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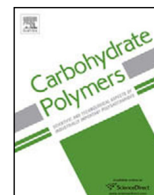

Purification, antioxidant activity and antiglycation of polysaccharides from *Polygonum multiflorum* Thunb

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ABSTRACT

Polysaccharides, one of the most important constituents in *Polygonum multiflorum* Thunb, a famous Chinese medicinal herb, were isolated by DEAE-52, Sepharose 4B and Sephacryl S-300 column chromatography. Two polysaccharides (PMP-1 and PMP-2) were identified as homogeneous in molecular weight with HPLC. The molecular weights were 4.8×10^2 and 6.1×10^2 kDa, respectively. Antioxidant activity tests were performed with two polysaccharides at concentrations of 0.1–1.5 mg/mL. The results indicated that the inhibitory activity on oxidation and glycation exhibited a dose-dependent response. PMP-2 exhibited a much stronger antioxidant capacity against free radical, lipid oxidation and protein glycation. The IC_{50} values of PMP-2 were 0.47, 0.6 and 0.93 mg/mL for superoxide anion scavenging, hydroxyl radical scavenging, and hydroxyl peroxide scavenging, respectively. The inhibitory ability of PMP-2 on lipid oxidation was most markedly in rat liver, followed by heart and kidney. Meanwhile, PMP-2 also showed satisfactory suppression of AGEs formation. This suggested that the polysaccharides present in *PM* can contribute to the biological effects.

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1. Introduction

Polygonum multiflorum Thunb (*PM*) is well-known in traditional Chinese herbal medicine and nowadays has been widely used as a popular functional food; which is one of the forth batch of Chinese herbal medicine that can be used on dietary supplement published by The Ministry of Health of the People's Republic of China. In addition to nutritional values, *PM* extract also exhibits some medicinal properties, which include: cardiovascular improvement, immune function enhancement, anti-tumor and anti-inflammatory effects, cholesterol reduction, and atherosclerosis inhibition (Lv, Gu, Tang, & Ho, 2007). Over long periods of time, majority of investigational focus has been centered around stilbenes and anthraquninones from *PM*. Starch and non-starch polysaccharides comprise more than 40% of *PM*'s weight; but surprisingly these have received very little focus, despite Chinese folk medicinal associations to biological function.

Polysaccharides, which are widely distributed in animals, plants, and microorganisms, have been demonstrated to exhibit antitumor, anticancer, antiviral, and immunological bioactivities (Li, Liu, Fan, Ai, & Shan, 2011). Generally, polysaccharides are nontoxic and have been reported to play the important roles as dietary free radical scavengers for oxidative damage prevention, and have been found in fungus, such as, *Grifola frondosa* (Chen, Ma, Liu, Liao, & Zhao, 2012), *Isaria farinosa* (Jiang, Jiang, Wang, & Hu, 2005), and *Chroogomphis rutilus* (Sun & Kennedy, 2010); Marine algae (Yoshizawa, Enomoto, Todoh, Ametani, & Kaminogawa, 1993), fruit, such as, *Zizyphus Jujuba* (Li et al., 2011) and *Mangifera pajang* (Ibrahim, Prasad, Ismail, Azlan, & Abd Hamid, 2010) (Al-Sheraji et al., 2012) and some plants, for example, *Dendrobium nobile* Lindl (Luo et al., 2010) and *Bryopsis plumosa* (Song, Zhang, Zhang, & Wang, 2010).

ROS are highly reactive oxidizing agents that have one or more unpaired electrons, and are classified as free radicals. ROS tend to induce chain reactions, in such a manner that "radical begets radical". Commonly known ROS, which have potential implications in reproductive biology include: superoxide $(O_2^-)^\bullet$ anion, hydrogen peroxide (H_2O_2), peroxyl $(ROO^-)^\bullet$ radical and the very reactive hydroxyl $(OH^-)^\bullet$ radical. ROS are capable of oxidizing biomolecules, resulting in cell death and tissue damage. Oxidative damage plays a significant pathological role in human disease, cancer emphysema, cirrhosis, arteriosclerosis, and arthritis (Halliwell, 1990).

Apart from ROS, α-oxoaldehydes such as methylglyoxal (MGO) and glyoxal (GO), which are formed from both glycoxidation, lipoxidation; the subsequent carbonyl modification of proteins is referred as "carbonyl stress" (Miyata, Kurokawa, & De Strihou, 2000), which is a highlighted phenomenon enhanced during diabetic complications. The carbonyl stress hypothesis outlines the functions of reactive dicarbonyl compounds; to modify pathogenic protein, lipid, and DNA, in addition to forming toxin adducts advanced

