International Journal of Biological Macromolecules 95 (2017) 778–787

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Antioxidant, antibacterial and anti-aging activities of intracellular zinc polysaccharides from *Grifola frondosa* SH-05

Chen Zhang[a,b,1], Zheng Gao[b,1], Chunlong Hu[c,1], Jianjun Zhang[b], Xinyi Sun[b], Chengbo Rong[a,*], Le Jia[b]

[a] *Institute of Plant and Environment Protection, Beijing Academy of Agriculture and Forestry Sciences, Beijing Engineering Research Center for Edible Mushroom, Key Laboratory of Urban Agriculture (North), Ministry of Agriculture, Beijing, PR China*
[b] *College of Life Science, Shandong Agricultural University, Taian 271018, PR China*
[c] *College of Forestry, Shandong Agricultural University, Taian 271018, PR China*

ARTICLE INFO

Article history:
Received 8 September 2016
Received in revised form
24 November 2016
Accepted 1 December 2016
Available online 5 December 2016

Keywords:
Antioxidant and anti-aging activities
Histopathology assay
Antibacterial activities
Grifola frondosa SH-05
Intracellular zinc polysaccharides

ABSTRACT

In present work, the strain of *Grifola frondosa* SH-05 was used as a vector of zinc biotransformation to produce the IZPS. The bioactivities including antioxidant and antibacterial activities *in vitro* and anti-aging properties *in vivo* of IZPS were investigated comparing with the IPS. The results which were in consistent with the results of histopathology assay demonstrated that the IZPS had superior antioxidant and anti-aging activities by scavenging the hydroxyl and DPPH radicals, increasing enzyme activities, decreasing the MDA contents and ameliorating the anile condition of mice. Besides, the IZPS also showed potential antibacterial activities. The IZPS with higher bioactivities was composed of were Rha, Ino and Glu with a molar ratio of 4.7:3.6:1. These conclusions indicated that the IZPS might be a potential source of natural antioxidant, antibacterial agent and anti-aging agent.

1. Introduction

The free radicals, generated during metabolic processes of oxygen, play important roles in the homeostasis of organism. However, the uncontrolled free radicals are considered to be deleterious because they can induce physiological damage to organism and indirectly cause diseases, such as heart diseases, certain types of cancer and aging [1]. Antioxidants, due to the potential effects in scavenging free radicals and alleviating oxidative stress to prevent the physiological damage on organism, have received more and more academic attentions currently [2]. Nevertheless, the synthetic antioxidants, widely used over the past few decades, are suspected to have potential damage to liver, stomach, even causing cancer. Recently, researchers take more focus on finding the natural and effective antioxidants to replace the synthetic antioxidants [3]. At present, polysaccharides, extracted from species of *Ganoderma*, *Lentinula*, *Hericium*, *Pachyme*, *Tremella etc.*, have been widely exploited and used in health products [4–7]. Meanwhile, the antibacterial activities of polysaccharide are also worthy of further researches.

Grifola frondosa, a *Basidiomycete* fungus which classified in the order *Aphyllopherales* of the family *Polyporaceae*, is one of the most infrequent mushrooms owing to its widely medicinal use. Reports have indicated that polysaccharides from *G. frondosa* fruiting body has the properties of diabetes control action, blood pressure regulation, cholesterol reduction, liver protection, and activities of anti-tumor, anti-viral and anti-HIV [8–10]. These activities suggested that *G. frondosa* is precious in pharmacology and health care industry.

Zinc, one of the most important trace elements in humans and animals, has been found in hundreds of enzymes such DNA and RNA polymerase. Zinc plays important roles in protein synthesis, cell division and proliferation, and many other metabolic processes [8,11]. Furthermore, zinc has been reported to have antioxidant properties through a series of indirect mechanisms which can inhibit the formation of ROS and abatement oxidative damage in

Abbreviations: Ara, arabinose; BHI, brain heart infusion; DG, dentate gyrus; DPPH, 1,1-diphenyl-2-picrylhydrazyl; FAAS, flame atomic absorption spectrometry; GC, gas chromatography; Glu, glucose; Gal, galactose; HE, hematoxylin-eosin; IZPS, intracellular zinc polysaccharides; IPS, intracellular polysaccharides; Ino, inositol; MDA, malondialdehyde; Man, mannose; MIC, minimal inhibitory concentration; PDA, potato dextrose agar; ROS, reactive oxygen species; Rha, rhamnose; Rib, ribose; SOD, superoxide dismutase; T-AOC, total antioxidative capacity; Xyl, xylose.

* Corresponding author.
E-mail addresses: woshiboer@163.com (C. Rong), jiale0525@163.com (L. Jia).
[1] Equal contributors.

http://dx.doi.org/10.1016/j.ijbiomac.2016.12.003
0141-8130/

certain pathological conditions [12,13]. In recent years, nearly a quarter of population has the risk of zinc deficiency, but synthetic zinc supplements are suspected to be toxic. It seems quite necessary in finding safer and more effective ways to solve this problem [14].

In present study, the strain of *G. frondosa* SH-05 was used as a vector of zinc biotransformation to produce the IZPS. The monosaccharide composition, biological antioxidant activities *in vitro* and anti-aging *in vivo* properties of IZPS were investigated comparing with the IPS. The results suggested that *G. frondosa* SH-05 can be used as a potential means of producing natural and effective antioxidants.

2. Materials and methods

2.1. Chemicals and reagents

DPPH and hydrogen peroxide were from Sigma Chemicals Company (St. Louis, USA). T-AOC box was from Nanjing Jiancheng Bioengineering Institute. The standard monosaccharides were provided by Merck Company (Darmstadt, Germany) and Sigma Chemical Company (St. Louis, USA). All the other chemicals were of analytical grade and purchased from local chemical suppliers in China.

2.2. Microorganism and cultural conditions

The strain of *G. frondosa* SH-05 was preserved in our laboratory and maintained on PDA slants at 4 °C. After cultivated in PDA plates for 7 days at 25 °C, the liquid cultivation was processed in filter flasks containing 100 mL of (g/L) potato 200, glucose 10, KH_2PO_4 1.5, $MgSO_4{\cdot}7H_2O$ 1 and VB_1 0.005 on a rotary shaker (160 rpm, Anting, Shanghai, China) for 14 d. Four food-borne bacteria including *Escherichia coli*, *Staphylococcus aureus*, *Bacillus megateriumand* and *Listeria monocytogenes* were provided by the Resources & Environ. Microbiol. Lab. (Taian, China).

2.3. Optimization of Zn concentration

The liquid cultivation media with different zinc concentrations (including 25, 50, 75, 100, 150 and 200 mg/L) were applied in present experiment, and each concentration had five parallels. After cultivating for 14 d, mycelia was filtered, washed three times and dried at 55 °C to constant weight to determine the biomass (g/L). The sample treatment method was according to Zheng et al. [15] and the zinc contents were determined by FAAS (novAA300, Analytik Jena AG, Jena, Germany). The accumulated-rate of zinc was calculated by the following formula (1):

$$\text{Accumulatedrateofzinc}(\%) = (A_1 - A_0) \times m_1/m_0 \times 100 \quad (1)$$

Where A_1 was zinc concentration in zinc-enriched mycelium (%), A_0 was the zinc concentration in mycelium without Zn, m_1 was dry weight of zinc-enriched mycelium (g), and m_0 was weight of Zn in media (g). The mycelium biomass (g/L) and accumulated-rate of zinc (%) were conducted to optimize the Zn concentration.

