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Characterization and antioxidant activities of extracellular and intracellular polysaccharides from *Fomitopsis pinicola*

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ABSTRACT

Fomitopsis pinicola (*F. pinicola*) is a kind of medicinal fungi, and few studies has been carried out on *F. pinicola* polysaccharides from liquid submerged cultivation. The characterization and antioxidant activities of extracellular polysaccharide (EPS) and intracellular polysaccharide (IPS) isolated from *F. pinicola* were investigated. The results showed that the molecular weight of EPS was 2.30×10^4 Da, and EPS was composed of mannose, rhamnose, xylose and galactose with the molar ratio of 0.1:1.0:0.3:0.5. The molecular weight of IPS was 4.07×10^5 Da, and the monosaccharide compositions included glucose, mannose, rhamnose, xylose and galactose with the molar ratio of 1.0:0.9:0.9:0.8:1.1. Antioxidant activities of both EPS and IPS including *in vitro* scavenging activities on 1-diphenyl-2-picrylhydrazyl (DPPH) and hydroxyl radicals, cellular protective effects on yeast cells from ultraviolet (UV) radiation and H_2O_2 oxidative damage were tested. Both EPS and IPS showed antioxidant activities in a dose dependent manner, and IPS had higher antioxidant activity than EPS. So EPS and IPS could be potential novel antioxidants for functional food.

1. Introduction

Reactive oxygen species (ROS) and free radicals are produced with the normal metabolism of oxygen in human body (Serviddio, Bellanti, & Vendemiale, 2013), and their high level especially under environmental stress conditions often cause tissue injury and diseases (Sies, 1997). Exotic antioxidants are usually employed to reduce the damage of free radical species to human body.

In recent years, medicinal edible fungi have been a research hotspot for exploiting natural antioxidants. Polysaccharides from fungi are usually being involved in scavenging free radicals and ROS (Ge, Duan, Fang, Zhang, & Wang, 2009; Sun et al., 2009). Some investigations have proved that polysaccharides from medicinal fungi (*Cordyceps sinensis, Agaricus bisporus, Dictyophora indusiata*) had significant antioxidant activity on 1-diphenyl-2-picrylhydrazyl (DPPH), superoxide anion and hydroxyls radical et al. (Huang, Siu, Wang, Cheung, & Wu, 2013). *Fomitopsis pinicola* (*F. pinicola*) is a member of basidiomycetes fungus, which is widely used as a medicinal mushroom in Asia (Cheng, Lin, Lur, Chen, & Lu, 2008). Choi, Park, Ding, and Cha (2007) has investigated the biological activities of *F. pinicola* extract by examining the antioxidant and antitumor activities *in vitro* and *in vivo*. Cheng et al. (2008) also conducted a thorough research on polysaccharide and ethanolic extract of cultured *F. pinicola* in regulating angiogenic process and inflammation. Although there have been several relevant reports about properties and biological activities of extract from *F. pinicola* fruiting body, few studies has been carried out on the comparison of characteristics and antioxidant activities of *F. pinicola* extracellular polysaccharide (EPS) and intracellular polysaccharide (IPS). Significant differences in structure or composition are expected in polysaccharides from different sources (Hsieh, Hsu, & Yang, 2005). In this study, the characterization and antioxidant activities of *F. pinicola* polysaccharides including IPS and EPS were investigated. Moreover, the protective effects of IPS and EPS on oxidative damage of yeast cells were also evaluated.

2. Materials and methods

2.1. Materials

F. pinicola used in this study was provided by the Quartermaster Equipment Institute of General Logistics Department of People's

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Liberation Army. The strain was inoculated at petri plate containing potato dextrose agar for 7 days at 30 °C. *F. pinicola* fruiting body was obtained from Changbaishan in Jilin province, China. Butylated hydroxytoluene (BHT) was purchased from Solarbio Co. Ltd (Beijing, China). 1-phenyl-3-methyl-4-benzene formyl pyrazolone (PMP) was from Ziyi Bio. Co. Ltd (Shanghai, China). 1-Diphenyl-2-picrylhydrazyl (DPPH) and reference monosaccharides (D-glucose, D-xylose, L-arabinose, D-mannose, L-rhamnose, D-galactose) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Preparation of crude polysaccharide extract