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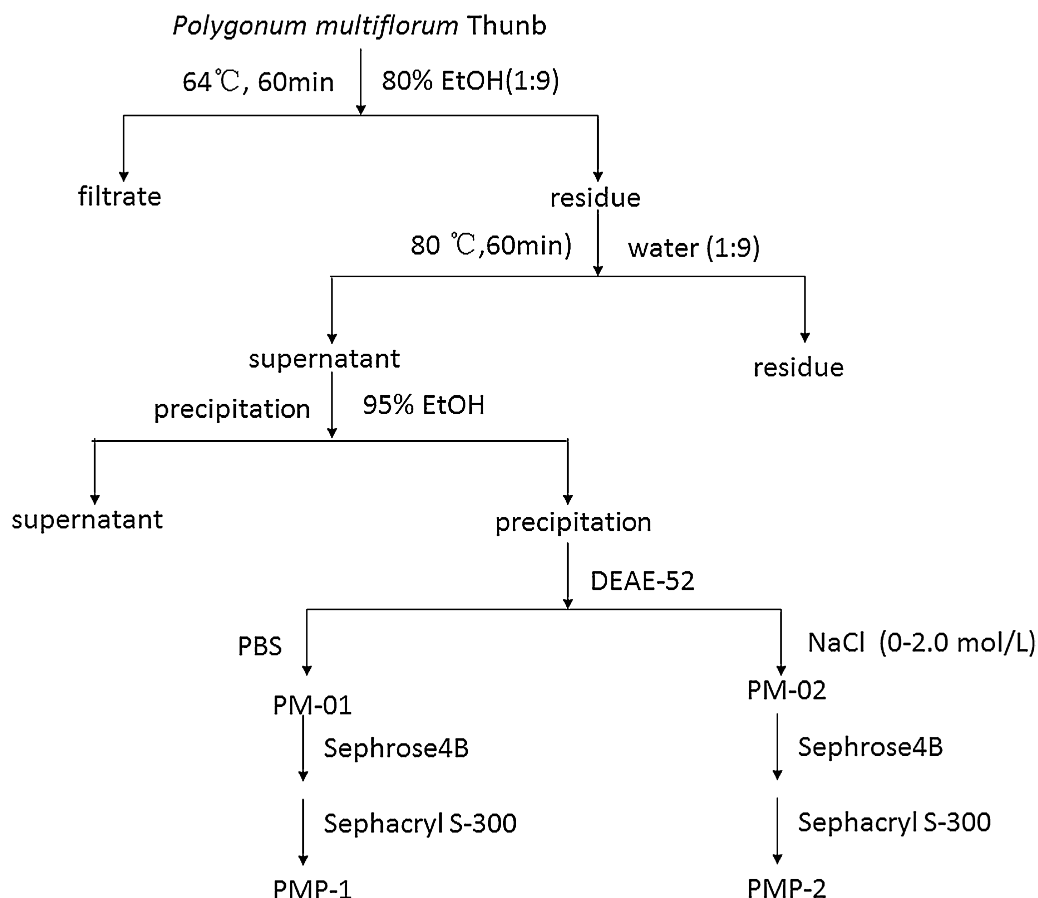

Fig. 1. Procedure of extraction and purification of polysaccharides from *Polygonum multiflorum* Thunbs.

glycation end products (AGEs). AGEs also known as glycotoxins, are a diverse group of highly oxidant compounds with pathogenic significance in diabetes and in several other chronic diseases (Kilhovd et al., 2009; Münch, Thome, Foley, Schinzel, & Riederer, 1997).

Antioxidants are compounds which dispose, scavenge, and suppress the formation of ROS. Thereby antioxidant supplements or foods containing antioxidants could help reduce oxidative damage within the human body. Despite certain claims that bioactive polysaccharides exist in *PM*, there is little, if any, conclusive evidence to support such claims. No detailed investigation has been carried out on the composition and antioxidative capacity of polysaccharides from *PM*. In the present study, we purified and investigated the properties of the major polysaccharides from *PM*, in addition to evaluating their antioxidant activities using different antioxidant tests, including: radical scavenging, lipid peroxidation, and protein glycation *in vitro*. These efforts were made to recognize the main compounds of this plant, which may be used as a functional factor used in food and pharmaceutical industries.

2. Materials and methods

Luminol, methylglyoxal (40% in water), bovine serum albumin, Sepharose 4B, Sephacyl S-300 and monosaccharides standards (D-glucose, D-mannose, L-rhamnose, D-galactose, D-xylose and D-arabinose) 1-phenyl-3-methyl-5-pyrazolone were purchased from Sigma (St. Louis, MO). Dextrans of different molecular weights were purchased from Pharmacia Co. (NY, USA); DEAE-52: Whatman (Maidstone, Kent, UK); and all other chemicals and reagents were purchased from Sinoreagent (Shanghai, China).

2.1. Extraction and purification of polysaccharides

Extraction and purification procedure was shown in Fig. 1. Dried root samples 1000 g were ground to a fine powder and extracted twice with 80% EtOH (1:9, v/w) at 64 °C for 60 min. The plant material was filtered off, the residue was extracted twice with water (1:9, w/v) at 80 °C for 60 min, the extract was then centrifuged at 4000 rpm/min for 20 min to remove the precipitate. The water extracts were concentrated and 95% EtOH was added (approximately four-fold water solution). The crude polysaccharide was precipitated from the extract after standing at 4 °C overnight. The formed precipitate was collected by centrifugation at 4000 rpm/min and repeatedly washed with ethanol, acetone and ether, respectively.

The crude polysaccharides were dissolved in deionized water and loaded on a DEAE-Cellulose column (2.6 cm × 20 cm) equilibrated with 20 mM PBS (Phosphate Buffered Saline, NaH_2PO_4/Na_2HPO_4, pH 6.8). The column was first washed with the same buffer at a flow rate of 1.0 mL/min, followed by a linear gradient of NaCl concentration (0–2.0 M). Neutral polysaccharides (F-1) and acidic polysaccharides (F-2) were collected in the test tube (6 mL/tube) with a fraction collector, concentrated using a rotary evaporator at 50 °C, dialyzed against deionized water for 3 days (molecular weight cutoff 10,000 Da) and lyophilized.