2.4. Preparation of IZPS

The dried mycelia (10 g) were homogenized and dissolved in three-fold amount of distilled water at room temperature, then heated at 80 °C for 3 h. After centrifugation at 3000 rpm for 10 min, the supernatant was precipitated with ethanol (1:4, v/v) at −4 °C overnight. The precipitation was centrifuged (3000 rpm, 20 min), lyophilized and collected to yield IZPS or IPS. The content of polysaccharide was determined by phenol-sulfuric acid method, using Glu as standard [16].

2.5. Monosaccharide composition analysis

The IZPS and IPS (0.5 g) were hydrolyzed with 1.8 mL trifluoroacetic acid (2 M) for 4 h at 120 °C in a sealed tube, respectively. After refrigeration, the residual TFA was removed with methanol, and the hydrolyzate was acetylated with hydroxylamine-hydrochloride (12 M) and sodium borohydride-ammonium hydroxide [17]. The standard monosaccharides including Rha, Rib, Ara, Xyl, Ino, Man, Glu and Gal were processed in the same way for analyzed. Monosaccharide composition was analyzed by GC (GC-2010, Shimadzu, Japan) equipped with a capillary column of Rtx-1 (30 m × 0.25 mm × 0.25 μm) with the mehod of our present work [18].

2.6. Experiment of antioxidant activities in vitro

2.6.1. Hydroxyl radical-scavenging assay

The hydroxyl radical-scavenging activity was determined according to Smirnoff and Cumbes [19]. The reaction system, contained 1 mL ferrous sulfate (9 mM), 1 mL sodium salicylate-ethano (9 mM) and 1 mL IPS or IZPS (200–1000 mg/L), was started by adding 1 mL H_2O_2 (8.8 mM). After incubated at 37 °C for 0.5 h and centrifuged at 3000 rpm for 10 min, the absorbance was measured at 510 nm against distill water as blank. The hydroxyl radical-scavenging activity was expressed as:

$$\text{Scavengingactivity}(\%) = (A_0 - A_1)/A_0 \times 100 \quad (2)$$

Where A_0 was the absorbance of the control group (water instead of sample) and A_1 was the absorbance of the sample. The EC_{50} values (mg/L) were defined as the effective concentrations at which the hydroxyl radicals were scavenged by 50%.

2.6.2. Reducing power assay

The reducing power of IPS and IZPS was measured according to Oyaizu [20] with a slight modification. The reaction mixtures contained 1 mL IPS or IZPS (200–1000 mg/L), 2.5 mL phosphate buffer solutions (pH 6.6, 0.2 M), and 1 mL potassium ferricyanide (1%, w/v). After incubating at 50 °C for 20 min, 2 mL trichloroacetic acid (10%, w/v) and 1.2 mL ferric trichloride (0.01 g/L) were added to the mixture. Then the absorbance was measured at 700 nm against distilled water as blank. The EC_{50} value (mg/L) was defined as the effective concentration of the sample at which reducing power reached 0.5.

2.6.3. DPPH radical-scavenging assay

The DPPH radical-scavenging activity was determined according to the method of Sun and Ho [21]. The reaction mixture contained 2 mL DPPH-ethanol (0.1 mM) and 2 mL IPS or IZPS (200–1000 mg/L). After shaking vigorously and incubating in the dark for 30 min, the absorbance was measured at 517 nm against the blank group (mixture of 2 mL ethanol and 2 mL distilled water). The DPPH scavenging ability was expressed as:

$$\text{Scavengingability}(\%) = (1 - (A_i - A_j)/A_c) \times 100 \quad (3)$$

Where A_c was the absorbance of the mixture of 2 mL ethanol and 2 mL DPPH-ethanol, A_i was the absorbance of the solution of 2 mL sample and 2 mL DPPH-ethanol, and A_j was the absorbance of the mixture of 2 mL sample and 2 mL ethanol. The EC_{50} value (mg/L) was defined as the effective concentration of the sample at which DPPH radicals were inhibited by 50%.

2.6.4. Superoxide radical-scavenging activity

The superoxide radical-scavenging activity was processed using the method of Wei et al. [22]. The mixture of sample (1 mL) and

Tris-HCl buffer (2 mL, 50 mM, pH 8.2) were incubated at 25 °C for 20 min, then the 1,2,3-phentriol (0.4 mL, 5 mM) was added. After fully shaken, the absorbance of the homogeneous solution was measured at 325 nm. The superoxide radical-scavenging rate was evaluated according to the formula:

$$\text{Scavengingrate}(\%) = (A_0 - A_1)/A_0 \times 100 \quad (4)$$

Where A_0 was the absorbance of the blank and A_1 was the absorbance of the polysaccharide samples. The EC_{50} value (mg/L) was defined as the effective concentration of the sample at which superoxide anion radicals were inhibited by 50%.

2.6.5. Hydrogen peroxide-scavenging activity

The hydrogen peroxide-scavenging activity was determined according to the reported method [23] with sight modifications. The reaction mixture was composed of 2 mL of phosphate buffer (0.1 M, pH 7.4), 0.4 mL of H_2O_2 solution (0.3%), and 1 mL of sample solution. Absorbance of the reaction mixture at 234 nm was determined after 10 min. The scavenging rate of H_2O_2 was expressed as:

$$\text{Scavengingrate}(\%) = (A_0 - A_1)/A_0 \times 100 \quad (5)$$

Where A_1 was the absorbance of the polysaccharide samples group and A_0 was the absorbance of the blank group.

2.6.6. Ferrous ion chelating activity

The chelating effect on Fe^{2+} ions was measured according to the reference method [24] with some modifications. Briefly, the reaction mixture, containing 2 mL of sample of different concentration, 0.1 mL of ferrous chloride ($FeCl_2$), 0.4 mL of ferrozine solution (5 mM), and 5 mL of distilled water, was shaken well and incubated for 10 min at room temperature. The absorbance of the mixture was determined at 562 nm, and the Fe^{2+} chelating activity was calculated according to the following formula:

$$Fe^{2+}\text{ chelating activity}(\%) = (A_0 - A_1)/A_0 \times 100\% \quad (6)$$

Where A_1 was the absorbance of the polysaccharide samples group and A_0 was the absorbance of the blank group.

2.7. Antibacterial activity

2.7.1. Antibacterial zone assay

Three pathogenic bacteria including *S. aureus*, *E. coli*, *B. megaterium*, and *L. monocytogenes* were used for investigating antibacterial activities by determining bactericidal zone according to a previous report [25]. The IPS and IZPS were diluted in sterilized NaCl (0.85%, w/v). After activating in nutrient broth for 24 h at 37 °C, the bacterial suspensions (0.5 mL) were cultivated in the BHI agar plates. Holes in the plates were made with a sterile steel cylinder (Diameter = 10 mm). Two hundred microlitre aliquots of polysaccharides (10 mg/mL or 20 mg/mL) were filtrated by filters (0.22 μm), and dropped into the holes, against NaCl (0.85%) as the blank. The aerobic incubation was processed at 37 °C for 24 h, and the inhibition zone was measured and recorded. All procedures were performed in triplicate.

2.7.2. Minimal inhibitory concentration

The MIC was defined as the lowest concentration of samples which inhibited the visible growth of tested microorganisms. The IPS and IZPS were dissolved in sterilized NaCl (0.85%), and a series concentrations of polysaccharides including 10, 5, 2.5, 1.25 and 0.625 mg/mL) were prepared for MIC tests, respectively, using sterilized NaCl (0.85%) as the blank control. The procedure was carried out uniformly as the antibacterial assays. The minimal concentrations that generate the visual inhibition zones were regarded as MIC.