IPS was extracted twice from *F. pinicola* fruiting body with hot water at 70 °C in a 1:30 (w/v) ratio for 2 h, and then four volumes of ethanol were added and precipitated overnight at 4 °C. The precipitate was collected by centrifugation at 10,000 × *g* for 15 min and lyophilized, thus yielded a crude polysaccharide extract. In the preparation of EPS, fermentation medium (sucrose 5%, yeast extract 1%, KH_2PO_4 0.2%, $MgSO_4{\cdot}7H_2O$ 2%, w/v) was inoculated, and then incubated for 5 d. Similar to IPS, the fermentation broth was processed by concentration, precipitation, centrifugation and lyophilization to obtain crude polysaccharide extract.

2.3. Isolation and purification of the polysaccharides

Both the crude EPS and IPS (100 mg each) were dissolved in 10 mL of distilled water and centrifuged. The supernatant was added onto an anion exchange column (2.6 cm × 50 cm) with DEAE-cellulose. Eluting process was performed with a linear gradient from 0 to 0.5 mol/L NaCl at a flow rate of 1 mL/min. Total sugar content of the eluent was determined by phenol-sulfuric acid method (DuBois, Gilles, Hamilton, Rebers, & Smith, 1956). The fractions eluted with linear gradient of NaCl solution were pooled, desalted and further purified on a Sephadex G-200 column eluting with distilled water at 0.5 mL/min. The major polysaccharide fractions were pooled and lyophilized. From this process, the purified polysaccharides were obtained for further identification of structure and monosaccharide compositions.

2.4. Analysis of monosaccharide compositions

Monosaccharide compositions were determined by high performance liquid chromatography (HPLC) after pre-column derivatization (Sun et al., 2009). In brief, 50 mg of purified polysaccharide powder was hydrolyzed with 2 mol/L of trifluoroacetic acid aqueous solution at 120 °C for 6 h in a sealed tube. After the hydrolysis was completed, the excess acid was removed by co-distillation with methanol to yield dry hydrolysate, which was dissolved in 0.3 mol/L of NaOH (100 μL), then 0.5 mol/L of methanol solution (200 μL) of PMP was added. The resulted solution was incubated at 70 °C for 1 h. After derivatization, the solution was cooled to room temperature followed by adjustment of pH to neutral with 0.3 mol/L HCl, then 1.5 mL of distilled water was added by vigorously shaking. Finally, the mixture was extracted with chloroform three times, and the aqueous phase was filtered through a 0.22 μm nylon membrane (Westborough, MA, USA). The resulted solution (10 μL) was injected onto a C18 column (4.6 mm × 250 mm) connected with a DAD-UV detector (Agilent Technologies, USA). The mobile phase was a mixture of 0.1 mol/L KH_2PO_4 (pH 10)–acetonitrile (83:17) at a flow rate of 1.0 mL/min. The column temperature was 30 °C. Sugar identification was done by comparison with reference monosaccharides (D-glucose, D-xylose, L-arabinose, D-mannose, L-rhamnose, D-galactose). Calculation of the molar ratio of monosaccharides was based on the peak area of the monosaccharides.

2.5. Determination of molecular weight

The molecular weights of EPS and IPS were determined on an Agilent 1200 HPLC system equipped with an evaporative light scattering detector (ELSD) and TSK-gel G5000 PWXL (7.8 mm × 30 cm, TOSOH Corp., Japan). The column was eluted with double-distilled water at a flow rate of 0.8 mL/min. Standard dextrans (T10, T40, T70, T100, T380, T500, Sigma, USA) were used for molecular weight measurement.

2.6. Infrared spectroscopy

The Fourier-transform infrared (FTIR) spectrum of the polysaccharides was detected on a Bruker-Vector 22 spectrometer (German). The polysaccharides were mixed with KBr powder, ground and pressed into 1 mm pellets in the frequency range of 4000–500 cm^{-1}.