Protein content was estimated by the method of binding of Coomassie Brilliant Blue G-250 to protein using bovine serum albumin as a standard (Bradford, 1976).

F-1 and F-2 were dissolved in a phosphate buffer (20 mM, pH 6.8) to a concentration of 1.0% (w/v), respectively, and were then loaded onto a Sepharose 4B gel column (1.6 cm × 100 cm) and eluted with deionized water at a flow rate of 0.2 mL/min. The elute obtained was

pooled and concentrated. The eluted fractions were further loaded on a Sephacryl S-300 column (2.6 cm × 50 cm), and eluted with deionized water at a flow rate of 0.2 mL/min. The elute obtained was pooled, concentrated and lyophilized. These are polysaccharides PMP-1 and PMP-2.

2.2. Molecular weights of polysaccharide

Molecular weights of the samples were determined by high performance gel permeation chromatography (HPGPC) with a Waters HPLC apparatus equipped with two serially linked Ultrahydrogel™ Linear (Φ7.8 mm × 300 mm ID) columns, a Waters 2410 interferometric refractometer detector and UV detector connected in series with a Millennium32 workstation. The conditions were mobile phase: 0.1 M $NaNO_3$; flow rate: 0.9 mL/min; column temperature: 45 °C. The molecular weights were estimated by reference to the calibration curve made under the conditions described above from Dextran T-series standards of known molecular weights.

2.3. Analysis monosaccharide composition of PM polysaccharide

2.3.1. Hydrolysis of polysaccharide

Polysaccharide sample 100 μL (5 mg/mL) was dissolved in 4 M trifluoroacetic acid (100 μL) in a 5 mL ampoule. The ampoule was sealed in a nitrogen atmosphere and incubated for 2 h at 110 °C. After the ampoule was cooled to room temperature, the sample was washed with 200 μL methanol and blown dry by N_2 stream. This process is typically repeated 3 times to remove trifluoroacetic acid.

2.3.2. Derivatization with the polysaccharide of PM

The derivatization method published by Honda et al. (1989) was used with some modification. 100 μL monosaccharide reference (each monosaccharide, m/v 0.36 g/L) and 100 μL 0.6 M NaOH were mixed in 1 mL a stoppered test tube. 50 μL mixture from the test tube was added to 50 μL 1-phenyl-3-methyl-5-pyrazolone (0.4355 g/5 mL) methanol solution. The mixture was shaken well, incubated for 100 min at 70 °C. The reaction mixture was then cooled to room temperature, and neutralized with 50 μL 0.3 M hydrochloric acid. One milliliter of chloroform was then added to the solution. The mixture was shaken well and centrifuged at 5000 rpm/min for 10 min. The chloroform layer was discarded and the aqueous layer was extracted twice with chloroform. The final aqueous layer was analyzed directly by HPLC.

2.3.3. Chromatography

Chromatographic conditions were as follows: column: ZORBAX Eclipse XDB-C_{18}, 250 mm × 416 mm i.d.; temperature: 30 °C; solvent: 0.1 M phosphate buffer solution (pH 6.7)–acetonitrile (83:17); UV detector: 250 nm.

2.4. Infrared (IR) spectra analysis of fractions

The IR spectra of polysaccharides were determined using a Fourier transform infrared spectrophotometer (Nexus 5DXC FT-IR, Thermo Nicolet, America). The sample was ground with spectroscopic grade potassium bromide (KBr) powder and then pressed into a 1 mm pellet for FT-IR measurement in the frequency range of 4000–400 cm^{-1}.

2.5. Superoxide anion scavenging activity

The superoxide anion scavenging abilities of polysaccharides of *PM* were assessed on a SH-G biochemistry chemiluminescence meter (BCM) (Shanghai Measurement Equipment Factory, Shanghai, China). For the superoxide anion assay (Tian et al., 2012), superoxide anion was generated by pyrogallol autoxidation. The reaction mixture contained 50 μL pyrogallol (1×10^{-3} M), 880 μL carbonate balanced salt solution (CBSS1 × 10^{-3} M) of pH = 10.2 and 20 μL luminol (1×10^{-3} M). A sample cell loaded with the mixture was first placed in the BCM. When the cell crossed the monitor, a known concentration of the sample was injected into the cell instantly. The chemiluminigenic was simultaneously recorded in the processor once every 6 s (CBSS replaced the sample in the control). The highest chemiluminigenic was applied to calculation. The scavenging rate was obtained according to the formula:

$$\text{Scavenging rate} \ (\%) = \frac{(\text{CL} \ [\text{control}] - \text{CL} \ [\text{sample}]) \times 100}{\text{CL} \ (\text{control})}$$

2.6. Hydroxyl radical scavenging activity

The hydroxyl radical scavenging abilities of polysaccharides of *PM* were assessed on an SH-G biochemistry chemiluminescence meter (BCM). The BCM is composed of three parts: an automatically rotating sample support, in which 12 sample cells (glass tubes, diameter = 10 mm, height = 20 mm) were placed, a chemiluminescence monitor and a data processor. Each sample cell rotates and crosses the monitor at a set time interval according to a self-set program. During testing, the chemiluminescence intensity (CL) of a reaction system was recorded in a data processor at the set time interval.

