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Characterization and antioxidant activities of the polysaccharides from mycelium of *Phellinus pini* and culture medium

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ABSTRACT

Two polysaccharides isolated and purified from the mycelium (PPM) and its culture medium (PPE) of *Phellinus pini* using gel filtration were subjected to composition analysis and valuated for the antioxidant activity. The average molecular weights of PPM and PPE were approximately 22.0 and 38.0 kDa, respectively. PPM and PPE were both neutral heteropolysaccharides consisting of mannose, galactose, and glucose with molecular ratios of 2.99:1.00:0.34 and 38.40:1.00:1.76, respectively. *In vitro* antioxidant assay, PPM and PPE could scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and hydroxyl radical, chelate ferrous ion and reduce ferric ion. The antioxidant activities of PPM were stronger than those of PPE, suggesting that PPM has significant potential as a natural antioxidant agent.

1. Introduction

Phellinus pini belonging to Polyporaceae grows mainly in the northeast, north, northwest and southwest of the primeval forest in China. It was a white-rot fungus which causes white pocket rot in many species of pine, and this process reduces drastically the mechanical properties of wood and consequently its economic value (Ayer, Muir, & Chakravarty, 1996). But it was also a rare and precious medicine used to fight against cancer, improve immunity, and lower blood sugar etc. (Hata, 1997; Tao, Zhang, Zhong, & Li, 2005). For several now, some active ingredients in *P. pini*, such as ceramides, lignan (+)-pinoresinol, the steroids and ergosterol peroxide have been isolated and identified (Lourenco, Lobo, Rodriguez, & Jimeno, 1996). However, little is known about the polysaccharides isolated from *P. pini*. It is well known that polysaccharides are important active components of mushrooms, and many polysaccharides from mushroom have been demonstrated to have various bioactivities, such as antitumor, antioxidant, anticancer, and immunological properties (Wang, Zhou, & Quan, 2014; Han et al., 1999; Kim et al., 2003). Hence, we extracted and purified polysaccharides from mycelium of *P. pini* and its culture medium, and their antioxidant activity were evaluated by various methods *in vitro*.

2. Materials and methods

2.1. Chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH), thiobarbituric acid (TBA), ethylenediaminetetraacetic acid (EDTA), ferrozine, trichloroacetic acid (TCA), deoxyribose (DR), 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), Vitamin C (Vc) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were of analytical grade.

2.2. Strains and fermentation

Phellinus pini (*P. pini*) was initially grown on PDA medium in a Petri dish for 10 days. A 5-mm^2 area of the agar-plate culture was excised with a sterilized cutter and inoculated into 250 mL basal liquid medium contained in a 500 mL flask. The basal liquid medium (pH 6.0) consisted of sucrose (30 g/L), peptone (3.0 g/L), yeast extract powder (3.0 g/L), vitamin B1 (VB1, 10 mg/L), vitamin B2 (VB2, 10 mg/L), KH_2PO_4 (3.0 g/L), and $MgSO_4{\cdot}7H_2O$ (1.5 g/L). The culture medium was cultured using 250 mL medium in 500 mL baffled flasks at 28 °C for 7 d on a shaking incubator at 120 r/min.

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2.3. Extraction and purification of polysaccharides

The *P. pini* mycelia were obtained by centrifugation at 4000 r/min for 15 min. Then the mycelia were washed twice with distilled water, defatted by ethanol, and extracted thrice (120 min each) with distilled water at 100 °C. All the aqueous extracts were combined, concentrated under a reduced pressure and then precipitated by adding 95% ethanol (4 volumes) to yield the crude mycelium polysaccharide (CPPM). The supernatant of submerged cultivation was collected, concentrated, mixed with three volumes of ethanol and then centrifuged to get the crude medium polysaccharide (CPPE). The crude polysaccharides (CPPM and CPPE) were deproteinizated by a combination of proteinase and Sevag method (Staub, 1965). Subsequently, the deproteinated polysaccharides were applied to Sepharose CL-6B column (2.5 cm × 90 cm) and equilibrated with 0.15 mol/L NaCl with a flow rate of 0.5 mL/min. The eluate was detected using phenol-sulphuric acid (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The main polysaccharide fractions were combined, concentrated and then lyophilised to obtain purified polysaccharides, named as PPM and PPE, respectively.