2.8. Experiment of anti-aging activities in vivo

Fifty-four male mice (Kunming strains), weighted 20 ± 2 g, were randomly separated into six groups of nine animals each. All mice were given sufficient food and water everyday. The normal group and model group were intragastrically administered with distilled water (0.01 mL/g), while the L-IPS, H-IPS, L-IZPS and H-IZPS groups were respectively treated with low (50 mg/kg) and high does (150 mg/kg) of IPS and IZPS by gastric gavage. At the same time, the normal group was injected physiological saline, while the other groups were intraperitoneally injected with D-galactose (150 mg/kg), respectively. At the end of the experiment (20 days), all the mice were sacrificed by exsanguinations under diethyl ether anesthesia to get the blood samples which were next anticoagulated by sodium citrate (stored at −2 °C). The samples of heart, liver, and kidney were collected respectively, and rapidly weighed, homogenized (1:6 w/v) in 0.2 M phosphate buffer (4 °C, pH 7.4). The homogenates were centrifuged (3000 rpm) for 15 min and the supernatants were stored at −20 °C for further analysis. The experiments were performed as approved by the institutional animal care and use committee of Shandong Agricultural University, and in accordance with the Animals (Scientific Procedures) Act. 1986 (amended 2013).

2.8.1. T-AOC determination

The T-AOC was analyzed by commercial kits which were purchased from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.8.2. SOD activity

The SOD activity was determined according to Bayer and Fridovich [26]. The reaction system contained 3.5 mL potassium phosphate buffer (pH 7.8, 0.05 M), 0.5 mL methionine (130 mM), 0.5 mL nitroblue tetrazolium (NBT, 750 μM), 0.5 mL disodium ethylene diamine tetraacetate (100 μM), 0.4 mL distilled water, 0.1 mL tissue sample, and 0.5 mL riboflavin (20 μM). The SOD activity was expressed as U (50% inhibition of photochemical reduction of NBT as 1 U) and calculated by

$$U = (A_0 - A_S)/A_0/50\% \times N \quad (7)$$

Where A_0 was the absorbance of control group, A_S was the absorbance of test group, and N was a constant.

2.8.3. MDA contents

The content of MDA was measured according to the method of Zhao et al. [27]. The mixture, contained 0.2 mL liver tissue sample and 2 mL 0.6% thiobarbituric acid (TBA, w/v), was heated in a boiling water for 15 min. After cooling rapidly, the mixture was centrifuged at 3000 rpm for 10 min, and the supernatant was collected for the determination of MDA contents according to the following formula.

$$\text{MDAcontents}(\text{mM}) = 6.45 \times (A_0 - A_1) - 0.56 \times A_2 \quad (8)$$

Where A_0–A_2 were the absorbance measured at 532 nm, 600 nm and 450 nm, respectively.

2.8.4. Histopathology analysis of brains

According to the method of Zhu et al. [28], the brains were removed and immersed in the 4% buffered neutral formalin overnight at 4 °C. Briefly, the specimens were processed through graded alcohols and xylene, embedded in paraffin blocks, cut into 5 mm sections and mounted on the glass slides. The sections were deparaffinating in xylene and rehydrated in graded ethanol. Finally, the slices were stained with HE staining or Nissl staining, washed with double distilled water, dehydrated in ethanol, cleaned with xylene, and examined with light microscopy.

Table 1
Antibacterial activity of inhibition zones of IPS and IZPS *in vitro*.

Microorganisms	Diameters of inhibition zone (mm)			
	H-IPS	L-IPS	H-IZPS	L-IZPS
E. coli	19.4 ± 2.3	13.2 ± 1.6	30.0 ± 4.3**	27.1 ± 1.8##
S. aureus	22.2 ± 3.1	18.1 ± 1.0	39.7 ± 2.5**	31.5 ± 1.3###
B. megaterium	16.3 ± 1.0	14.6 ± 1.9	26.3 ± 1.4**	22.1 ± 2.1#
L. monocytogenes	17.4 ± 1.2	15.5 ± 1.1	28.6 ± 3.2**	26.3 ± 1.5###

** Significant difference compare to H-IPS Group, $P < 0.05$.
# Significant difference compare to L-IPS Group, $P < 0.01$.
Significant difference compare to L-IPS Group, $P < 0.05$.
Significant difference compare to L-IPS Group, $P < 0.001$.

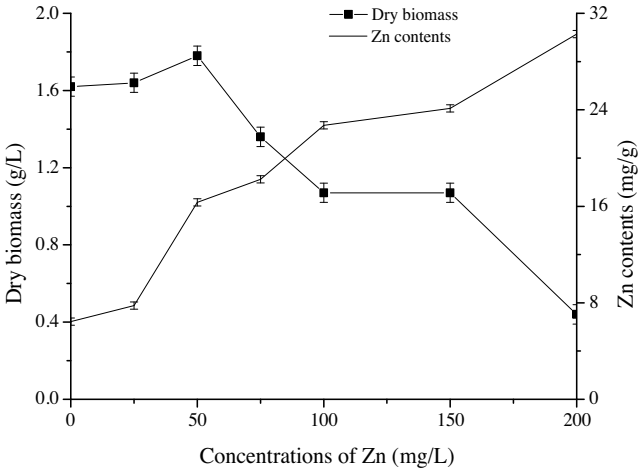

Fig. 1. Biomass and Zn content under different concentrations of Zn.

2.9. Statistical analysis

All experiments were carried out in triplicates and results were recorded as means ± SD (standard deviation). The results were analyzed by one-way analysis of variance (ANOVA, SPSS 16.0). $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Biomass, Zn content and monosaccharide composition

Through the atomic absorption, the Zn content in mycelia was demonstrated in Fig. 1. These results showed that Zn content in mycelia was correlated with the original concentrations of Zn. The Zn content of dry mycelia was improved with the increase of Zn concentration in liquid medium with dose-dependent. Meanwhile, the dry biomass of mycelia was increased at first and decreased dramatically after maximizing at the Zn concentration of 50 mg/L. The maximum biomass of the mycelia was 1.77 ± 0.07 g/L and the Zn content was 16.5 ± 0.03 mg/g. In order to obtain zinc mycelia with a reasonable production rate, 50 mg/L of Zn concentration in liquid medium was chosen to produce IZPS.

The result of monosaccharide composition was shown in Fig. 2. The percentage of each monosaccharide was calculated by the relevant peak area. Monosaccharide composition was confirmed using the retention time of chromatographic peak. Obviously, GC analysis revealed that IPS was composed of Rha, Ino and Man in a mass percentage of 67.0%, 22.4%, and 10.6% with a molar ratio of 6.9:2.1:1, while IZPS was composed of Rha, Ino and Glu in a mass percentage of 48.2%, 40.5% and 11.3% with a molar ratio of 4.7:3.6:1.

3.2. *Antioxidant ability* in vitro

As shown in Fig. 3A, both IZPS and IPS expressed the increased activities of scavenging hydroxyl radical significantly with the increase of samples concentration from 0 to 1000 mg/L. The hydroxyl radical scavenging rates of IZPS and IPS were 94.4 ± 2.8% and 87.9 ± 2.5%, respectively. Within the range of tested concentrations, the EC_{50} values of IZPS and IPS were 203.7 ± 6.8 mg/L and 510.3 ± 11.7 mg/L, indicating that IZPS had superior effects in scavenging hydroxyl radicals.

As could be seen from Fig. 3B, the reducing power of IPS and IZPS reached 0.59 ± 0.05 and 0.38 ± 0.11 at the concentration of 1000 mg/L, indicating IPS had higher reducing power ability.

The scavenging effects on DPPH radicals were presented in Fig. 3C. When compared with IPS, IZPS showed stronger scavenging capacity on DPPH radicals, and the EC_{50} level was 211.2 ± 12.6 mg/L. Meanwhile, the scavenging abilities of IZPS reached 67.85 ± 0.09%, which was 26.15 ± 0.23% higher than that of IPS at the concentration of 1000 mg/L.