Hydroxyl radical was produced by a copper-catalyzed Haber–Weiss reaction, with zymosan used as a CL amplifier (Rowley & Halliwell, 1983). Various polysaccharides fractions (0.25–3 mg/mL) were dissolved in phosphate buffer (20 mM, pH 7.8) to obtain samples. Additionally, 200 μL of L-ascorbic acid (Vc) solution (2 mM), 400 μL $CuSO_4$ solution (2 mM), 100 μL zymosan solution (75 mg/mL), 50 μL luminol solution (0.1 mM), 100 μL samples and 550 mL phosphate balance solution (PBS) were added, in their given order (samples were replaced by the corresponding buffer solution in the control group) to the test glass cuvette. A sample cell loaded with the mixture was first placed in the BCM as a control. When the cell crossed the monitor, 600 μL H_2O_2 (68 mM) was injected into the cell instantly. The chemiluminigenic was simultaneously recorded in the processor once every 6 s (PBS replaced the sample in the control). The highest chemiluminigenic was applied to calculation. The scavenging rate was obtained according to the formula:

$$\text{Scavenging rate} \ (\%) = \frac{(\text{CL} \ [\text{control}] - \text{CL} \ [\text{sample}]) \times 100}{\text{CL} \ (\text{control})}$$

2.7. Hydrogen peroxide scavenging activity

The ability of the polysaccharides of *PM* to scavenge hydrogen peroxide was determined according to the method described by our previous paper (Tian et al., 2012). The reaction mixtures were added in turn: 50 μL polysaccharides of *PM*, 850 μL carbonate balanced salt solution (CBSS 5×10^{-2} M) of pH = 9.0 and 50 μL luminol (1×10^{-3} M). A sample cell loaded with the mixture was first placed in the BCM. When the cell crossed the monitor, 50 μL H_2O_2 (0.15%) was injected into the cell instantly. The chemiluminigenic was simultaneously recorded in the processor once every 6 s (CBSS replaced the sample in the control). The highest chemiluminigenic was applied to calculation. The scavenging rate was obtained according to the formula:

$$\text{Scavenging rate} \ (\%) = \frac{(\text{CL} \ [\text{control}] - \text{CL} \ [\text{sample}]) \times 100}{\text{CL} \ (\text{control})}$$

2.8. Inhibition of lipid peroxidation

The liver, heart and kidney of Sprague-Dawley (SD) rats (Pukou Breeding Center, Nanjing, China) were dissected and homogenized with a Polytron in ice-cold phosphate buffer (20 mM, pH 7.4) to produce a 1:10 (w/v) liver, heart and kidney tissue homogenate. A 0.20 mL aliquot of the supernatant homogenate was incubated with 0.50 mL of PMP sample solutions (0.1–1.0 mg/mL) in the presence of 0.05 mL of 1.00 mM $FeSO_4$ (AnalaR) and 0.02 mL of 0.01 mM L-Cys at 37 °C for 30 min. The reaction was stopped by the addition of 0.5 mL trichloroacetic acid (TCA; 20%, w/v), then centrifuged at 4000 rpm/min for 10 min to remove the precipitated protein. One milliliter of the supernatant was taken in a cuvette, followed by 1 mL thiobarbituric acid (TBA; 0.67%, w/v), and the mixture was then boiled for 10 min. The color intensity of thiobarbituric acid reactive substance (TBARS) with TBA was measured by its absorbance at 532 nm using a spectrophotometer (Spectronic Genesys, Milton Roy Co., Ivyland, PA). The inhibition ratio (%) was calculated using the following formula:

$$\text{Inhibition ratio} \quad (\%) = \left[\frac{A - B}{B}\right] \times 100$$

where A and B were the absorbance of the control and the sample, respectively.

2.9. Inhibition effects on the formation of AGEs by PM polysaccharide

BSA (1.4 mg/mL) was incubated with MGO (500 μM) in PBS buffer, pH 7.4 at 37 °C, respectively. *PM* polysaccharide (0.1–1.5 mg/mL) was also incubated with MGO (500 μM) in the presence of BSA (0 and 1.4 mg/mL) for the background screen. 0.3 mL of streptomycin and penicillin mixed solution was added before incubation to prevent the growth of bacterial. 400 μL of reaction mixture was collected and frozen at different time points (0, 4, 8, 16, 24, 72, 168 h). AGEs levels were quantified using fluorescence at an excitation/emission wavelength of 370/440 nm, which is characteristic of AGEs.

2.10. Statistical analysis

The experiments used completely randomized block designs and the analyses were carried out in triplicate. All data are expressed as means ± SEM. One-way analysis of variance (ANOVA; SPSS 10.0 for Windows) was used to test for differences between sample treatments. The significance of difference among mean values was determined at ($p < 0.05$).

3. Result and discussion

3.1. Extraction and characterization of polysaccharide from PM

The crude polysaccharide was extracted from *PM* according to the procedure shown in Fig. 1. The yield of crude polysaccharides was 4.9%. Next the crude polysaccharide was separated through an anion-exchange chromatography of DEAE-52, resulting in two independent elution peaks (F-1 and F-2; Fig. 2). The yields of F-1 and F-2 were 29.4% and 47.4% based on crude polysaccharides, respectively. The total recovery of the eluted polysaccharides (F-1 and F-2) was 76.8%. The protein content of F-1 and F-2 was very low. The two fractions were collected, concentrated and further purified sequentially by gel filtration chromatography of Sepharose 4B and Sephacryl S300, respectively. As a result, purified polysaccharide PMP-1 was obtained from F-1, and PMP-2 was obtained from F-2 (Fig. 2). The yields of PMP-1 and PMP-2 were 79.5% and 81.2%. PMP-1 and PMP-2 were subjected to further chemical analysis.

