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Antioxidant activities of different fractions of polysaccharide purified from *Gynostemma pentaphyllum Makino*

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Abstract

The water-soluble crude polysaccharide GM, obtained from the *Gynostemma pentaphyllum Makino* by boiling-water extraction and ethanol precipitation, was fractionated by DEAE–Sepharose CL-6B column chromatography, and purified by Sephadex G-100 column chromatography, giving three polysaccharide fractions termed GMA, GMB and GMC. The monosaccharide components of them were studied by PC and GC. On the basis of superoxide radical assay, hydroxyl radical assay and self-oxidation of 1,2,3-phentriol assay, the antioxidant activities of GM, GMA, GMB and GMC were investigated. Among these contents, GMC had the higher scavenging effects on superoxide radicals and inhibitory effects on self-oxidation of 1,2,3-phentriol, and so should be explored as a novel potential antioxidant.

Keywords: *Gynostemma pentaphyllum Makino*; Polysaccharide; Purification; Antioxidant activity

1. Introduction

Oxidation is essential to many organisms for the production of energy to fuel biological processes. However, the uncontrolled production of oxygen derived free radicals is involved in onset of many diseases such as cancer, rheumatoid arthritis, and atherosclerosis as well as in degenerative processes associated with aging (Mau, Lin, & Song, 2002). In order to reduce damage to the human body, synthetic antioxidants are used for industrial processing at the present time. However, the most commonly have been suspected of being responsible for liver damage and carcinogenesis (Grice, 1988; Qi et al., 2005). Thus, it is essential to develop and utilize effective and natural antioxidants so that they can protect the human body from free radicals and retard the progress of many chronic diseases (Kinsella, Frankel, German, & Kanner, 1993; Nandita & Rajini, 2004). Published data indicates that plant polysaccharides in general have strong antioxidant activities and can be explored as novel potential antioxidants (Hu, Xu, & Hu, 2003; Jiang, Jiang, Wang, & Hu, 2005; Ramarahnam, Osawa, Ochi, & Kawaishi, 1995). The extraction and characterization of active compounds from medicinal plants and the search for these new pharmacologically active agents has led to the discovery of many clinically useful drugs that play a major role in the treatment of human disease (Colegate & Molyneux, 1993; Donehower & Rowinsky, 1993).

Gynostemma pentaphyllum Makino is a well known edible and medicinal plant in oriental countries (Hu, Chen, & Xie, 1996). Recently, *Gynostemma pentaphyllum Makino* has attracted great attention owing to its anti-tumor activities (Zhou, Liang, & Hu, 2001), anti-gastric ulcer effect (Rujjanawate, Kanjanapothi, & Amornlerdpison, 2004), immunomodulatory effect (Qian, Wang, & Tang, 1998), and treating hyperlipidaemia (Birgitte, Per, & Zhao, 1995). The cultures of *Gynostemma pentaphyllum Makino* or their extracts processed in health care have been put into production on a large scale. To date, no investigation has been

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carried out on polysaccharides that may account for the textural properties and antioxidant activities of *Gynostemma pentaphyllum Makino*. Identification of the polysaccharides is necessary to better effectively exploit the structure and functional properties of these substances.

In this study, we report on the extraction and purification of the major polysaccharides of *Gynostemma pentaphyllum Makino* using a DEAE–Sepharose CL-6B column chromatography and a Sephadex G-100 column chromatography. In addition, the properties and antioxidant activities of these major polysaccharides are also identified.

2. Materials and methods

2.1. Materials and chemicals

Dried *Gynostemma pentaphyllum Makino* was purchased from a local store (Quanzhou, Fujian Province, China).

Nitro blue tetrazolium (NBT), phenazine methosulfate (PMS), dihydronicotineamidadenine dinucleotide (NADH), thiobarbituaric acid (TBA), deoxyribose, L-rhamnose, D-glucuronic, D-arabinose, D-xylose, D-fructose, D-galactose and D-mannose were purchased from Sigma Chemical Co. (St. Louis, MO, USA), while DEAE–Sepharose CL-6B and Sephadex G-100 were from the Pharmacia Co. (Sweden). All other reagents used were of analytical grade.