The results of superoxide radical-scavenging activity examination were shown in Fig. 3D. Apparently, the superoxide radical-scavenging abilities of the three samples increased dose-dependently. At the concentration of 1000 mg/L, IPS and IZPS exhibited the scavenging effects of 39.21 ± 2.61% and 71.20 ± 4.02%, respectively. The EC_{50} value of IZPS was 525.27 ± 17.93 mg/L. As conclusion, the superoxide radical-scavenging activity of IZPS was stronger than that of IPS.

For the H_2O_2 scavenging activities, as exhibited in Fig. 3E, all polysaccharides demonstrated dose-dependent manners. Meantime, the IPS and IZPS showed superior H_2O_2 scavenging activity. At the concentration of 1000 mg/L, the scavenging rate of IPS and IZPS were 90.31 ± 4.24% and 95.23 ± 3.75%.

As demonstrated in Fig. 3F, the Fe^{2+} chelating activity of the two samples increased dose-dependently and presented a smooth growth. At the concentration of 1000 mg/L, the Fe^{2+} chelating rate of IPS and IZPS were 27.09 ± 2.61% and 50.92 ± 3.08%, respectively. IZPS showed better Fe^{2+} chelating activity than IPS.

3.3. *Antibacterial activity* in vitro

The *in vitro* antibacterial activities of the polysaccharide were shown in Table 1. The H-IZPS had the most potential effects in inhibiting the tested microorganisms by analyzing the inhibition zones of 30.0 ± 4.3 mm (*E. coli*), 39.7 ± 2.5 mm (*S. aureus*), 26.3 ± 3.4 mm (*B. megaterium*) and 28.6 ± 3.2 mm (*L. monocytogenes*). In addition, for each group, the antibacterial activities against the four tested microorganisms were in the order of *S. aureus* > *E. coli* > *L. monocytogenes* > *B. megaterium*, beyond that the inhibition zone of L-IPS on *E. coli* (13.2 ± 1.6 mm) was smaller than that of *L. monocytogenes* (15.5 ± 1.1 mm). For the same kind of bacteria, polysaccharides with the higher concentrations showed the stronger antibacterial activity. The results showed that the antibac-

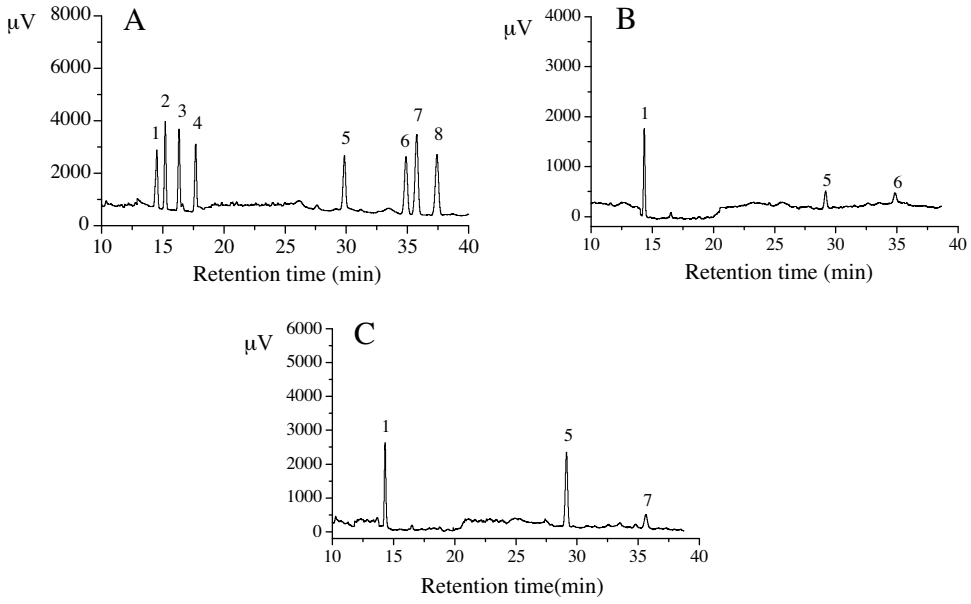

Fig. 2. GC chromatograms of monosaccharides composition with a HP-5 fused silica capillary column. (A) standard samples, (B) IPS, and (C) IZPS. (Peaks:1. Rha 2. Rib 3. Ara 4. Xyl 5. Ino 6. Man 7. Glu 8. Gal.).

Table 2
Minimal inhibitory concentration (MIC) of IPS and IZPS.

Microorganisms	Diameters of inhibition zone (mm)	
	IPS (mg/mL)	IZPS (mg/mL)
E. coli	5.0	1.25
S. aureus	2.5	<0.625
B. megaterium	10.0	2.5
L. monocytogenes	5	2.5

terial activities of IZPS were stronger than that of IPS, indicating that zinc-enriched could be an effective modification method to improve the antibacterial activities of IPS from *G. frondosa*. The results of MIC (Table 2) were consistent with the inhibition zones. According to the MIC analysis, IZPS was found to be more effective against four tested microorganisms which had the lowest MIC for *S. aureus* (<0.625 mg/mL), *E. coli* (1.25 mg/mL), *L. monocytogenes* (2.5 mg/mL), *B. megaterium* (2.5 mg/mL) than that of IPS.

3.4. *Anti-aging ability* in vivo

When compared to the normal group, the model group showed unnormal behaviors of slow-responsing, less-eating, and hair-lossing, and the death ratio was higher, suggesting the success of aging model construction. The mice in the control groups, which were given different concentrations of IPS and IZPS, showed better manners when compared with that in the model group.

The results of the T-AOC, SOD and MDA levels in liver and serum were distinctly exhibited in Table 3. In accordance with above reports, when compared with the normal group, the T-AOC and SOD activities of mice in model group decreased dramatically both in liver and serum, which were contrary to the levels of MDA. After 28 d of lavage administration, the liver and serum T-AOC activities of H-IPS (29.41 ± 1.34 U/g in liver and 15.16 ± 0.76 U/g in serum), L-IZPS (34.33 ± 0.70 U/g in liver and 19.03 ± 0.23 U/g in serum) and H-IZPS (31.46 ± 5.45 U/g in liver and 22.41 ± 0.54 U/g in serum) groups increased significantly ($P < 0.001$) compared with those of model group (19.27 ± 1.07 U/g in liver and 10.21 ± 0.33 U/g in serum), especially the H-IZPS group which was the nearest to those of the normal group. The administration of IPS and IZPS significantly ($P < 0.05$ or $P < 0.001$) enhanced SOD activities in dose-dependent manners, especially in H-IZPS group, the SOD activities increased by 60.09% comparing with the normal group. At the meantime, the liver and serum MDA contents of the model group reached 3.51 ± 0.32 mM/g and 7.74 ± 0.61 mM/g, which was 112.12% and 277.56% higher than the normal group respectively. The content of MDA in liver and serum were decreased significantly ($P < 0.001$) in the L-IZPS (2.16 ± 0.08 μM/L in liver and 4.03 ± 0.17 μM/L in serum) and H-IZPS (1.96 ± 0.12 μM/L in liver and 3.28 ± 0.19 μM/L in serum) groups than those in the model control group (3.51 ± 0.32 μM/L in liver and 7.74 ± 0.61 μM/L in serum). And for H-IZPS, the inhibition ratios of liver MDA and serum MDA were 38.46% and 47.29%, respectively.

