolonged the survival period of H22-bearing mice and suppressed tumor generation of the H22-tumor bearing mice. In addition, no significant loss of body weight, anorexia, listlessness, abdominal distention, liver function, renal function or other apparent adverse effects were observed among the animals, with the exception of 5-FU treated group (data not shown).

3.4. Effect of two polysaccharides fractions on spleen index and splenocyte proliferation in H22-bearing mice

It is reported that the anticancer activities of polysaccharide–protein complexes could be associated with their immunostimulating properties (Ooi & Liu, 2000). To investigate whether two polysaccharides fractions enhance humoral immunity or cell-mediated immunity, we measured the effect of GUMP-1-1 and GUMP-1-2 on spleen index and mitogen-stimulated splenocyte proliferation in tumor-bearing mice. As shown in Fig. 4A, an increase in relative spleen weight of GUMP-1-1 or GUMP-1-2-treated groups was observed, compared with control group ($p < 0.05$ and $p < 0.01$). While spleen index of 5-FU-treated group decreased significantly ($p < 0.05$). Furthermore Con A- and LPS-induced splenocyte proliferation was significantly enhanced by GUMP-1-1 or GUMP-1-2 at the doses of 100 and 200 mg/kg compared to the model control ($p < 0.05$ and $p < 0.01$) (Fig. 4B).

4. Conclusions

In the paper, two main polysaccharide fractions (GUMP-1-1 and GUMP-1-2) were purified from *G. umbellata* mycelia by fractionation on DEAE-52 cellulose and Sephacryl S-400 column chromatography. Chemical analysis indicated that two polysaccharide fractions contained different contents of neutral sugar, uronic acid and protein, as well as varying monosaccharide compositions and average molecular weight. These properties provided basis for the differences of antioxidant, antitumor and immunopotentiating activities of these polysaccharide fractions. Several in vitro assays, such as hydroxyl radical scavenging assay, superoxide radical scavenging assay, and chelating effects on ferrous ion, were applied to evaluate the antioxidant potential of the two polysaccharides. Among two fractions, GUMP-1-2 had the stronger free radicals scavenging and ferrous ion chelating abilities than GUMP-1-1, implying some factors including the content of uronic acid, protein content and molecular weight may influence their antioxidant activities. Furthermore, GUMP-1-1 and GUMP-1-2 could significantly inhibit the growth of H22 tumor in BALB/c mice and extended the survival time of mice bearing H22 tumor.

GUMP-1-1 and GUMP-1-2 could also remarkably increase the spleen weights and splenocyte proliferation of H22 tumor bearing mice, which indicated that they could improve the immune response. Interestingly, a high inhibition ratio of H22 tumor growth was observed in GUMP-1-2-treated group at very dose point compared to that of GUMP-1-2-treated group. This different antitumor potency probably depended on their monosaccharide composition, protein content, molecular mass and chain conformation. The above results suggested that the antitumor activity of these two polysaccharides might be achieved by improving immune response. However, the antitumor mechanisms of GUMP-1-1 and GUMP-1-2 remain unclear. Further studies are needed to elucidate the structure-function relationships and mechanisms responsible for its antitumor activities.

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