2.4. Homogeneity and molecular weight analysis

The polysaccharides (10 mg) was dissolved in distilled water (1 mL), and then the homogeneity and the molecular weight distribution of them were determined by high-performance lipid chromatography (HPLC), which was performed on a SHIMADZU HPLC system (Shimadzu, Japan) fitted with one TSK-G3000 PWXL columns (7.8 mm ID × 30.0 cm L) and a RID-10A detector. The column was calibrated by molecular mass markers (T-130, 80, 40, 20, 10). The mobile phase was 0.7% Na_2SO_4, and the flow rate was 0.5 mL/min at 40 °C, with 1.6 mPa.

2.5. Chemical analysis

The sugar content of samples was determined by the phenol-sulphuric acid method (Dubois et al., 1956). The content of protein was determined by Bradford's method (Bradford, 1976). The content of uronic acids was measured by the method of m-hydroxydiphenyl method (Blumenkrantz & Asboe-Hansen, 1973). The total phenols content in samples was determined using the Folino–Ciocalteau assay (Sabir & Rocha, 2008). Gallic acid was used in a standard curve and the results were expressed in microgram of gallic acid equivalents per milliter of dry extract.

2.6. Analysis of monosaccharide composition

The monosaccharides of PPM and PPE were analyzed by gas chromatography (GC) of acetylated alditols. Briefly, the sample (10 mg) was hydrolyzed with 2 mL of 2 M trifluoroacetic acid (TFA) in an ampoule (2 mL). The ampoule was sealed under a nitrogen atmosphere and kept at 120 °C for 2 h, and the excess acid was completely removed by co-distillation with ethanol. The hydrolyzed product was reduced with KBH_4 (30 mg) and converted into the alditol acetates according to the method in the literature (Johnes & Albersheim, 1972). The resulting alditol-acetates were analyzed by GC using a Vavian 3400 instrument (Hewlett-Packard, Lake Forest, CA, USA), equipping with DM2330 column (30 m × 0.32mm × 0.2 μm). The column temperature was kept at 170 °C for 2 min and then increased to 250 °C for 20 min at the rate of 10 °C/min.

2.7. Ultraviolet–visible (UV) analysis

In order to investigate whether there were protein and/or nuclear acid in the samples, the polysaccharides (0.5 mg) was dissolved in distilled water (5 mL). Then UV–vis absorption spectra were recorded with a Shimadzu MPS-2000 spectrophotometer between 190 and 290 nm.

2.8. FT-IR spectrum analysis

The FT-IR spectra were recorded on SPECORD in a range 4000–400 cm^{-1}. The samples were analyzed as KBr pellets.

2.9. Assay antioxidant activity in vitro

2.9.1. Assay of DPPH radical scavenging activity

The DPPH quenching ability was measured by the method proposed by Konrath et al. (Yamaguchi, Takamura, Matoba and Terao, 1998) with slight modifications. 1.5 mL freshly prepared DPPH solution (0.1 mM in methanol) was mixed with 4.5 mL of sample (1–5 mg/mL). The mixture was incubated at 25 °C for 30 min in the dark, and the absorbance of reaction liquid was measured at 517 nm. Vc was used as the positive control. The percentage scavenging radical was calculated using the following equation:

Scavenging rate $(\%) = (A_0 - A_1)/A_0 \times 100$, where A_0 is the absorbance of the control (water instead of sample), and A_1 is the absorbance of the sample.

2.9.2. Assay of hydroxyl radical scavenging activity

The assay was performed as described by the method of Chung et al. with minor changes (Chung, Osawa, & Kawakishi, 1997). 0.2 mL solution of various concentrations of sample (0.1–15 mg/mL) was added to 1.2 mL of 10 mM phosphate buffer (PBS) at pH 7.4 containing 2.67 mM 2-deoxyribose and 0.13 mM EDTA. 0.2 mL of iron ammonium sulfate (0.4 mM) was added. Samples were kept in a water bath at 37 °C, the reaction was started by adding 100 μL of vitamin C (1.0 mM) and 10 μL of H_2O_2 (0.1 M). Samples were maintained at 37 °C for 15 min, and then 2 mL of 1% TBA and 2 mL of 2% CCl_3COOH was added to the resulting mixture. Finally the mixture was heated in boiling water for 15 min and cooled by ice water. The absorbance was determined against a blank at 532 nm with spectrophotometer. Vc was used as the positive control. The scavenging percentage was calculated as following:

Scavenging rate $(\%) = (A_0 - A_1)/A_0 \times 100$, where A_0 is the absorbance of the control (water instead of sample), and A_1 is the absorbance of the sample.