3.5. *Histopathology analysis of brains*

Effects of IPS and IZPS on brains of aging mice were performed to investigate the morphology of necrotic cells, and the results were illustrated in Fig. 4. In the normal group, morphology of neurons in cortex (Fig. 4A) hippocampus region (Fig. 4B) remained intact, the nucleus and cytoplasm were centered with clear staining and the neurons remained well-arranged. In the model group, necrotic cells with loosened and vacuolar neural fiber (triangles and arrows) were arranged confused and disorderly. While, the extent of degeneration and necrosis of nerve cells in four does groups was lower at different degrees. Especially in the H-IZPS group, there was much higher number of intact neurons than those in the model group.

Current study revealed age-associated pathological changes in dentate gyrus (DG) with increased levels of stained dark and degenerative neurons, shrunken and irregular cytoplasm when compared to normal group, while H-IZPS administration showed a significant decrease in the number of dead cells. Furthermore, after H-IZPS administration, the neuronal density also slightly increased in this region of aging mice. (Fig. 5A) Meanwhile, a significant damage was observed in CA2 (Fig. 5B) region of the hippocampus proper in aging mice when compared to those in normal group. Increased levels of dark neurons and lack cytoplasmic staining cells (red arrows) were observed during aging. After gavage with the IZPS, the damage and deterioration of neurons was significantly alleviated.

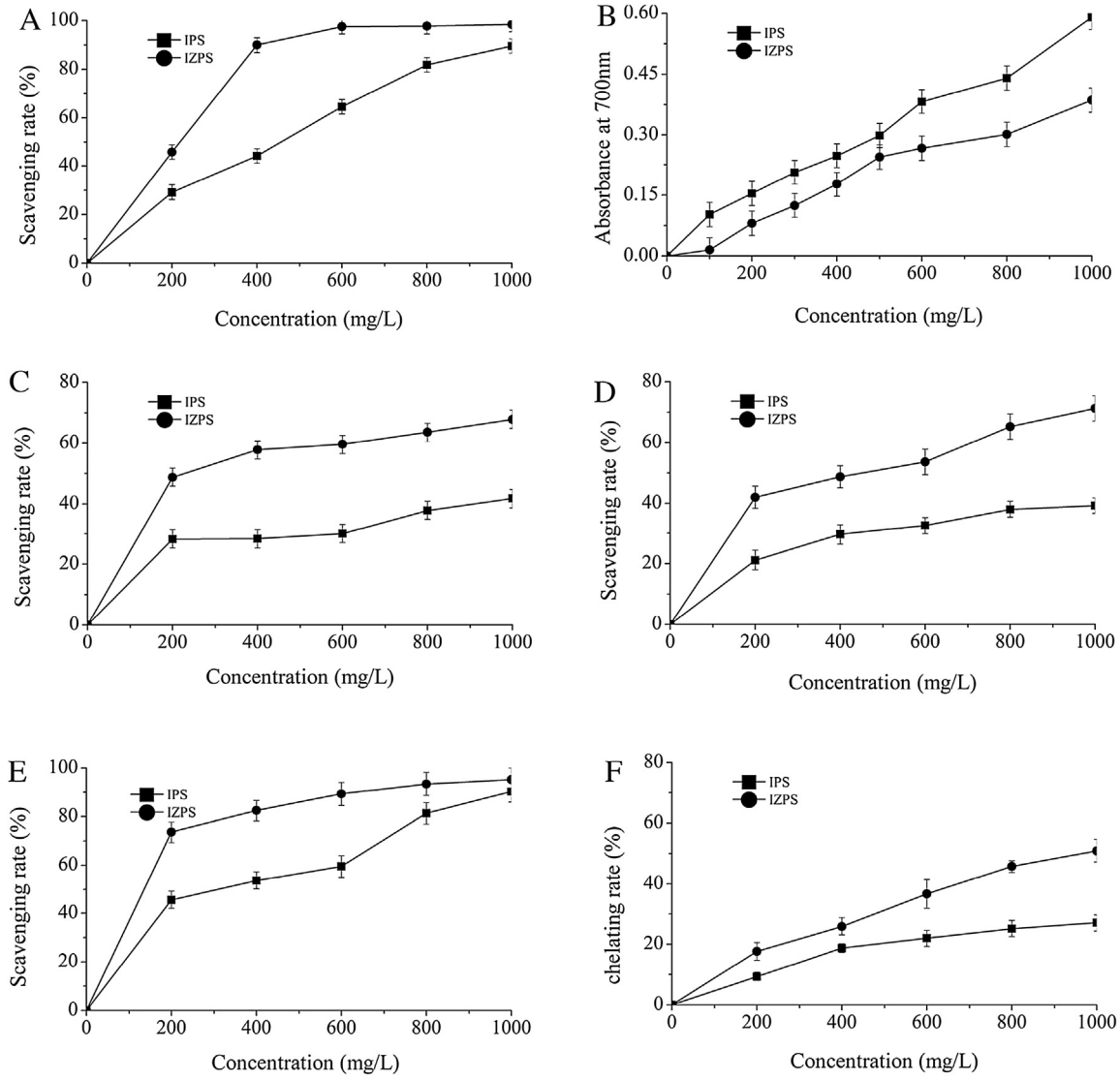

Fig. 3. The *in vitro* antioxidant activities of IPS and IZPS from *G. frondosa* SH-05. (A) Scavenging effect on hydroxyl radical, (B) Reducing power, (C) Scavenging effect on DPPH, (D) Scavenging effect on superoxide radical, (E) Scavenging effect on hydrogen peroxide and (F) Ferrous ion chelating activity.

Table 3
Anti-aging activities of IPS and IZPS *in vivo*.

Index	Normal	Model	L-IPS	H-IPS	L-IZPS	H-IZPS
Heart						
T-AOC (U/g)	17.52 ± 1.21	7.36 ± 0.52$^{###}$	9.94 ± 0.36***	12.37 ± 0.96***	13.59 ± 1.58***	14.26 ± 1.09***
SOD (U/g)	20.19 ± 1.18	12.76 ± 0.51$^{###}$	14.95 ± 0.71**	18.46 ± 0.85***	19.21 ± 0.93***	20.62 ± 1.01***
MDA (μM/L)	1.04 ± 0.02	2.32 ± 0.08$^{###}$	2.12 ± 0.13	1.77 ± 0.21**	1.54 ± 0.06***	1.31 ± 0.03***
Liver						
T-AOC (U/g)	35.62 ± 1.89	19.27 ± 1.07$^{###}$	31.18 ± 2.36***	29.41 ± 1.34***	34.33 ± 0.70***	31.46 ± 5.45***
SOD (U/g)	19.39 ± 0.12	11.4 ± 0.51$^{###}$	12.94 ± 0.74**	13.64 ± 0.61***	15.89 ± 0.33***	18.25 ± 0.11***
MDA (μM/L)	1.12 ± 0.09	3.51 ± 0.32$^{###}$	2.95 ± 0.13*	2.64 ± 0.26**	2.16 ± 0.08***	1.96 ± 0.12***
Kidney						
T-AOC (U/g)	43.42 ± 2.21	29.03 ± 1.61$^{###}$	34.39 ± 0.96***	32.37 ± 1.63***	35.29 ± 1.87***	37.12 ± 1.23***
SOD (U/g)	19.83 ± 1.21	11.61 ± 0.58$^{###}$	13.91 ± 0.67***	16.17 ± 0.84***	17.16 ± 1.34***	18.28 ± 1.62***
MDA (μM/L)	10.21 ± 0.55	20.51 ± 1.64$^{###}$	12.93 ± 1.36***	11.10 ± 0.33***	11.45 ± 0.50***	11.04 ± 0.89***
Serum						
T-AOC (U/g)	24.87 ± 0.72	10.21 ± 0.33$^{###}$	11.61 ± 0.57**	15.16 ± 0.76***	19.03 ± 0.23***	22.41 ± 0.54***
SOD (U/g)	140.16 ± 1.74	32.53 ± 0.15$^{###}$	111.39 ± 0.91***	132.3 ± 16.87***	128.94 ± 5.91***	138.67 ± 3.00***
MDA (μM/L)	2.05 ± 0.21	7.74 ± 0.61$^{###}$	5.16 ± 0.24***	4.09 ± 0.05***	4.03 ± 0.17***	3.28 ± 0.19***

$^{###}$ Significant difference compare to Normal Group, $P < 0.001$.
* Significant difference compare to Model Group, $P < 0.01$.
** Significant difference compare to Model Group, $P < 0.05$.
*** Significant difference compare to Model Group, $P < 0.001$.