2.7. Assay of antioxidant activities in vitro of EPS

2.7.1. DPPH radical scavenging activity

The methods of Shimada et al. was employed with some modifications (Shimada, Fujikawa, Yahara, & Nakamura, 1992). Different concentrations of EPS and IPS (2.0–10.0 mg/mL) were prepared with distilled water. A total of 0.5 mL each sample with different concentrations was mixed with 1.5 mL of 0.4 mmol/L methanol solution of DPPH, and the mixed solution was reacted for 30 min in darkness. Then absorbance was measured at 517 nm with BHT as control. The activity of scavenging the DPPH radical was calculated using the following equation: $Y\% = [1 - (A_1 - A_2)/A_0] \times 100$, where A_0 was the absorbance of the blank group (distilled water + DPPH), A_1 was the absorbance of the sample reaction (sample + DPPH), and A_2 was the background absorbance of the sample (distilled water replaced DPPH).

2.7.2. Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity was measured according to Fenton method described before (Zhong, Jin, Lai, Lin, & Jiang, 2010). Polysaccharides of different concentrations (2.0–10.0 mg/mL) were prepared. Then added 5 mmol/L of $FeSO_4$ (2 mL), 5 mmol/L of salicylic acid-ethanol solution (2 mL), 2 mL of polysaccharide sample solution sequentially. Then the reaction started with 5 mmol/L H_2O_2 (2 mL) in water bath at 37 °C. Absorbance was measured at 510 nm with distilled water as reference and BHT as positive control. The hydroxyl radical scavenging effect was calculated as follows: $Y\% = [1 - (A_1 - A_2)/A_0] \times 100$, where A_0 was the absorbance of blank, A_1 was the absorbance of the sample, and A_2 was the background absorbance of the sample.

2.7.3. Protective effect on yeast cells from UV

Firstly, the yeast cells were cultured to the early stage of the logarithm in yeast extract peptone dextrose medium. Then 20 mL of fermentation broth was centrifuged for 5 min at 4000 × *g*, and the supernatant was discarded for yeast paste collection. Then the cells were washed with phosphate buffer solution twice, and the yeast paste was mixed with 20 mL of saline. A total of 1 mL yeast suspension was mixed with 4 mL of different concentrations of EPS and IPS (5–25 mg/mL) in petri dish. Then the cells were disposed with UV radiation for lethal time (1 min treatment could kill all yeast cells in the control group) respectively, then the blank group was mixed with distilled water. Finally, the mixture after UV radiation were diluted to appropriate concentration for spread plate viable count method at 28 °C for 2 d with BHT as control. The survival rate was calculated as follows: $Y\% = [(A_0 - A_1)/A_0 \times 100]$, where A_0 was the amount of cells which had not been disposed by UV radiation,