2.9.3. Assay of ferrous ion-chelating activity

The ferrous ion-chelating potential of sample was determined as in Dinis, Maderia, and Almeida (1994). The reaction mixture contained 1.0 mL of sample (0.5–4.5 mg/mL), 0.1 mL of $FeCl_2$ (2 mM) and 0.2 mL of ferrozine (5 mM). After incubating at 25 °C for 10 min, the absorbance was measured at 562 nm using EDTA as positive control. A lower absorbance indicates a higher chelating ability. The chelating ability was calculated using the following equation:

Chelating activity $(\%) = (A_0 - A_1)/A_0 \times 100$, where A_0 is the absorbance of the control (water instead of sample), and A_1 is the absorbance of the sample.

2.9.4. Assay of ferric reducing power

The ferric reducing ability of plasma (FRAP) assay was carried out according to the procedure of Veenashri and Muralikrishna (2011). Briefly, the FRAP reagent was prepared daily from 300 mmol/L acetate buffer (pH 6.3), 10 mmol/L TPTZ solution (in mmol/L HCl) and 20 mmol/L $FeCl_3 \cdot 6H_2O$ solution in a proportion of 10:1:1 (v/v). 0.3 mL of sample was added to 2.7 mL of FRAP reagent.

The mixture was incubated at 3 °C for 10 min. The absorbance was measured at 593 nm.

Fresh working solutions of $FeSO_4$ were used for calibration. The antioxidant capacity based on the ability to reduce ferric ions of sample was calculated from the linear calibration curve and expressed as mmol $FeSO_4$ equivalents per gram of the texted samples. Increased absorbance of FRAP reflected increased reducing power.

2.10. Statistical analysis

All values are expressed as the mean ± SD. Comparison between any two groups was evaluated using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons tests. Differences was considered to be statistically significant if $P < 0.05$.

3. Result and discussion

3.1. Characteristic of PPM and PPE

Two polysaccharides, namely, PPM and PPE were purified from *P. pini* mycelium and its culture medium by hot-water extraction, ethanol precipitation, deproteinatin, and Sepharose CL-6B gel permeation chromatography. The yields of PPM and PPE were determined as 11.33% and 8.47%, respectively. The total sugar contents evaluated in PPM and PPE were 94.24% and 92.84%, respectively. PPM and PPE both had a negative response to the Bradford test and there was no absorption peak at 200–280 nm in the UV spectrum, indicating the absence of protein and nucleic acid. Furthermore, uronic acid was not detected in the two polysaccharides, suggesting they belonged to neutral polysaccharides. The total phenols contend was relatively lower in PPM (456 mg/100 g) than PPE (1450 mg/100 g).

The HPLC profile (Fig. 1) showed that each polysaccharide had a single and symmetrically sharp peak revealing that they were homogeneous polysaccharides. According to the retention time, the average molecular weights of PPM and PPE were estimated to be 22.0 kDa and 38.0 kDa, respectively. As summarized in Table 1, GC analysis showed difference of the two polysaccharides, with the presence of Mannose, Galactose and Glucose in the molar ratio of 28.57:1.00:1.47 for PPM; Mannose, Galactose and Glucose in the molar ratio of 2.99:1.00: 0.34 for PPE.

As shown in the FTIR spectra of PPM and PPE (Fig. 2), the broad and intense band at 3384 cm^{-1} was the stretch vibration of O—H. The signal at 2935 cm^{-1} was attributed to the stretch vibration of the C—H bond. The band at 1650 cm^{-1} was assigned to the bending vibration of O—H, and the signal at 1541 cm^{-1} was attributed to the vibration of C—O. The band at 1416 cm^{-1} was assigned to C—H bending vibration. The characteristic absorption band at 814 cm^{-1} 875 cm^{-1} suggested α- and β-configurations simultaneously existing in PPM and PPE.