3.2. Characterization of the polysaccharide from PM

Biological properties of polysaccharides are linked to their composition and structure; the molecular weight (MW) also plays an important role. First, the apparent molecular mass was determined by size exclusion chromatography. The elution profile of this macromolecule suggests that this polymer is homogeneous. Based on calibration with standard dextrans, the average molecular weight of PMP-1 was 4.8×10^2 kDa (Mp: 434,635, Fig. 3A), and M_W of PMP-2 was 6.1×10^2 kDa (Mp: 460,604, Fig. 3A). Regarding the monosaccharide composition of polysaccharide PMP-1 and PMP-2 isolated from *PM*, glucose is the main sugar unit for both of these compounds (Fig. 3B).

To have a more precise characterization of the polysaccharides present in *PM*, the FTIR spectra in the region 3750–400 cm^{-1} was performed. As is shown in Fig. 3C, the band 3420 cm^{-1} (PMP-1), 3404 cm^{-1} (PMP-2), represents the stretching of the hydroxyl groups. The small band at around 2927 cm^{-1} (PMP-1), 2928 cm^{-1} (PMP-2) was attributed to the C—H stretching and bending vibrations. The bands at 1635 cm^{-1} (PMP-1), 1637 cm^{-1} (PMP-2) were due to the bond stretching vibrations of C=O bonds in the acylamino group. The broad absorption bands at 1409, 1383 cm^{-1} (PMP-1) and 1411, 1383 cm^{-1} (PMP-2) could be assigned to deforming vibrations of C—H bond. Each particular polysaccharide has a specific band in the 1200–1000 cm^{-1} region. This region is dominated by ring vibrations overlapped with stretching vibrations of (C—OH) side groups and the (C—O—C) glycosidic bond vibration. The absorptions at 1154, 1079, 1022 cm^{-1} (PMP-1) and 1154, 1081, 1022 cm^{-1} (PMP-2), indicated a pyranose unit (Zhao, Kan, Li, & Chen, 2005). A characteristic peak at around 930 cm^{-1} (PMP-1), 929 cm^{-1} (PMP-2), indicates the β-configuration of the C—H deforming vibrations in pyran ring (Coimbra, Barros, Barros, Rutledge, & Delgadillo, 1998). Absorptions at 855, 762 cm^{-1} (PMP-1), 854, 764 cm^{-1} (PMP-2) were typical for α-dominating configuration in pyranose form (Barker, Bourne, Stacey, & Whiffen, 1954).

3.3. Superoxide anion scavenging activity

Superoxide anion plays important roles in the formation of other ROS. The unpaired electron in the valence shell of the superoxide radical makes it reactive and it subsequently reacts with other molecules to form secondary radicals such as the hydroxyl radical ($OH^•$), peroxynitrate ($ONOO^•$), hydrogen peroxide H_2O_2 and the peroxyl radical ($LOO^•$), and can also be split to form singlet oxygen ($O^•$) (Wootton-Beard & Ryan, 2011). Superoxide anion could induce oxidative stress damage, tumor promotion, cell growth, and DNA synthesis, by which superoxide anion utilizes the cell signaling pathway of Ras (the protein encoded by the protooncogene ras). Our results indicated that PMP-1 and PMP-2 inhibited superoxide radicals in a dose-dependent manner (Fig. 4). As shown in Table 1, PMP-2 is more powerful on scavenging the superoxide radicals, and the IC_{50} of PMP-2 and PMP-1 were 0.47 and 1.41 mg/mL, respectively. In recent years, some of the polysaccharides that were reported to have higher scavenging effects on superoxide radicals, were obtained from *Bryopsis plumosa* (Song et al., 2010), *Chroogomphis rutilus* (Sun & Kennedy, 2010) and *Zizyphus Jujuba* (Li et al., 2011).

3.4. Hydroxyl radical scavenging activity

Among the reactive oxygen species, the hydroxyl radical is the most reactive and induces severe damage to adjacent biomolecules. Hydroxyl radicals can be produced from O_2 under a variety of