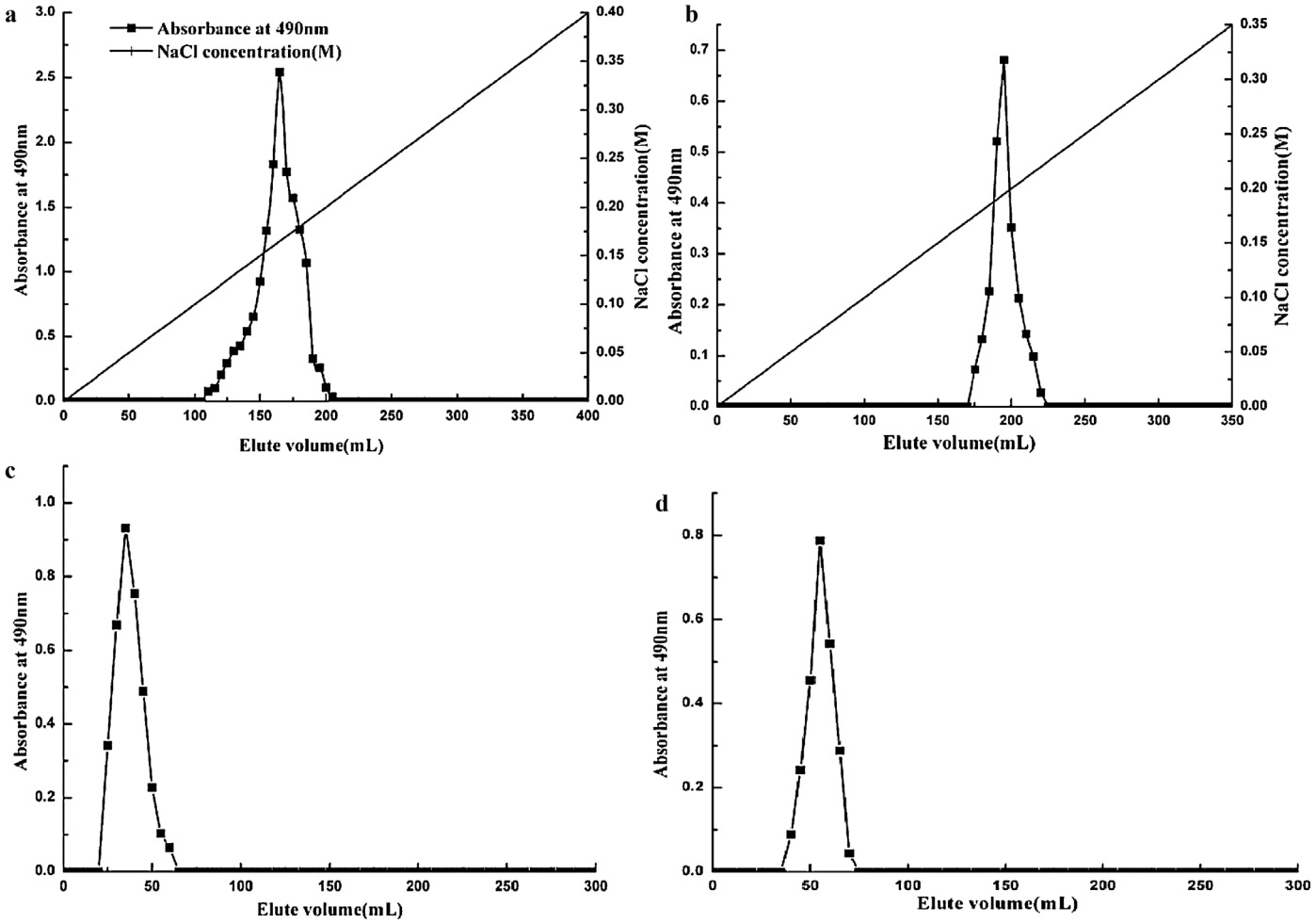

Fig. 1. Elution profile of the polysaccharides extracted from *F. pinicola*. (a) Linear elution curve of crude EPS on DEAE-cellulose column; (b) Linear elution curve of crude IPS on DEAE-cellulose column; (c) EPS elution curve of polysaccharides fraction on Sephadex G-200 column; (d) IPS elution curve of polysaccharides fraction on Sephadex G-200 column.

and A_1 was the amount of cells after UV radiation. Each sample was done in three replicates.

2.7.4. Protective effect on yeast cells from H_2O_2

Similar to the oxidative injury of UV-irradiation above, the yeast cells were cultured to the early stage of logarithm, centrifuged and the supernatant was discarded for collecting yeast paste. Then the cells were washed for yeast suspension preparation. A total of 1 mL yeast suspension was mixed with 4 mL different concentrations of *F. pinicola* EPS and IPS (5–25 mg/mL), and then disposed with 1 mL H_2O_2 for lethal concentration (2%), respectively. The survival rate was calculated as follows: $Y\% = [(A - A_0)/A] \times 100$, where A was the amount of cells which had not been disposed by H_2O_2, and A_0 was the amount of cells after disposing by H_2O_2.

2.7.5. Statistical analysis

The data of all the antioxidant activities were repeated three times and the results were presented as means ± standard deviations of three replicates.

3. Result and discussion

3.1. Isolation and purification of EPS and IPS

Crude polysaccharides were firstly fractionated using a DEAE-cellulose column. The polysaccharides fraction of EPS and IPS eluted with a linear gradient of 0–0.5 mol/L NaCl were the major component of total saccharide (Fig. 1 a and b). Then the fraction was further purified by Sephadex G-200, and each of them showed a single and symmetrical sharp peak (Fig. 1c and d). The major fractions were collected and lyophilized, thus the purified EPS and IPS were obtained, respectively. UV spectrum showed that both the two polysaccharides had no absorption at 280 nm, indicating the absence of protein.