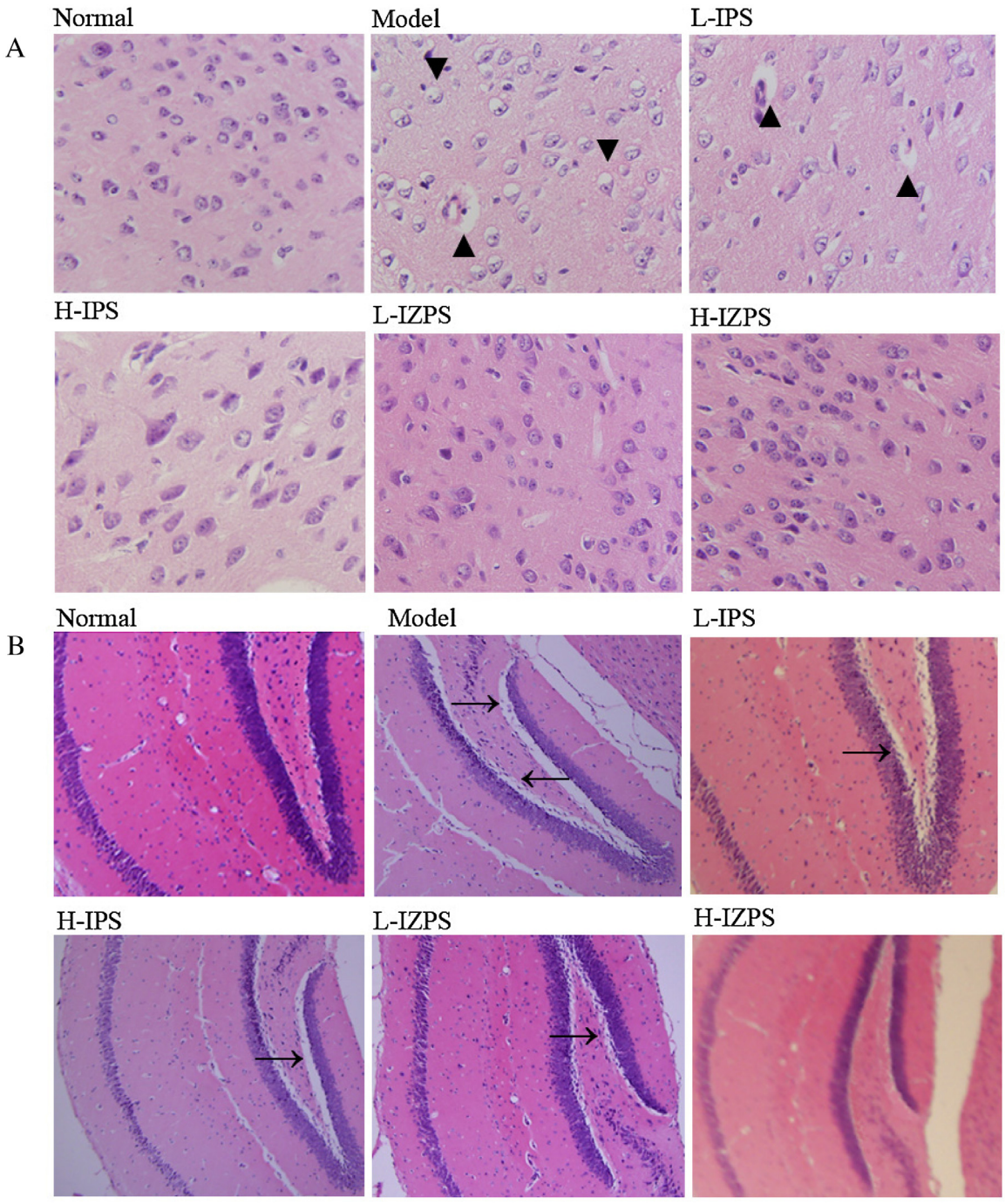

Fig. 4. Effects of IPS and IZPS on (A) the cortex of mice brain and (B) the hippocampus of mice brain with HE staining.

4. Discussion

Naturally, many fungi had the ability to accumulate trace elements [29,30] whatever environment they live, the ability to take up and enrich trace elements was species-specific and influenced by the availability of the elements [29]. In this experiment, the original resource of zinc was available and controlled by different concentrations of zinc sulphate, which has been considered as the best inorganic source [31,32]. Previous articles had reported that high concentrations of elements could inhibit mycelium growth and low concentrations might stimulate mycelium growth [33]. In present work, the zinc content of dry mycelia was improved with the increase of zinc concentration in liquid medium while the dry biomass was decreased. Recently, more attentions had been concerned about zinc-enriched polysaccharides owing to its higher antioxidant activity than regular polysaccharides [34]. However, there was no report about the polysaccharides from *G. frondosa* SH-05 submerged with zinc acetate.

Many literatures had demonstrated that the excess radicals played vital roles in accelerating the aging process. Hence, the radical scavenging abilities seemed to be important on the evaluations of antioxidant [35,36]. The excess hydroxyl radical and hydrogen peroxide in human body could attack biological molecules, such as lipids, proteins, enzymes, DNA and RNA, leading to cell or tissue injury which could also be produced artificially *in vitro* [37,38]. At the concentration of 200 mg/L, the scavenging rate of IZPS was