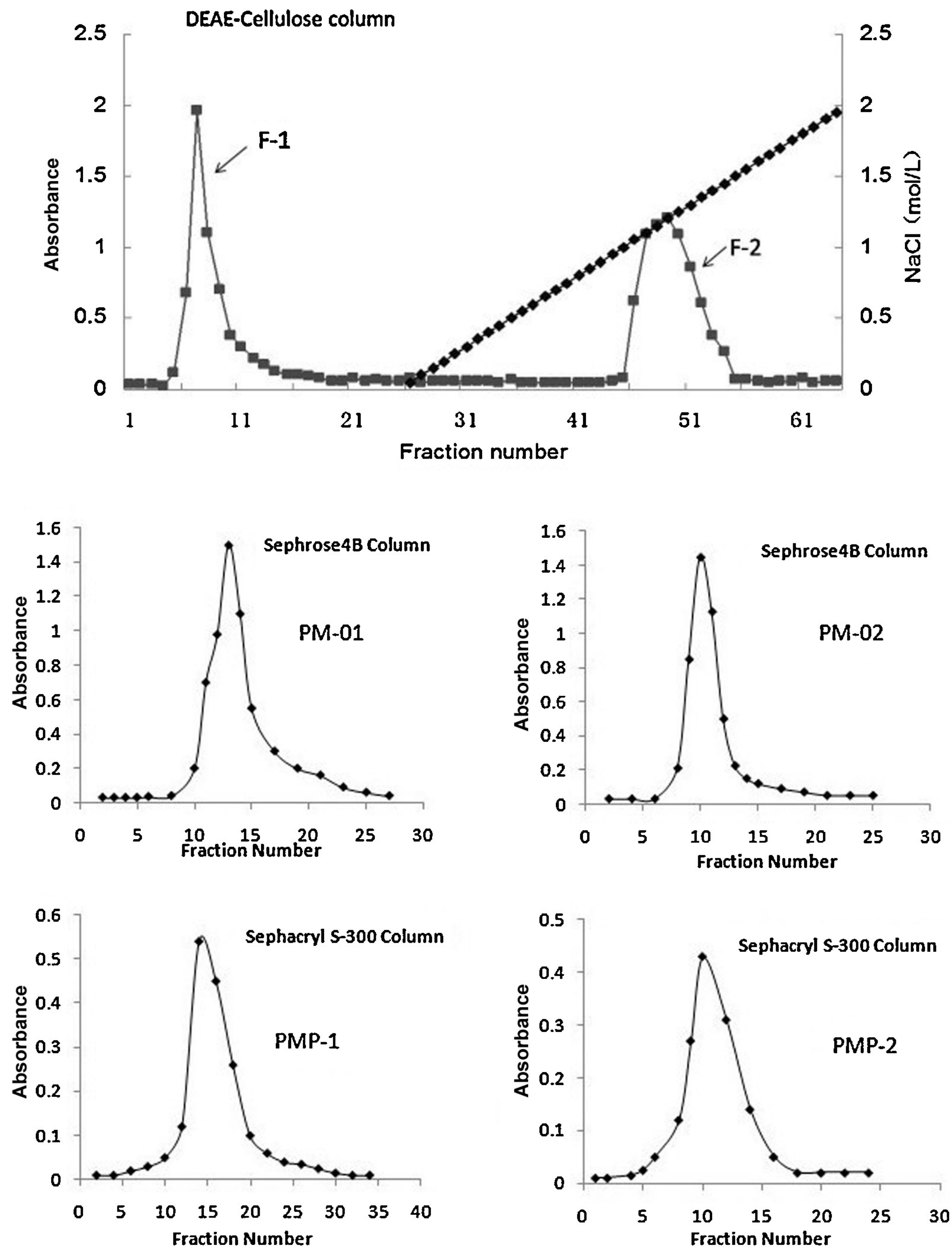

Fig. 2. Two fractions (F-1 and F-2) obtained from the crude polysaccharide by the DEAE-52 column, followed purified by Sephrose 4B and Sephacryl S300 column sequentially, respectively.

Table 1
The lowest half maximum inhibitory concentrations (IC_{50}) for radical scavenging activity and inhibitory activity on lipid peroxidation of polysaccharides from *Polygonum multiflorum* Thunbs.

	IC_{50}PMP-1 (mg/mL)	IC_{50}PMP-2 (mg/mL)
Superoxide anion	1.41 ± 0.25	0.47 ± 0.05
Hydroxy radical	2.65 ± 0.26	0.93 ± 0.07
Hydroxy peroxide	1.39 ± 0.12	0.60 ± 0.09
Lipid		
Liver	0.29 ± 0.06	0.16 ± 0.03
Heart	0.63 ± 0.09	0.51 ± 0.011
Kidney	0.82 ± 0.16	0.8 ± 0.08

stress conditions and are involved in numerous cellular disorders such as inflammations, embryo teratogenesis, herbicide effects, cell death and killing of micro-organisms in pathogen-defense reactions (Chen & Schopfer, 1999). In this study, the scavenging effects of polysaccharide PMP-1 and PMP-2 on hydroxyl radical are shown in Fig. 4. The IC_{50} of PMP-2 and PMP-1 was determined as 0.93 and 2.45 mg/mL, which suggested that hydroxyl radical scavenging activity of PMP-2 was higher than that of PMP-1. Nevertheless, the ability of inhibiting hydroxyl radical of polysaccharides of *PM* is lower than that of inhibiting superoxidate anion. The polysaccharide exhibited hydroxyl radical scavenging activity was also reported by Luo et al. (2010), which reported polysaccharides from *Dendrobium nobile* Lindl. Dalonso and Petkowicz (2012) studied Guarana powder polysaccharides at a higher concentration;

Fig. 3. Molecular Weight distribution of PMP-1 and PMP-2 (A). Neutral sugar composition of PMP-1 and PMP-2 (B). The IR spectra of PMP-1 and PMP-2 (C).

the methanolic extract and the polysaccharide exhibited similar hydroxyl radical scavenging effects (70%).