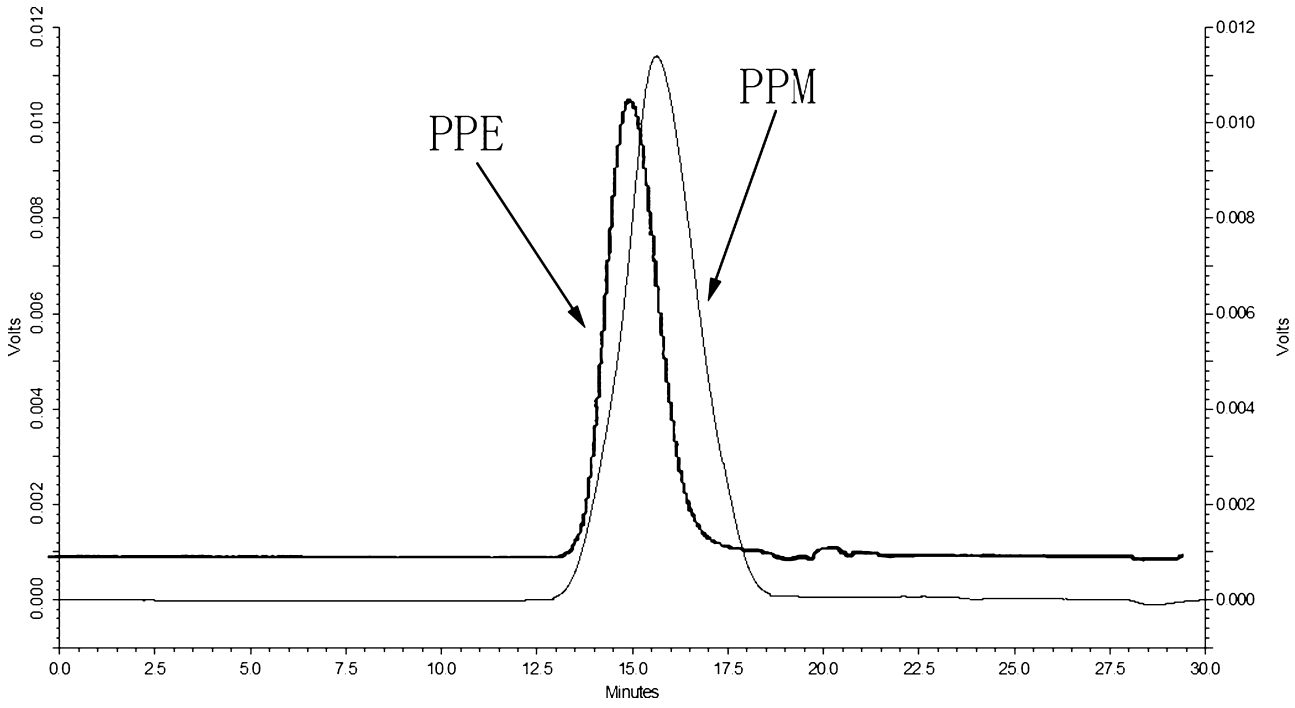

Fig. 1. HPLC elution profiles of PPM and PPE.

Table 1
The total sugar content, monosaccharide composition and molecular weight of PPM and PPE.

Fraction	Monosaccharide composition (molar ratio)			Prptein content[a]	Mw (kDa)
	Mannose	Galactose	Glucose		
PPM	2.99	1.00	0.34	Tr	22.0
PPE	38.40	1.00	1.76	Tr	38.0

[a] Percentage weight in the fraction.
Tr: trace < 1%.

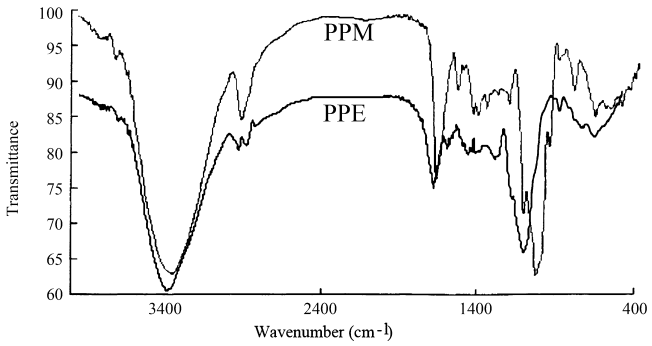

Fig. 2. IR spectra of PPM and PPE.