2.2. Isolation and purification of polysaccharides

The *Gynostemma pentaphyllum Makino* (250 g) was extracted with 95% ethanol at 50 °C for 6 h, dried, and then extracted with distilled water at 95 °C for 1.5 h twice. After each extraction, the soluble polymers were separated from residues by filtration, and extracts were combined, concentrated and dialyzed against running water for 48 h. The above extract was submitted to graded precipitation with four volumes of ethanol and the mixture was kept overnight at 4 °C to precipitate the polysaccharides. The precipitate was collected by centrifugation, washed successively with ethanol and ether, and dried at reduced pressure, giving GM as a crude polysaccharide.

Size-exclusion and anion-exchange chromatography were used for the fractionation of this preparation. The sample GM (800 mg) was dissolved in 10 mL distilled water, centrifuged, and then the supernatant was injected to a column (4.6 × 30 cm) of DEAE–Sepharose CL-6B equilibrated with distilled water. After loading with sample, the column was eluted with distilled water for 500 mL at 4 mL/6 min/tube, followed stepwise by NaCl aqueous solution (0 and 2 M) for 400 mL, respectively, at 8 mL/12 min/tube. The major polysaccharide fractions were collected with a fraction collector, dialyzed against tap water and distilled water for 48 h, respectively, and then purified by gel-filtration chromatography on a column of Sephadex G-100 (2.6 × 70 cm).

2.3. Monosaccharide composition and properties

Total carbohydrate and protein of these polysaccharides were determined by the phenol–sulfuric acid (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) and Bradford (Bradford, 1976), respectively. Paper chromatography (PC, Wang, Luo, & Liang, 2004) and gas chromatography (GC) were used for identification and quantification. GC was performed on a HP5890 instrument (Hewlett-Packard Component, USA) with a column HP-5 (30 m × 0.32 mm × 0.25 μm). First, the polysaccharide (5 mg) was hydrolyzed with 1 mL of HCl–methanol at 80 °C for 20 h, then hydrolyzed products were neutralized to pH 6.0 by KOH–methanol and dried at reduced pressure. Derivation was then carried out using the trimethylsilylation reagent according to the method of Guentas et al. (2001) with some modifications. Above dried product was dissolved with 0.2 mL pyridine of at 75 °C for 30 min, added 0.2 mL hexamethyl disilazane and 0.1 mL trimethychlorosilane, and mixed rapidly. The derivatives were loaded onto a HP 5 capillary gas chromatography (GC) column equipped with flame-ionization detector (FID), using mannitol as the internal standard. The operation was performed using the following conditions: H_2: 20 mL/min; air: 200 mL/min; N_2: 20 mL/min; injection temperature: 250 °C; detector temperature: 250 °C; column temperature programmed from 160 to 180 °C at 20 °C/min, then increasing to 220 °C at 8 °C/min and holding for 1 min at 220 °C (Yang et al., 2006).

The IR spectrum of the polysaccharide was determined using a Fourier transform infrared spectrophotometer (FTIR, Bruker, Germany) equipped. The purified polysaccharide was ground with KBr powder and then pressed into pellets for FTIR measurement in the frequency range of 4000–500 cm^{-1} (Kumar, Joo, Choi, Koo, & Chang, 2004). The molecular weight was calculated by the calibration curve obtained by using various standard dextrans (Wang, Liang, & Zhang, 2001).

2.4. Determination of the polysaccharides purification

The sample were dissolved in 0.9% sodium chloride, centrifuged, and then the filtrate was applied to a Sephadex G-100 column (1.6 × 80 cm), which was eluted with 0.9% sodium chloride at 2.2 mL/12 min/tube. Polysaccharides were detected by the phenol–sulfuric acid method (Dubois et al., 1956). Elution curve was drawn by tube number as abscissa and absorbance as vertical coordinate. In addition, the polysaccharides purification was also identified by cellulose acetate pellicle electrophoresis (borax–sodium hydroxide buffer, pH 10) at 40 V for 50 min with detection using Toluidine Blue.

2.5. Assay for antioxidant activities

2.5.1. Superoxide radical assay

The superoxide radical assay was measured by the method of Robak and Gryglewski (1988) with a minor

modification. Samples were dissolved in distilled water at 0 (control), 0.625, 1.25, 2.5, 5, or 10 mg/mL. A 0.1-mL aliquot of each sample solution was mixed with 1 mL of 16 mM Tris–HCl (pH 8.0) containing 557 μM NADH, 1 mL of 16 mM Tris–HCl (pH 8.0) containing 45 μM PMS, and 1 mL of 16 mM Tris–HCl (pH 8.0) containing 108 μM NBT. After 5 min of incubation at 25 °C, the absorbance was measured at 560 nm. The superoxide radical effect was calculated as scavenging activity (%) = (1 − absorbance of sample/absorbance of control) × 100.