3.5. Hydrogen peroxide scavenging activity

Hydrogen peroxide can be formed *in vivo* by many oxidizing enzymes such as superoxide dismutase (SOD). Hydrogen peroxide itself is not very reactive; however it can sometimes be toxic to cells due to the ability to give rise to hydroxyl radical in the cells. Addition of hydrogen peroxide to cells in culture can lead to transition metal ion dependent OH radicals mediated oxidative DNA damage. Levels of hydrogen peroxide at or below about 20–50 mg seem to have limited cytotoxicity to many cell types. Cancer cells rely on an increased level of oxidative stress, primarily the generation of H_2O_2, to signal the transcription of genes involved in their survival and to regulate the balance between proliferation and apoptosis (Loo, 2003). Thus, removing hydrogen peroxide as well as superoxide radical is very important. Fig. 4 depicts the H_2O_2 scavenging capacity of PMP-2 and PMP-1. Our results indicated that polysaccharides from *PM* inhibited H_2O_2 in a dose-dependent manner. At

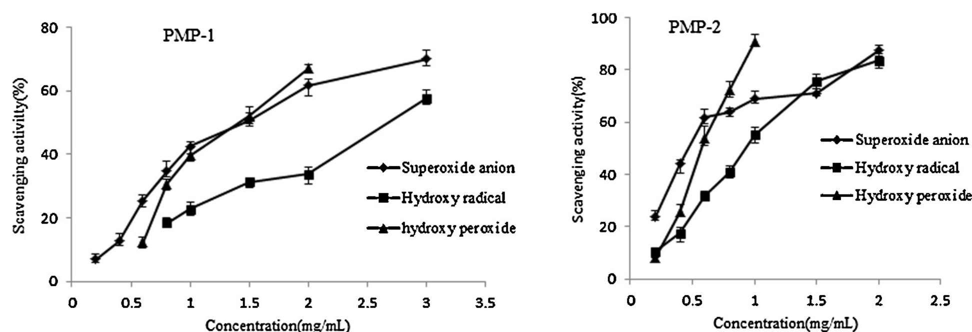

Fig. 4. Scavenging effects of PMP-1 and PMP-2 on superoxide anion, hydroxyl radical, and hydrogen peroxide. Results were presented as mean value (n = 3).

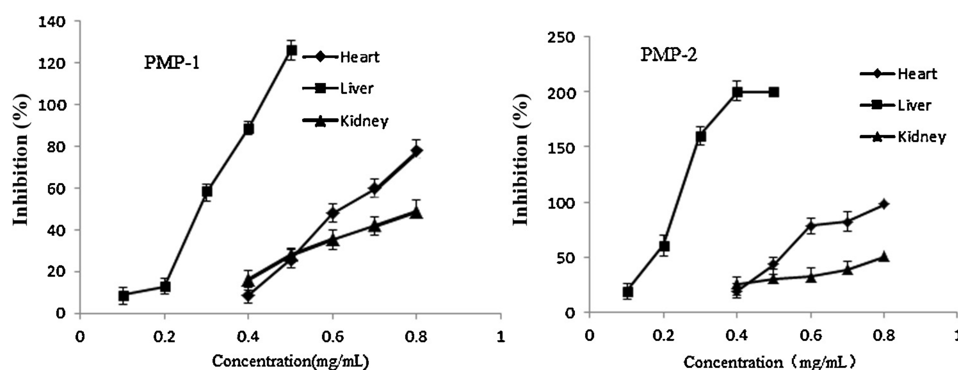

Fig. 5. Inhibition effects on lipid peroxidation with PMP-1 and PMP-2. Results were presented as mean value ($n=3$).

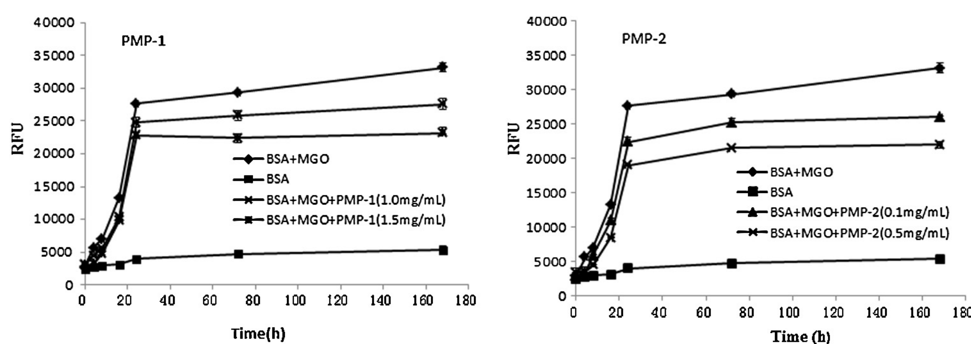

Fig. 6. Inhibition effects on the formation of AGEs with PMP-1 and PMP-2. Results were presented as mean value ($n=3$).

1.0 mg/mL, the PMP-2 scavenged H_2O_2 more than 90%. The IC_{50} of PMP-2 (0.6 μg/mL) exhibited higher antioxidant activity than PMP-1 (1.39 μg/mL).