3.2. Monosaccharide compositions of EPS and IPS

The chromatogram of a standard mixture of these six monosaccharides was shown in Fig. 2. The HPLC results indicated that EPS consisted of mannose, rhamnose, xylose, galactose with a molar ratio of 0.1:1.0:0.3:0.5, while IPS consisted of glucose, mannose, rhamnose, xylose, galactose with a molar ratio of 1.0:0.9:0.9:0.8:1.1. Compositional analysis of polysaccharides from 35-day-cultured *F. pinicola* showed that myo-inositol, fucose, galactose, glucose, mannose, and fructose were neutral sugars in the polysaccharide (Cheng et al., 2008). The monosaccharide compositions of EPS and IPS from *F. pinicola* were relatively similar, but IPS had glucose while EPS did not. This might suggest that the sources of polysaccharides affected the monosaccharide compositions of polysaccharides. Moreover, monosaccharide compositions of EPS and IPS here were different from cultured *F. pinicola*

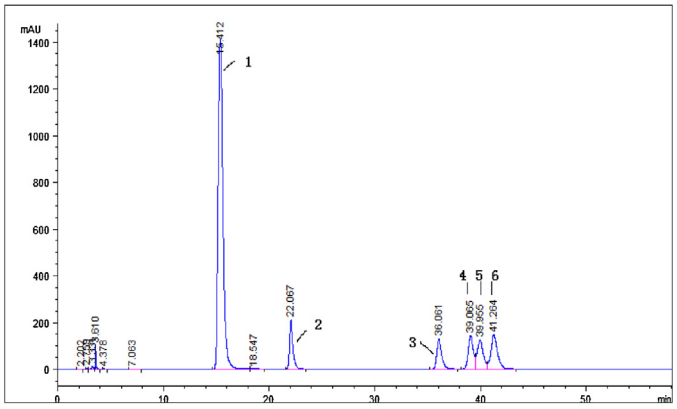

Fig. 2. HPLC chromatograph of monosaccharide standards (1-glucose, 2-mannose, 3-rhamnose, 4-galactose, 5-xylose, 6-arabinose).

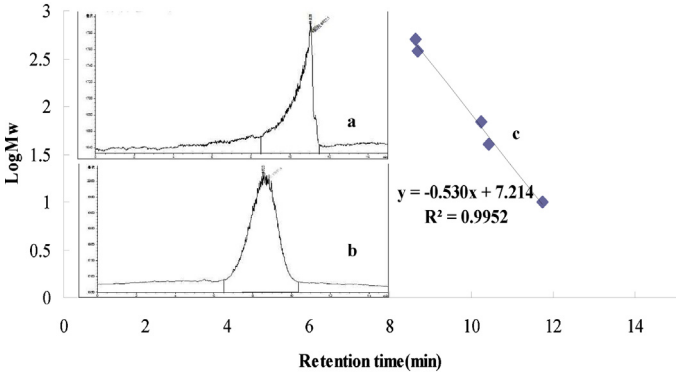

Fig. 3. HPGPC chromatogram of EPS and IPS from *F. pinicola* and standard curve of molecular weight. (a) HPGPC chromatogram of EPS and (b) HPGPC chromatogram of IPS on TSK-gel G5000 PWXL (7.8 mm × 30 cm). (c) The standard curve of molecular weight.

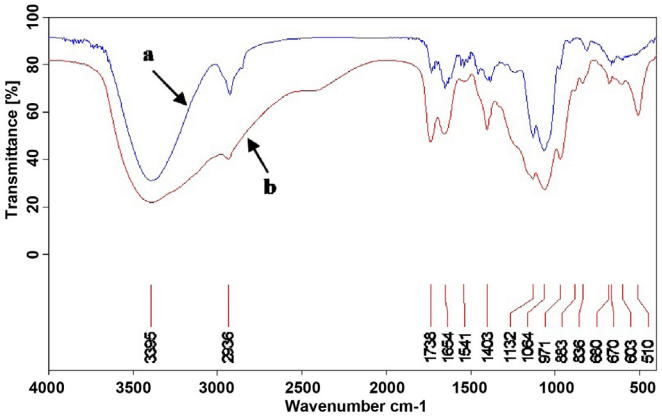

Fig. 4. IR spectrum of EPS (a) and IPS (b) from *F. pinicola*.