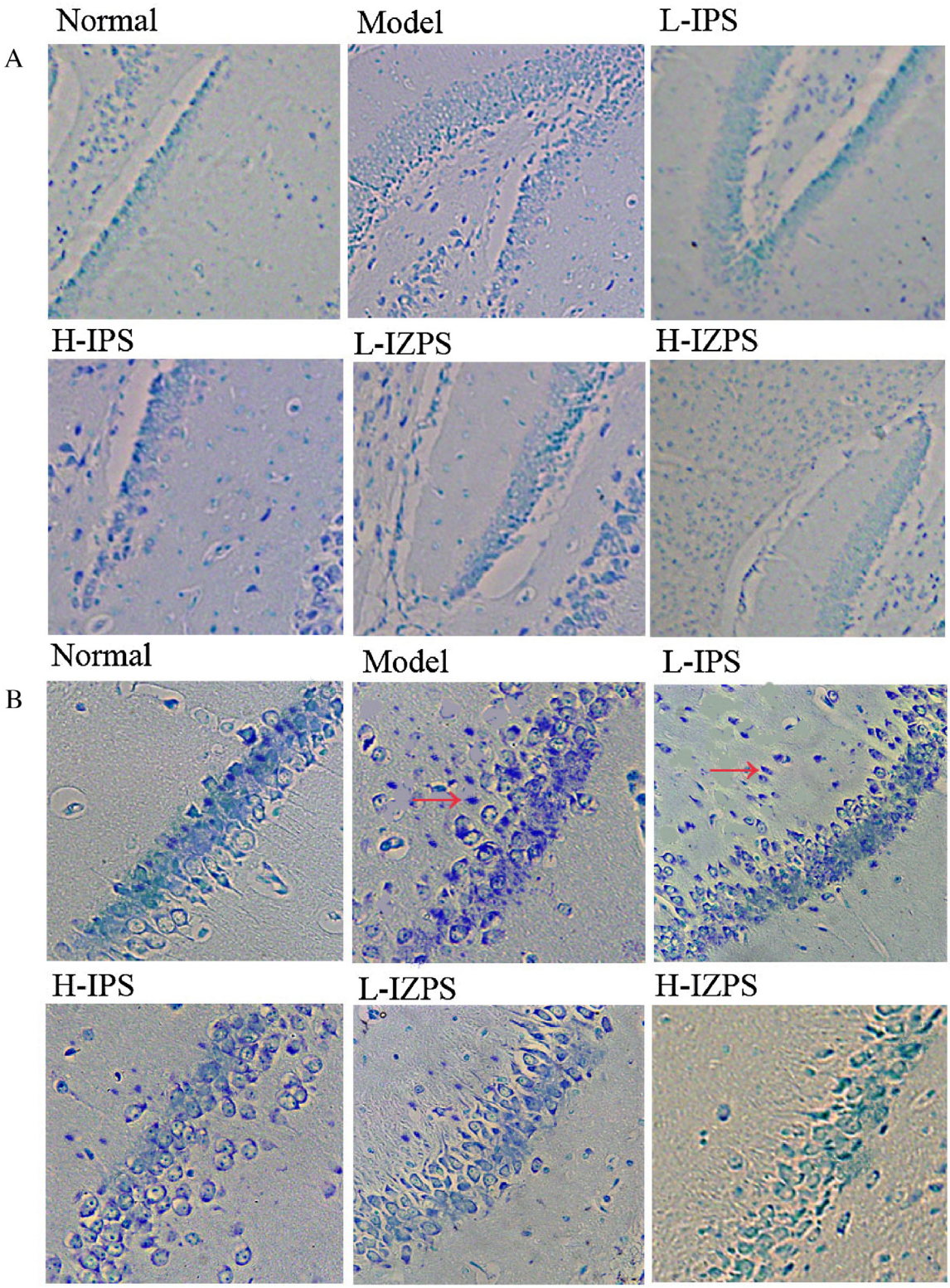

Fig. 5. Effect of IPS and IZPS on (A) DG of hippocampus and (B) the CA2 region of hippocampus in mice brain with Nissl staining. Red arrows showed the dark neurons. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

50%, while the concentration of IPS was 500 mg/L to achieve the same scavenging activity. The hydroxyl radical scavenging abilities of IPS and IZPS were much higher than polysaccharides from *Cordyceps sinensis* and *Ganoderma lucidum* [37,39]. The DPPH, a relatively stable radical, was widely used to investigate the scavenging activity of some antioxidants and many literatures had reported that fungi polysaccharides had the scavenging effects on DPPH radical [40–42]. Furthermore, Fe^{2+} chelation might ensue in important antioxidative effects by retarding metal-catalyzed oxidation because ferrous iron can stimulate lipid oxidation by generating reactive free radicals [43]. Currently, IZPS showed higher DPPH· scavenging ability and Fe^{2+} chelating ability than IPS. While at the

experiment of reducing power, IPS exhibited better capacity than IZPS which may due to the addition of element zinc had changed the properties of polysaccharides. Documented literatures had been reported that the element Zn could access with the polysaccharide to change the electron of the polysaccharides because zinc sulphate ($ZnSO_4$) had no antioxidant capacity [44]. Moreover, as description of Han et al. [45], the antioxidant properties of polysaccharides were mainly associated with their monosaccharide compositions. IZPS and IPS were heteropolysaccharides and the main sugar unit was Rha with the mass percentage of 67% and 48.2%. However, IZPS contained Glu and higher ratio of Ino when compared with IPS. The difference of their effects on the *in vitro* antioxidant activity might assist with the different monosaccharide composition and the concentration of Zn.

It had been reported that polysaccharides extracted from mushrooms or spent mushroom substrate had antibacterial activities on food-borne pathogenic microorganisms [46,47]. However, there was scarcely published report about antibacterial activities of polysaccharides and zinc-enriched polysaccharides from *G. frondosa*. Currently, the precise antibacterial mechanism of the polysaccharide was still unclear. It was proposed that the polysaccharides could disrupt the bacterial cell walls and cytoplasmic membranes leading the dissolution of proteins and leakages of essential molecules [47]. In this work, the results indicated that the antibacterial activities of IPS were significantly ($P < 0.05$) improved after zinc-rich, suggesting that zinc-enrichment might be an effective modification method to improve the bioactivities of polysaccharides [48].

In present work, we further examined the anti-aging activities *in vivo* of the IPS and IZPS for the investigation of zinc in increasing the antioxidant properties. It had been reported that the D-galactose had potential effects in inducing the senility of animals in laboratory with lower immune system [49]. Presently, we successfully built the aging model as the data shown in Table 1. As free radicals and lipid peroxidation had been associated with a number of diseases [50,51], the T-AOC activities, SOD activities and MDA contents were determined. The T-AOC represented the non-enzyme original antioxidative capacity in the body which could reduce the ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}), which could combine with phenanthroline and produced a stable chelate monitored by spectrophotography at 520 nm [52]. The SOD, converted the superoxide radical to H_2O_2, formed the first antioxidant enzyme defense system against reactive oxygen species. The lipid peroxidation was evaluated by measuring the formation of MDA, the main decomposition product of peroxides derived from polyunsaturated fatty acids [52,53]. Compared with the normal group, the T-AOC and SOD levels of the model group decreased significantly while the contents of MDA increased prominently both in liver and serum. Moreover, the administration of IPS and IZPS could cause significant increase in SOD and T-AOC activities, as well as decrease in MDA contents, indicating that IPS and IZPS had potential abilities in scavenging various types of free radicals and their products. Meanwhile, the comparison between IPS and IZPS concluded that the latter showed superior performance on anti-aging and antioxidant activities (Table 3). However, detailed characterizations of IPS and IZPS should be explored in further study, such as the bond types and the detailed mechanism of their antioxidation.

During the process of aging, the most susceptible organ was hippocampus, which played an important role in spatial learning and memory [54]. Significant damage and deterioration in hippocampus of brain was observed in D-galactose induced aging mice. After gavage with the IZPS, the damage and deterioration in hippocampus was significantly alleviated in H-IZPS groups. The results were consistent with the examination of the anti-aging activities *in vivo* (Table 3).

5. Conclusion

In present work, the IZPS extracted from *G. frondosa* showed higher antioxidant activities *in vitro* and anti-aging capability *in vivo* than IPS, indicated that the IZPS was more valuable for future applications. Results also showed that zinc-enrichment enhanced the antibacterial activities of IPS. The superior performance of IZPS might attribute to its monosaccharide compositions. The current study demonstrated a process to enrich trace element from inorganic to organic for better absorption without toxicity, which provided a novel way to supplement zinc for bioavailability.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

The authors gratefully acknowledge the financial supports by Mushroom Technology System of Shandong Province (SDAIT-07-05).