2.5.2. Hydroxyl radical assay

The hydroxyl radical assay was measured by the method of Ghiselli et al. with a minor modification (Ghiselli, Nardini, Baldi, & Scaccini, 1998; Halliwell, Gutteridge, & Aruoma, 1987). Samples were dissolved in distilled water at 0 (control), 0.5, 1, 2, 4, or 8 mg/mL. The sample solution (0.1 mL) was mixed with 0.6 mL of reaction buffer [0.2 M phosphate buffer (pH 7.4), 2.67 mM deoxyribose, and 0.13 mM EDTA], 0.2 mL of 0.4 mM ferrous ammonium sulfate, 0.05 mL of 2.0 mM ascorbic acid, and 0.05 mL of 20 mM H_2O_2. The reaction solution was incubated for 15 min at 37 °C, and then 1 mL of 1% thiobarbituric acid and 1 mL of 2.0% trichloroacetic acid were added to the mixture. The mixture was boiled for 15 min and cooled on ice. The absorbance of the mixture was measured at 532 nm. Percent inhibition of hydroxyl radical was calculated as (1 − absorbance of sample/absorbance of control) × 100.

2.5.3. Self-oxidation of 1,2,3-phentriol assay

The scavenging ability for self-oxidation of 1,2,3-phentriol of all different contents were investigated according to the method of Marklund and Marklund (1974) with a minor modification. Briefly, samples were dissolved in distilled water at 0 (control), 10, 20 or 40 mg/mL. The sample solution (0.1 mL) was mixed with 2.8 mL of 0.05 M Tris–HCl buffer (pH 8.0) containing 1 mM EDTA and 1,2,3-phentriol (0.2 mL, 6 mM) was shaken rapidly at room temperature. The absorbance of the mixture was measured at 325 nm per 30 s for 4 min against a blank, and a slope was calculated as absorbance/min. The ability of different scavenging ability for self-oxidation of 1,2,3-phentriol of all fractions was calculated using the equation (1 − slope of sample/slope of control) × 100.

3. Results and discussion

3.1. Isolation, purification and composition of polysaccharides

The crude polysaccharide GM was isolated from the hot-water extract of *Gynostemma pentaphyllum Makino* by a yield of 12.75%. After fractionation on DEAE–Sepharose CL-6B column, GMA (12.5%) GMB (3.3%), and GMC (8.6%) were obtained from distilled water elute and NaCl elute, respectively (Figs. 1 and 2). The three fractions were purified by gel chromatography on Sephadex G-100 column, respectively. We came to a conclusion that they were homogeneous by the following tests. They were showed both only one symmetrical peak from gel-filtration chromatography on Sephadex G-100 column, indicating that no other polysaccharide was present in the sample, and gave a single spot on cellulose acetate pellicle electrophoresis.

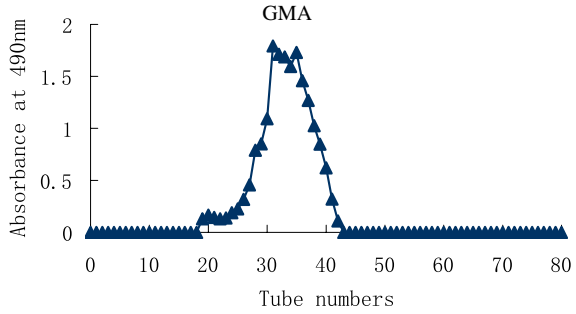

Fig. 1. DEAE–Sepharose CL-6B column chromatogram of GM from distilled water elute.

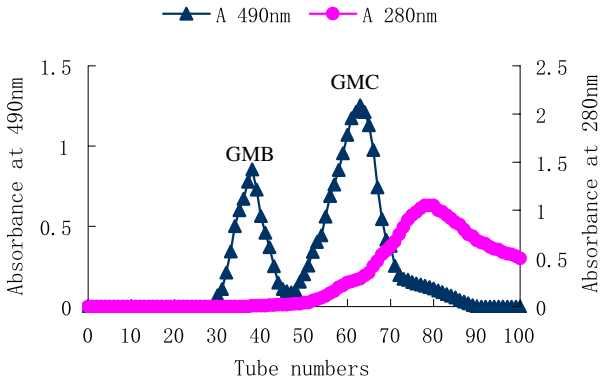

Fig. 2. DEAE–Sepharose CL-6B column chromatogram of GM from NaCl stepwise elute.