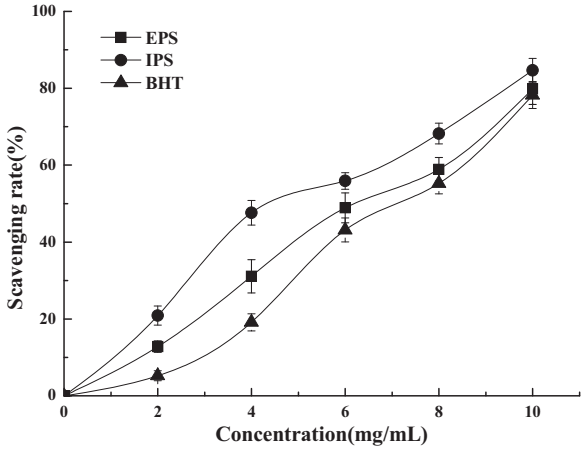

Fig. 5. Scavenging effects of EPS and IPS on DPPH free radical.

polysaccharides by Cheng et al. (2008), and the reason might be due to the varied fungus origins and different incubation time.

3.3. Determination of molecular weight

The molecular weights of EPS and IPS were shown in Fig. 3. Both EPS and IPS showed a symmetrical peak on HPGPC chromatogram, which also showed that their purity was high. According to the calibration curve of the standards $\mathrm{Log}M_w = -0.530X + 7.214$, the molecular weights of EPS and IPS were estimated to be 2.30×10^4 and 4.07×10^5 Da, respectively. Polysaccharides extracted from 35-day-cultured *F. pinicola* are composed of three polysaccharides, with the molecular weight of 5.37×10^6 Da (19.6%), 10.6×10^6 Da (19.3%) and 1.47×10^4 Da (53.6%), respectively (Cheng et al., 2008). There is a significant difference of molecular weights between EPS and IPS, with the molecular weight of IPS was nearly 20 times larger than that of EPS. Hence the degree of IPS polymerization was much higher than that of EPS, and the reason might be that IPS from the fruiting body had longer time of synthesis.

3.4. Analysis of IR spectroscopy

As shown in Fig. 4, both EPS and IPS had similar sugar characteristic absorption peaks in the IR spectrum. The broad and intense band at 3395 cm^{-1} was the stretch vibration of hydroxyl group. The signal at 2936 cm^{-1} was assigned to the stretch vibration of C—H bond (Chen et al., 2012), and the signal at 1738 cm^{-1} was the absorption of C=O. The signal at 1654 cm^{-1} was due to the bending vibration of O—H, and the signal at 1541 cm^{-1} was attributed to the vibration of C—O (Chen et al., 2012). The signal at 1403 cm^{-1} was due to the bending vibration of C—H, and the signal at 1064 cm^{-1} was attributed to the stretch of C—O—C linkages (Guo et al., 2013). In addition, the absorption at 883 cm^{-1} suggested a β-anomeric configuration (Sun et al., 2009).

3.5. Antioxidant properties

3.5.1. Scavenging ability on DPPH radical

The model of scavenging the stable DPPH radical has been widely used to evaluate the antioxidant activities of different materials. Antioxidants can be paired with single electron of DPPH and make it fade, and the degree of fading is positively related to the capacity of the antioxidants (Musa, Abdullah, Kuswandi, & Hidayat, 2013). As shown in Fig. 5, both EPS and IPS showed more significant effects on scavenging DPPH radical than BHT, and the scavenging abilities were positively correlated with the linear dose. The linear relationship between concentrations and scavenging rates was calculated as follows: $y = 8.195x + 5.240 (R^2 = 0.972)$ for IPS and $y = 7.932x - 1.065 (R^2 = 0.994)$ for EPS, respectively, where y was the scavenging rate (%), and x was the sample concentration (mg/mL). Thus EC_{50} values were determined to be 5.46 mg/mL for IPS and 6.44 mg/mL for EPS, respectively. It could also be concluded that the scavenging ability of IPS was slightly higher than that of EPS, and their scavenging abilities were getting closer with concentrations increased. The difference of scavenging abilities for EPS and IPS might be due to their different molecular weights.