3.6. Inhibition of lipid peroxidation

Lipid peroxidation (LPO) is the most extensively studied manifestation of oxygen activation in biology. LPO is broadly defined as "oxidative deterioration of PUFA" which are fatty acids that contain more than two carbon double bonds (Halliwell, 1990). The most common types of LPO are: (a) nonenzymatic membrane LPO, and (b) enzymatic (NADPH and ADP dependent) LPO. In spermatozoa, production of malondialdehyde (MDA), an end product of LPO induced by ferrous ion promoters, has been reported. This can be assayed by the thiobarbituric acid (TBA) reaction, which is a simple and useful diagnostic tool for the measurement of LPO for *in vitro* and *in vivo* systems (Ernster, 1993). In the present study, polysaccharides PMP-1 and PMP-2 possessed significant inhibitory effects on lipid oxidation in liver, heart and kidney in rats. From Fig. 5, with increasing concentration, the inhibition capacity of the polysaccharides were enhanced, especially in the liver tissue, in the small concentration range (0.1–0.3 mg/mL, PMP-2), and the inhibition surge to more than 100%. Meanwhile, as shown in Table 1, PMP-1 and PMP-2 exhibited more lipid oxidation inhibition than hydroxy peroxide, superoxide anion and hydroxy radical. Of those, PMP-2 showed the lowest half maximal inhibitory concentration (IC_{50}) of 0.16 mg/mL on lipid oxidation in liver tissue within a 30 min incubation time, followed by 0.51 mg/mL in heart tissue, and 0.80 mg/mL in kidney tissue. We found that PMP-2 was more effective than PMP-1 in inhibiting lipid oxidation in three organ tissues (Table 1). We also learned inhibition lipid oxidation by *G. frondosa* polysaccharide in the recently literature (Chen et al., 2012). This result suggested that the two fractions of *PM* polysaccharides had great capacity for inhibiting lipid peroxidation. Therefore, the polysaccharides from *PM* may serve as possible functional foods in diets to help the human body reduce oxidative damage.

3.7. Inhibition of formation AGEs

Carbonyl stress is an important mechanism of tissue deterioration in several pathological conditions, such as diabetes complications, Alzheimer's disease, and aging. The reactive dicarbonyl compounds such as glyoxal (GO), methyglyoxal (MG), and 3-deoxyglucosone (3-DG), which are generated from Mallard reactions, are irreversibly and progressively modified lysine, arginine, and cysteine residues of proteins. Over time and the formation of AGEs, are thought to contribute to the development of diabetes mellitus and its complications. Recently, it has been proposed that antioxidants may play a role in either increasing insulin sensitivity or modulating the rise in blood glucose following carbohydrate (CHO) consumption, through their interactions with digestive enzymes (Bryans, Judd, & Ellis, 2007). Early animal studies indicated that supplementation with achyranthes bidentata and lycium barbarum polysaccharides inhibited nonenzyme glycation in D-galactose induced mouse aging models *in vivo* (Deng et al., 2003). Further, *in vitro* studies revealed inhibition of the formation of advanced glycation end product by polysaccharides from *Punica granatum* and longan (*Dimocarpus longan* Lour.) (Rout & Banerjee, 2007).

The formation of MGO-mediated protein glycation was determined by fluorescence formation. We were able to conclude, that with the extension of time, the level of AGEs increased. Addition of these two polysaccharides with different concentrations in the incubation mixtures, revealed a gradual decrease of the formation of AGEs and that the inhibitory capacity is dose-dependent (Fig. 6). Of those, PMP-2 exhibited better inhibition by suppressing 31.1%

and 19.0% of the formation of AGEs at the concentration of 0.5 and 0.1 mg/mL during 24 h incubation, respectively. PMP-1 also performed certain inhibition by decreasing 17.4% and 10.5% of the formation of AGEs at the concentration of 1.5 and 1.0 mg/mL during 24 h incubation, respectively. In the literature, we also found that three polysaccharides from *Misgurnus anguillicaudatus* showed inhibition of the formation of glycation-BSA (Zhang, Wang, & Dong, 2011).

4. Conclusion

In the present study, we successfully obtained two purified homogeneous polysaccharides, PMP-1 and PMP-2, both being composed of D-glucose. The antioxidant activity of polysaccharides from *PM* in *in vitro* methods, clearly indicated that PMP-2 was more efficiently active than PMP-1 against free radical, lipid oxidation and protein glycation. Furthermore, the free radical scavenging activity of PMP-2 was exhibited in the order: superoxide anion (IC_{50} 0.47 mg/mL) > hydrogen peroxide (IC_{50} 0.60 mg/mL) > hydroxyl radical (IC_{50} 0.93 mg/mL). Whereas the inhibitory activity of PMP-2 on lipid oxidation in rat tissue showed as: liver tissue (IC_{50} 0.16 mg/mL), heart tissue (IC_{50} 0.51 mg/mL), and kidney tissue (IC_{50} 0.8 mg/mL). In addition, PMP-2 also displayed better inhibition by suppressing 31.1% of the formation of AGEs at the concentration of 0.5 mg/mL.

Although, it is unclear whether active compounds indeed remain active after being absorbed and metabolized in the body, the interest in plant antioxidants is increasing among scientists, food manufacturers, and consumers. The bioactivities of polysaccharides and their conjugates can be affected by many factors including chemical components, molecular mass, structure, conformation, and even the extraction and isolation methods (Xu et al., 2009). In the present study, the two polysaccharides were composed of glucose, whereas Kayali et al. (2005) found β-glucan works like a scavenger and has an antioxidant effect on lipid peroxidation in spinal cord injury in rats. A published study (Song & Moon, 2006) reported the antioxidant profiles of β-glucan, extracted from *Saccharomyces cerevisiae* KCTC 7911 by five different *in vitro* evaluation methods. Moreover, our study suggested that higher antioxidant activities were found when the molecular weight increased ($M_{PMP-2} > M_{PMP-1}$, Table 1). These results were in accordance with (Song et al., 2010), on the contrary, Zhao et al. (2005) showed that in the antioxidant assay, the low molecular weight products are more effective than high molecular weight products. Thus, the antioxidant activity of polysaccharides is influenced by various factors combined rather than one single factor. Further detailed structural characterization of polysaccharides should be carried out to elucidate the structure–function relationship.

The results of this study show that the polysaccharides of *PM* can be used as an easily accessible source of natural antioxidants and as a possible dietary supplement.

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