3.2. Antioxidant activity of polysaccharides

3.2.1. DPPH radical scavenging activity

The antioxidant properties of *P. pini* polysaccharides were investigated by DPPH assay. DPPH, a stable N-centered free radial, has been well used to employ the ability of free-radical scavenging properties or hydrogen donation of compounds and medicine materials (Hatano et al., 1989). As shown in Fig. 3, DPPH scavenging activities of the two polysaccharides increased with increasing concentrations. At the concentration of 3.5 mg/mL, the DPPH radical scavenging rate of PPM and PPE was 85.61% and 69.95%, respectively, with IC_{50} value of 1.80 mg/mL and 3.06 mg/mL, respectively. Between the two polysaccharides, PPM possessed stronger scavenging activity than that of PPE. GC analysis revealed that PPM and PPE contained the same neutral monosaccharide composition of mannose, galactose and glucose. However, the ratio of the three monosaccharides greatly differed from one another. The mannose contents in the two polysaccharides were determined as PPE > PPM, with being 93.29% and 69.91%, respectively. In addition, there was a significant difference in the average molecular weights between PPM (22.0 kDa) and PPE (38.0 kDa). Of the two, PPM exhibited higher antioxidant activity than PPE, which probably due to the difference in the ratio of monosaccharide, as well as molecular weight between them. In addition, the total phenolic content in them was determined as PPM < PPE, indicating that the DPPH radical scavenging activities of the two polysaccharides were probably owing to their carboxyl group in hexuronic acid, instead of phenolic compounds (Wang, Mao, & Wei, 2012).

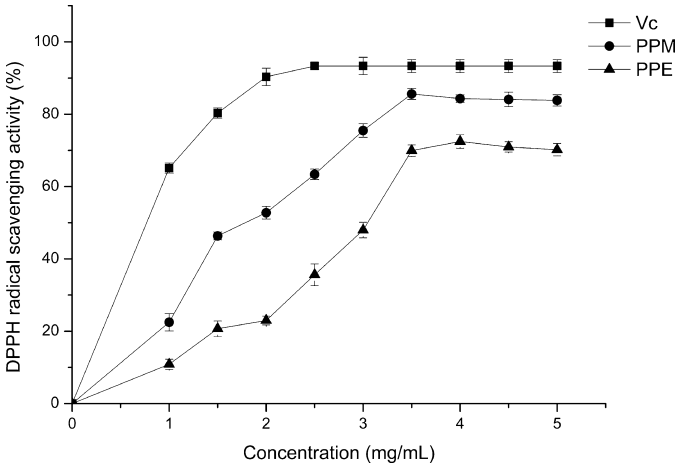

Fig. 3. DPPH radical scavenging activity of PPM and PPE. Each value represents the mean ± SD ($n = 3$).

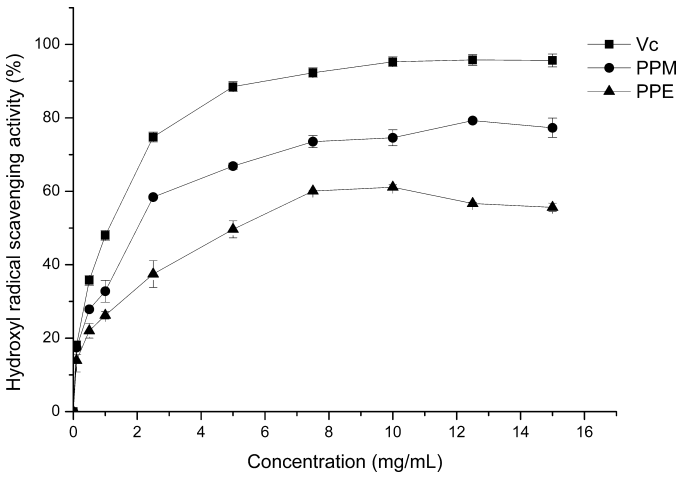

Fig. 4. Hydroxyl radical scavenging activity of PPM and PPE. Each value represents the mean ± SD ($n = 3$).

3.2.2. Hydroxyl radicals scavenging activity

The hydroxyl radical is accepted to be the most reactive and poisonous free radical, and induce severe damage to adjacent biomolecules as an active initiator for lipids peroxidation (Chance, Sies, & Boveris, 1979; Ke et al., 2009). An accepted reaction system containing Fe^{3+}-EDTA-H_2O_2-deoxyribose in the aqueous phase was used to generate •OH and measure inhibitory activity of *P. pini* polysaccharides, and the results were summarized in Fig. 4. Both PPM and PPE were able to scavenge hydroxyl radical. At the concentrations ranging from 0.1 to 12.5 mg/mL, the hydroxyl radical scavenging ability of PPM increased markedly and dose-dependently, and reached 79.27% at 12.5 mg/mL. Compared with PPM, PPE exhibited a lower scavenging activity in the tested concentration range (0.1–15 mg/mL), and with the highest scavenging rate as 61.13% at 10 mg/mL. The IC_{50} values of PPM and PPE were 1.99 mg/mL, 4.99 mg/mL, respectively. In addition, both the IC_{50} values of PPM and PPE of hydroxyl radicals were higher than that of Vc (1.11 mg/mL).