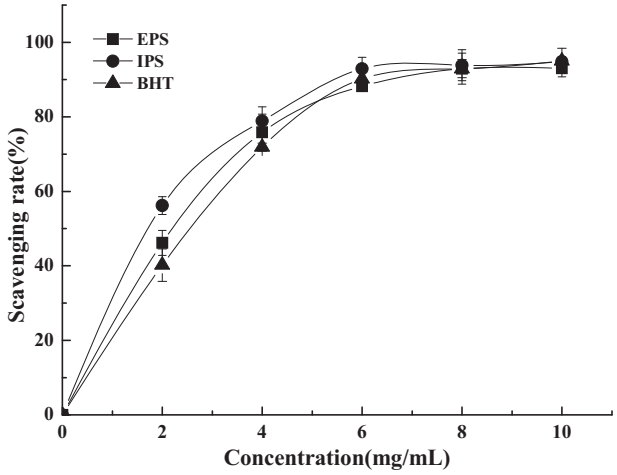

Fig. 6. Scavenging effects of EPS and IPS on hydroxyl free radical.

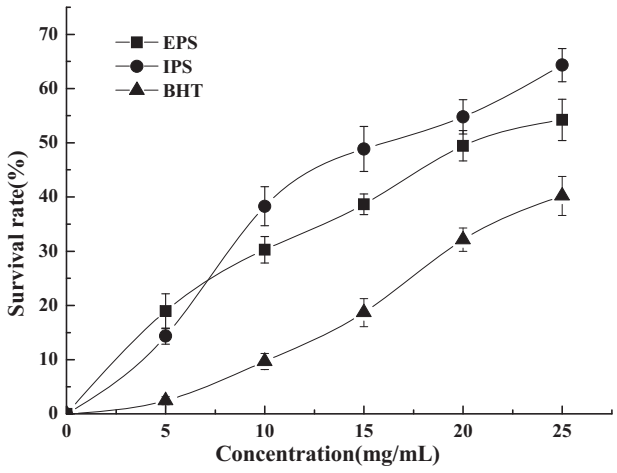

Fig. 7. Protective effects of EPS and IPS on yeast cells from UV oxidative damage.

3.5.2. Scavenging ability on hydroxyl radical

Hydroxyl radical is the strongest oxidizer which could cause oxidative damage to the biomolecules such as lipids, proteins and nucleic acids. As shown in Fig. 6, the hydroxyl radical generated by Fenton reaction was scavenged by EPS and IPS. In the range of 0–6 mg/mL, both the scavenging abilities of polysaccharides were enhanced with increased concentrations. When the concentrations exceeded 6 mg/mL, both EPS and IPS tended to completely scavenge hydroxyl radical. Moreover, the scavenging ability of IPS was found to be slightly higher than those of EPS and BHT. At 2 mg/mL, the scavenging ability of EPS, IPS and BHT were 46.17%, 56.24% and 40.08%, respectively. At 4 mg/mL, their scavenging abilities of them were 75.86%, 78.82% and 71.05%, respectively. All of them exceeded EC_{50}, and the scavenging abilities might be due to the strong hydrogen donating capability of hydroxyl substitutions of polysaccharides.

3.5.3. Protective effect on yeast cells from UV

Studies have proved that UV injury to cells is mainly due to DNA damage, which is induced by the generation of reactive oxygen species ($O_2^{\bullet -}$, O_2, H_2O_2, $^{\bullet}OH$, $ROO^{\bullet}$) and other excessive free radicals (Herrling, Fuchs, Rehberg, & Groth, 2003). Antioxidants supplements can effectively reduce the UV radiation damage to DNA. Yeast cells was chosen for they were the simplest eukaryote and closest to the human body cells. As shown in Fig. 7, the protective effects of EPS and IPS on yeast cells were positively correlated with linear dose response relationships, and their linear equations were $y = 2.120x + 5.422$($R^2 = 0.964$) and $y = 2.592x + 4.375$($R^2 = 0.952$) respectively, where y was the survival rate (%), and x was the sample concentration (mg/mL). Meanwhile, both the two polysaccharides had more significant effects on the survival of yeast cells than BHT, and their corresponding EC_{50} values were 17.60 mg/mL for IPS and 21.03 mg/mL for EPS, respectively. Moreover, the protective effect of IPS on yeast cells was higher than that of EPS. Despite concentrations at 5 mg/mL, the protective effects of IPS on yeast cells was a little lower than that of EPS, which might be due to the protective effect of IPS was not shown under 5 mg/mL.