References

[1] H. Barry, Annu. Rev. Nutr. 16 (1996) 33–50.
[2] L. Ronald Prior, X.L. Wu, Am. J. Biomed. Sci. 5 (2) (2013) 126–139.
[3] B. Li, S. Liu, R. Xing, K.C. Li, R.F. Li, Y.K. Qin, X.Q. Wang, Z.H. Wei, P.C. Li, Carbohydr. Polym. 92 (2013) 1991–1996.
[4] X.W. Zhou, J. Lin, Y.Z. Yin, J.Y. Zhao, X.F. Sun, K.X. Tang, A.J.C.M 35 (4) (2007) 559–574.
[5] Y.W. Chen, D.J. Hu, K.L. Cheong, J. Li, J. Xie, J. Zhao, S.P. Li, J. Pharm. Biomed. 78–79 (9) (2013) 176–182.
[6] Z.J. Wang, D.H. Luo, Z.Y. Liang, Carbohydr. Polym. 57 (2004) 241–247.
[7] H.C. Lo, F.A. Tsai, P.W. Solomon, J.G. Yang, B.M. Huang, Life Sci. 78 (2006) 1957–1966.
[8] M.B. Jeremy, Y.G. Shi, Science 23 (1996) 1081–1085.
[9] N.A. Talpur, B.W. Echard, A.Y. Fan, O. Jaffari, D. Bagchi, H.G. Preuss, Mol. Cell Biochem. 237 (2002) 129–136.
[10] I.Y. Bae, H.Y. Kim, S. Lee, H.G. Lee, Carbohydr. Polym. 83 (2011) 1298–1302.
[11] C. Andreini, I. Bertini, J. Inorg. Biochem. 111 (2012) 150–156.
[12] Y.M. Harmaza, E.I. Slobozhanina, Biophys. Asp. Biophys. 59 (2014) 322–337.
[13] W.E. Stehbens, Exp. Mol. Pathol. 75 (2003) 265–276.
[14] W. Maret, H.H. Sandstead, J. Trace Elem. Med. Biol. 20 (2006) 3–18.
[15] L. Zheng, M. Liu, G.Y. Zhai, Z. Ma, L.Q. Wang, L. Jia, J. Sci. Food Agric. 95 (15) (2015) 3117–3126.
[16] M.F. Chaplin, J.F. Kennedy, Carbohydrate Analysis: A Practical Approach, IRL Press Ltd, New York, 1994.
[17] L. Lennart, P.E. Jansson, J. Chromatogr. A 767 (1–2) (1997) 325–329.
[18] Z. Ma, C. Zhang, X. Gao, F.Y. Cui, J.J. Zhang, M.S. Jia, S.H. Jia, L. Jia, Int. J. Biol. Macromol. 73 (2015) 236–244.
[19] N. Smirnoff, Q.J. Cumbes, Phytochemistry 28 (4) (1989) 1057–1060.
[20] M. Oyaizu, J.S.N.D 44 (6) (1986) 307–316.
[21] T. Sun, C.T. Ho, Food Chem. 90 (2005) 743–749.
[22] Q. Wei, X.H. Ma, Z. Zhao, S.S. Zhang, S.C. Liu, J. Anal. Appl. Pyrol. 88 (2010) 149–154.
[23] S.A. Ganie, E. Haq, A. Masood, A. Hamid, M.A. Zargar, Evid. Based Complement. Altern. Med. 8 (2011) 1–12.
[24] T.C.P. Dinis, V.M.C. Madeira, L.M. Almeida, Arch. Biochem. Biophys. 315 (1994) 161–169.
[25] D. Kremer, I. Kosalec, M. Locatelli, F. Epifano, S. Genovese, G. Carlucci, M. Zovko Končić, Food Chem. 131 (4) (2012) 1174–1180.
[26] W.F. Bayer, I. Fridovich, Anal. Biochem. 161 (1987) 559–566.
[27] S.J. Zhao, T.C., Li, L.P. Zhu, China Agricultural Technology Press, Beijing (2002), P 142–143.
[28] X.Y. Zhu, P.S. Ma, W. Wu, R. Zhou, Y.J. Hao, Y. Niu, T. Sun, Y.X. Li, J.Q. Yu, Brain Res. Bull. 124 (2016) 295–305.
[29] U. Thomet, E. Vogel, U. Krähenbühl, Eur. Food Res. Technol. 209 (1999) 317–324.
[30] M. Tuzen, Microchem. J. 74 (2003) 289–297.
[31] L. Rojas, L. McDowell, R. Cousins, F. Martin, N. Wilkinson, A. Johnson, J.B. Velasquez, J. Trace Elem. Med. Biol. 10 (1996) 139–144.
[32] K. Beutler, O. Pankewycz, D. Brautigan, Bio. Trace Elem. Res. 61 (1998) 19–31.
[33] D. Figlas, M. Oddera, N. Curvetto, J. Med. Food 13 (2) (2010) 469–475.
[34] J.J. Zhang, Z. Ma, L. Zheng, G.Y. Zhai, L.Q. Wang, M.S. Jia, L. Jia, Carbohydr. Polym. 111 (2014) 947–954.
[35] E. Cadenas, K.J. Davies, Free Radic. Biol. Med. 29 (3–4) (2000) 222–230.
[36] A. Sanz, R.K. Stefanatos, Curr. Aging Sci. 1 (1) (2008) 10–21.
[37] C.H. Dong, Y.J. Yao, Food Sci. Technol. –Brazil 41 (2008) 669–677.

[38] C.L. Ye, Q. Huang, Carbohydr. Polym. 89 (4) (2012) 1131–1137.
[39] X.P. Chen, Y. Chen, S.B. Li, Y.G. Chen, J.Y. Lan, L.P. Liu, Carbohydr. Polym. 77 (2009) 389–393.
[40] L.P. Fan, J.W. Li, K.Q. Deng, L.Z. Ai, Carbohydr. Polym. 87 (2012) 1849–1854.
[41] W.C. Zeng, Z. Zhang, H. Gao, L.R. Jia, W.Y. Chen, Carbohydr. Polym. 89 (2012) 694–700.
[42] R.S. Lin, H.H. Liu, S.Q. Wu, L.F. Pang, M.S. Jia, K.M. Fan, S.H. Jia, L. Jia, Int. J. Biol. Macromol. 51 (2012) 153–157.
[43] S.M. Sabir, S.D. Ahmad, A. Hamid, M.Q. Khan, M.L. Athayde, D.B. Santos, A.A. Boligonb, J.B.T. Rocha, Food Chem. 131 (2012) 741–747.
[44] P.A.F. Vieira, D.C. Gontijo, B.C. Vieira, E.A.F. Fontes, L.S. Assunção, J.P.V. Leite, M.G.A. Oliveira, M.C.M. Kasuya, Food Sci. Technol. 54 (2) (2013) 421–425.
[45] Q. Han, Q. Yu, J. Shi, C. Xiong, Z. Ling, P. He, J. Food Sci. 76 (2011) C462–C471.
[46] S.Q. Li, N.P. Shah, Food Chem. 165 (2014) 262–270.
[47] F. He, Y. Yang, G. Yang, L. Yu, Food Control 21 (9) (2010) 1257–1262.
[48] Y. Feng, W. Li, X. Wu, L. He, S. Ma, Carbohydr. Polym. 82 (3) (2010) 605–612.
[49] G.Q. Gong, F.B. Xu, J. China Pharm. Univ. 22 (2) (1991) 11–13.
[50] S. Perrone, M.L. Tataranno, S. Negro, M. Longini, B. Marzocchi, F. Proietti, F. Iacoponib, S. Capitanib, G. Buonocore, Early Hum. Dev. 86 (2010) 241–244.
[51] M. Valko, D. Leibfritz, J. Moncol, M.T.D. Cronin, M. Mazur, J. Telser Int. J. Biochem. Cell Biol. 39 (2007) 44–84.
[52] N. Xiao, X.C. Wang, Y.F. Diao, R. Liu, K.L. Tian, Shock 21 (3) (2004) 276–280.
[53] I.N. Zelko, T.J. Mariani, R.J. Folz, Free Radic. Biol. Med. 33 (3) (2002) 337–349.
[54] P.F. Jobim, T.R. Pedroso, A. Werenicz, R.R. Christoff, N. Maurmann, G.K. Reolon, N. Schröderc, R. Roesler, Behav. Brain Res. 228 (1) (2012) 151–158.