3.2.3. Fe^{2+} chelating activity of polysaccharides

Iron (Fe) and copper (Cu) ions are important elements for the human body, whereas these metal ions have potentially dangerous. Fe^{2+}, with high reactivity, can stimulate lipid peroxidation and accelerate lipid peroxidation, thereby driving the chain reaction of lipid peroxidation (Benedet and Shibamoto, 2008). So the Fe^{2+} chelating activity is considered as an important antioxidant property of materials. In this paper, the Fe^{2+} chelating ability was determined by the reduction of absorbance at 562 nm, the red color is quantitatively formed by the reaction of ferrozine with Fe^{2+}. As shown in Fig. 5, PPM exhibited higher chelating activity than PPE. At the concentrations ranging from 0.5 to 4.5 mg/mL, the Fe^{2+} chelating activity of PPM was measured as 13.35–90.26%, with IC_{50} value of 1.47 mg/mL. The Fe^{2+} chelating activity of PPE was 78.71% at 4.5 mg/mL, with the IC_{50} values of 3.25 mg/mL. The results indicated that PPM possessed a much stronger ferrous chelating activity than PPE. However, the metal chelating activity of the two polysaccharides was significantly lower than that of EDTA, which had the strongest chelating capacity, and achieved 100% at concentration of 0.5 mg/mL. As we know, bioactivities of polysaccharides are closed related to their molecular weight, monosaccharide composition, configuration of glycosidic linkages, position of glycosidic linkages, as well as the degree of substitution on alcohol or phenolic hydroxyl groups (Bohn & BeMiller, 1995). Thus, the structural features and structure–function relationships involved in Fe^{2+} chelating activities of *P. pini* polysaccharides needed to be further studied.

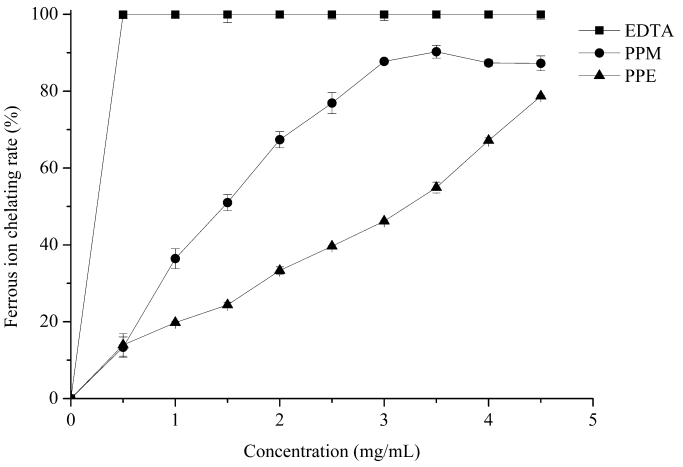

Fig. 5. Fe^{2+} chelating activity of PPM and PPE. Each value represents the mean ± SD ($n = 3$).

3.2.4. Ferric reducing powder of polysaccharides

FRAP assay is one of the widely simple and convenient method for determining the antioxidants activity (Li, Ren, & Lin, 2002). The ferrous ions from FRAP reagent was reduced by tested antioxidants in the presence of TPTZ, and formed an intense blue Fe^{2+}–TPTZ complex with an absorption maximum at 593 nm (Benzie & Strain, 1996). In this work, the FRAP value was used to measure the antioxidant activities of *P. pini* polysaccharides. The FRAP values was estimated by reference to a linear calibration curve (regression equation: $y = 0.113x - 0.006$, $R2 = 0.9991$) made from different concentration of $FeSO_4$ solution. The antioxidant capacity based on the ability to reduce ferric ions of sample was calculated from the linear calibration curve and expressed as mM $FeSO_4$ equivalents per gram of samples. The FRAP values of the PPM and PPE (2.5 mg/mL) were obtained as 2.12 and 1.02 mM $FeSO_4$/g, respectively.

On the basis of the results above, it can be obviously concluded that PPM had stronger scavenging activity of DPPH and hydroxyl radical, chelating activity of ferrous and reducing power than PPE *in vitro*. However, the content of total phenolic in PPE was higher than that of PPM, suggesting that polysaccharides in them played key role in their antioxidant property. In addition, the difference in their antioxidant activity might be ascribed to their different ratios of monosaccharide and molecular weights. Therefore, the antioxidant activities of the polysaccharide were not a function of a single factor but a coincidence of many factors.

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