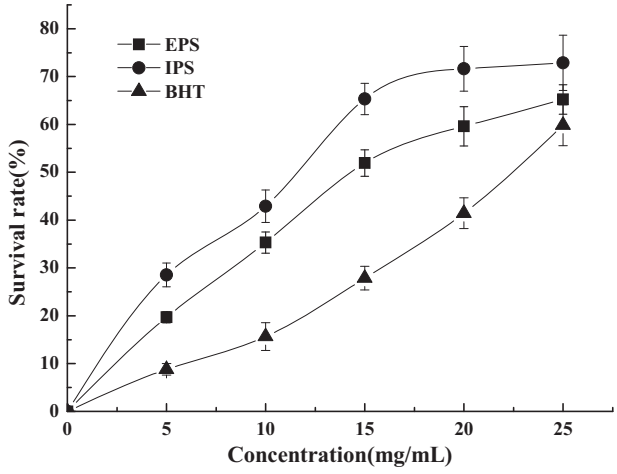

Fig. 8. Protective effects of EPS and IPS on yeast cells from H_2O_2 oxidative damage.

3.5.4. Protective effect on yeast cells from H_2O_2

Hydrogen peroxide (H_2O_2) is an interesting class of cytotoxic agents that can arise naturally from a wide variety of chemical and biochemical oxidations, and it is commonly used to study the production and repair of DNA single-strand breaks (Giandomenico, Cerniglia, Biaglow, Stevens, & Koch, 1997). When antioxidants are present, they can react directly with H_2O_2, and the intermediate products of H_2O_2 or inhibit the peroxidase in conjunction with H_2O_2 can reduce the damage to cells. As shown in Fig. 8, the survival rate of yeast cells was increased significantly with the addition of *F. pinicola* polysaccharides, and the protective effect of IPS was more significant than that of EPS. The linear equation were $y = 2.950x + 10.00$ for IPS and $y = 2.643x + 5.590$ for EPS, where y was the survival rate (%), and x was the sample concentration (mg/mL). EC_{50} values were 13.56 mg/mL for IPS and 16.80 mg/mL for EPS, respectively. Similar to the results of DPPH, hydroxyl and UV oxidative damage experiment, both EPS and IPS had more significant effects on yeast cells than that of BHT, and IPS exhibited a relatively higher antioxidant activity than EPS.

The activity of polysaccharides could be affected by their molecular weights (Zhang, Li, Xu, & Zeng, 2005), and the primary molecular structure of polysaccharide with smaller molecular weight might be destroyed during the extraction process. Thus IPS with larger molecular weight showed higher antioxidant activity than EPS. Monosaccharides composition and glycosyl linkages are another factors affecting antioxidant activity (Lo, Chang, Chiu, Tsay, & Jen, 2011). Different monosaccharides are potential reducing agents as hydrogen donors which can combine with radicals, but the detail mechanism is still needed to be investigated further. These results suggested that both EPS and IPS from *F. pinicola* were effective scavenger for free radicals and protective agents which could help human body reduce damages induced by oxidative damages.

4. Conclusion

In this study, the characteristics and antioxidant activities of EPS and IPS from *F. pinicola* were compared. EPS contained mannose, rhamnose, xylose, galactose, and the molecular weight of EPS was 2.30×10^4 Da. IPS consisted of glucose, mannose, rhamnose, xylose, galactose, and its molecular weight was 4.07×10^5 Da. IR analysis showed the typical polysaccharide absorption of the two polysaccharides. Both EPS and IPS showed strong scavenging abilities of DPPH, hydroxyl radical, and protective effects on yeast cells by UV and H_2O_2 induced oxidative damage. IPS had a higher antioxidant activity than EPS. In summary, polysaccharides from *F. pinicola* were proved to be novel antioxidants. Further study should be focused on the structure characteristics such as glycosidic linkage, *in vivo* antioxidant activity and the structure-activity relationship of the two polysaccharides.

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