The polysaccharide content, protein content, and sugar compositions of GM, GMA, GMB and GMC were determined and given in Table 1. The infrared spectrums of

Table 1
Components of monosaccharide and properties of polysaccharides from *Gynostemma pentaphyllum Makino*

Samples[a]	GM	GMA	GMB	GMC
Protein (wt%)	3.1	nd[b]	nd	nd
Carbohydrate (wt%)	19.41	99.8	99.5	98.9
Average molecular weights	nd	9.4×10^4	12×10^4	7.2×10^4
Sugar components[c] (mol%)				
Glu	1.54	11.45	1.30	1
Gal	3.05	nd	1.31	2.17
Man	1	nd	1	1.25
Fru	1.10	1	nd	1.02

[a] GM: crude polysaccharides by hot-water extraction; GMA: distilled water elute fraction purified by a DEAE–Sepharose CL-6B chromatography; GMB, GMC: NaCl elute fraction by DEAE–Sepharose CL-6B chromatography.
[b] nd, not detected.
[c] Glu, glucose; Gal, galactose; Man, mannose; Fru, fructose.

GMA, GMB and GMC all displayed a broad stretching intense characteristic peak at around 3407 cm^{-1} for the hydroxyl group, and a weak C–H stretching band at 2931 cm^{-1}. Two stretching peaks at 1077 and 1154 cm^{-1} suggest the presence of C–O bonds.

3.2. Antioxidant activities

3.2.1. Scavenging activity of superoxide radical by GM, GMA, GMB and GMC

Superoxide radicals were generated in a PMS/NADH system for being assayed in the reduction of NBT. Fig. 3 shows that the inhibitory effect of four polysaccharide contents extracted and purified from *Gynostemma pentaphyllum Makino* indicated a concentration-dependent, radical-scavenging activity at all tested concentrations of GM, GMA, GMB and GMC. The purified fraction GMC from crude polysaccharides of *Gynostemma pentaphyllum Makino* exhibited a relatively high level of radical-scavenging activity at lower amounts. At the amount over about 0.2 mg, scavenging activity of GMA, GMB and GMC were weaker than that of GM. At the amount of 1 mg, namely 0.33 mg/mL, the effects on scavenging superoxide radical of GM, GMB and GMC were 93.3%, 49.5% and 57.9%, respectively. Qi et al. (2006) reported that scavenging activity of Vitamin C for superoxide radical was about from 30% to 40% at 0.5 to 0.75 mg/mL. Compared to this result, GM, GMB and GMC had stronger scavenging activity for superoxide radical than Vitamin C. Our data on the activities of scavenging superoxides of different contents suggested that it was likely to contribute towards the observed antioxidant effect.

3.2.2. Scavenging activity of hydroxyl radical by GM, GMA, GMB and GMC

Hydroxyl radicals, generated by reaction of iron–EDTA complex with H_2O_2 in the presence of ascorbic acid, attack deoxyribose to form products that, upon heating with 2-thiobarbituaric acid under acid conditions, yield a pink tint. Added hydroxyl radical scavengers compete with deoxyribose for the resulted hydroxyl radicals and diminish tint formation (Cheng, Ren, Li, Chang, & Chen, 2002). The above-mentioned model was used to measure inhibitory activities of all fractions on hydroxyl radicals. As shown in Fig. 4, the four polysaccharides were found to have the ability to scavenge hydroxyl radicals at amounts between 50 and 800 μg. The scavenging effects increased with increasing concentration. Scavenging activities of GMB and GMC were weaker, while those of GM were stronger, and that of GMA was between them. All purification fractions showed scavenging activities of hydroxyl radicals, indicating that they were main contents to execute antioxidant functions in crude polysaccharides from *Gynostemma pentaphyllum Makino*. Scavenging effects of GM, GMA and GMB were 57.0%, 43.8%, and 25.6% at amount of 800 μg, namely 800 μg/mL, respectively. It was reported that scavenging effect on hydroxyl radical of Vitamin C was about 20% at 1.0 mg/mL (Qi et al., 2006). This result proved that polysaccharides from *Gynostemma pentaphyllum Makino* had significant effect on scavenging hydroxyl radical, and some fractions was more pronounced than that Vitamin C.

3.2.3. Scavenging activity of self-oxidation of 1,2,3-phentriol by GM, GMA, GMB and GMC

Table 2 depicted the scavenging power of self-oxidation of 1,2,3-phentriol of different polysaccharide contents extracted and purified from *Gynostemma pentaphyllum Makino*. The scavenging powers of GM and GMC correlated well with increasing concentrations, but that of GMA and GMB were not detected. Moreover, the scavenging power of GMC was relating more pronounced than that of GM. These results indicate that GMC have strong scavenging power for self-oxidation of 1,2,3-phentriol and should be explored as novel potential antioxidants.

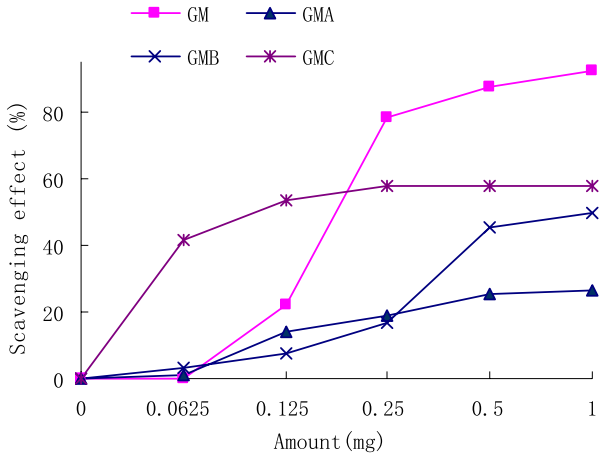

Fig. 3. Scavenging effects of GM and different polysaccharide fractions on superoxide radicals. Results are presented as means ± standard deviations ($n = 3$). Differences were considered to be statistically significant if $P < 0.05$ when compared to standard.

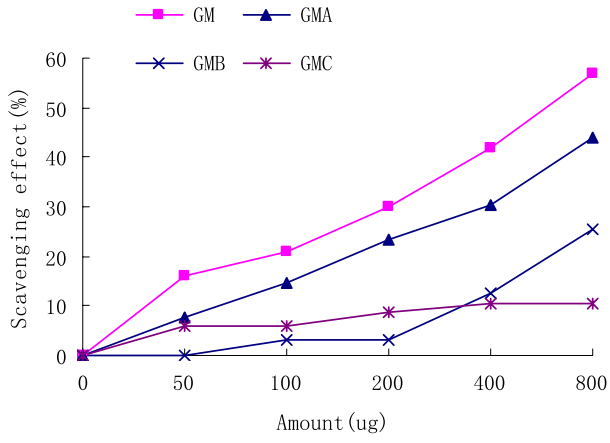

Fig. 4. Scavenging activities of GM and different polysaccharide fractions on hydroxyl radicals. Results are presented as means ± standard deviations ($n = 3$). Differences were considered to be statistically significant if $P < 0.05$ when compared to standard.

Table 2
Inhibitory effects of GM, GMA, GMB and GMC on self-oxidation of 1,2,3-phentriol

Samples	Amount (mg)	Scavenging effects (%)		
		1	2	4
GM		nd[a]	82.75 ± 0.2	98.38 ± 0.2
GMA		nd	nd	nd
GMB		nd	nd	nd
GMC		83.65 ± 0.2	98.85 ± 0.4	98.97 ± 0.5

Data are presented as means ± standard deviations ($n = 3$). Means with different letters within a row are significantly different ($p < 0.05$).
[a] nd, not detected.

4. Conclusions

According to the results above, it was concluded that the water extracting crude polysaccharide (GM) of *Gynostemma pentaphyllum Makino* contained predominantly three polysaccharide fractions (GMA, GMB and GMC) purified by DEAE–Sepharose CL-6B and Sephadex G-100 column chromatography, and purification polysaccharide prepared are confirmed of high purity. Antioxidation test in vitro shows that GMC possesses strong scavenging effect of superoxide radical and inhibiting activity of self-oxidation of 1,2,3-phentriol, which may be comparable to vitamin C, but GMA are confirmed having higher scavenging activity of hydroxyl radical. We may rationally assume that *Gynostemma pentaphyllum Makino* playing its curative effect in traditional medicine partly by the mechanism of antioxidation of polysaccharides in it, and so they should be explored as a novel potential antioxidant.

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