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Full Length Research Paper

Antioxidant activity of polysaccharides extracted from *Athyrium multidentatum* (Doll) Ching

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In this paper, total polysaccharides were respectively prepared from rhizome and aerial part of *Athyrium Multidentatum* (Doll) Ching. (AMC) and had their antioxidant activities investigated employing various established *in vitro* systems, including superoxide/hydroxyl radical scavenging activity and reducing power. Sulfate content and molecular weights of polysaccharides from rhizome (PR) and aerial part (PA) were determined. PR and PA exhibited stronger scavenging capacity on superoxide radicals than vitamin C (Vc) at concentrations from 22.22 to 44.44 µg/ml. The sulfate content in PR and PA were 3.45 and 4.68%, respectively. PR and PA consisted of different polysaccharide fractions. Available data obtained with *in vitro* models suggested that the ratio of sulfate content was an effective indicator to antioxidant activity of the samples.

Key words: Antioxidant activity, *Athyrium Multidentatum* (Doll.) Ching., polysaccharide, radical scavenging activity, reducing power.

INTRODUCTION

With the constant development of preclinical medicine and life science, studies on free radical have been developed. Reactive oxygen species (ROS) in the forms of superoxide anion ($\bullet O_2^-$), hydroxyl radical (•OH) and hydrogen peroxide (H_2O_2) are generated from metabolism or environmental sources interact continuously in biological systems, and their uncontrolled generation correlate directly with molecular level of many diseases (Lee and Lee, 2006). Antioxidants can terminate or retard the oxidation process by scavenging free radicals. They work in two ways: Scavenging ROS and inhibiting the generation of ROS. There are some synthetic antioxidant compounds, such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), *tert*-butylhydroquinone (TBHQ) and propyl gallate (PG), which are commonly used in processed foods. However, some synthetic antioxidants such as BHT and BHA are restricted by legislative rules because they are suspected to have some toxic effects and as possible carcinogens (Soubra et al., 2007). Therefore, evaluation of the functional properties of naturally occurring substances, especially those that are present naturally in human diets, has been of interest in recent years.

AMC is a traditional Chinese herbal medicine for its extensive pharmacological effects, such as tranquilization, lowering blood pressure and diuresis etc. (Liu et al., 2010). In addition, AMC has been used for a potherb in Changbai Mountain area of China since ancient times, and exported to South Korea, Japan, Hong Kong and other countries as

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Abbreviations: AMC, *Athyrium Multidentatum* (Doll) Ching.; **PR,** polysaccharide from rhizome; **PA,** polysaccharide from aerial part.

a delicious and nutritious potherb. In our previous study, polysaccharides were isolated from rhizome and aerial part of AMC. In the last decades, much attention has focused on the biological properties of polysaccharides and their chemical derivatives, especially sulfated derivatives (Lee et al., 2003; Han et al., 2005; Xing et al., 2005; Yang et al., 2005). Sulfated polysaccharides have been reported to possess diverse biological activity of potential medicinal value, such as anticoagulant, antitumour, anti-inflammatory, antiviral and antioxidant activity (Feldman et al., 1999). Evidence from different studies suggested that the antioxidant and anticoagulant activities of polysaccharides were strongly dependent on the degree of sulfation, the molecular weight, the sulfation pattern and glycosidic branches (Li et al., 2010). However, as far as our literature survey could ascertain, little information was available on chemical property and biological activity of polysaccharides from AMC.

In this study, PR and PA were prepared and their *in vitro* antioxidant activities were investigated, including superoxide/hydroxyl radical scavenging activity and reducing power. The *in vitro* antioxidant activity and the relationship between chemical property and antioxidant activity were reported in this paper.

MATERIALS AND METHODS

The rhizome and aerial part of AMC were harvested in Changbai Mountain area of China (September, 2009), air-dried and kept in plastic bags at room temperature for use. Pyrogallol, trichloroacetic acid (TCA), H_2O_2, glucose and Vc were purchased from Sigma Chemicals Co. Other reagents were of analytical grade and purchased from China National Pharmaceutical Group Corporation.

Preparation of polysaccharides

A total of 200 g of AMC rhizome was crushed and refluxed in 6000 ml water for 3 h. The hot solution was separated from rhizome residues by successive filtration through gauze and siliceous earth. The solution was concentrated to about 600 ml under reduced pressure, and then 1800 ml of anhydrous ethanol was added. The resultant precipitate was filtrated and dialyzed against tap water for 48 h and against distilled water for 24 h using 3000 Da *M*w cutoff dialysis membranes, sequentially. PR was obtained and kept in vacuum desiccator for use. PA was prepared in the same method.

Chemical analysis

Total sugar content was determined according to the method of Du et al. (2008) using glucose as standard. Sulfate content was analyzed by the barium chloride-gelatin method of Kawai et al. (1969). Molecular weights of PR and PA were determined by high performance size exclusion chromatography. Shodex GPC HT-804 column (30 cm × 0.8 cm i.d., Japan) and a Waters Model 590 pump were used. The column was maintained at 35°C and the mobile phase was 2.84% (w/v) Na_2SO_4 buffer with flow rate of 1.0 ml/min. Samples were filtered through 0.45 µm filter membrane before analysis. The samples were injected with an injector (Perkin-Elmer ISS100) and the injection volume was 25 µl. The detection was carried out at 35°C with a refractive index detector (Agilent 1100 Series). Column calibration was performed with standard dextran (National Institute for the Control of Pharmaceutical and Biological Product, China), and calculations of molecular weights were carried out using Turbochrom software (Perkin-Elmer, Norwalk, CT, USA).

Reducing power assay

The reducing power of all samples was determined by the method described by Yamaguchi et al. (1998), modified slightly. All solutions were used on the day of preparation. Briefly, 6.0 ml of reaction mixture, containing different concentrations of samples (50.0 to 400.0 µg/ml) in phosphate buffer (0.2 M, pH 6.8), was incubated with potassium ferricyanide (1%, w/v) at 50°C for 20 min. The reaction was terminated by TCA (10%, w/v) solution. Then the solution was mixed with distilled water and $FeCl_3$ (0.1%, w/v) solution. The reaction mixture was shaked vigorously and left still for 1 h. Increased absorbance at 700 nm indicated increased reducing power. Vc was used as positive control.

Hydroxyl radical scavenging assay

The hydroxyl radical scavenging ability of all samples was assessed by the method of Cao et al. (2008) with minor modification. The reaction mixture, containing H_2O_2 (1.0 mM) and $FeSO_4$ (2.0 mM), was incubated with salicylate (6.0 mM) at 37°C for 15 min. The absorbance A_1 of the mixture was determined at 510 nm against the blank. The absorbance A_2 was determined after 1.0 ml of sample was added. In order to offset the reduced absorbance caused by the adding of sample, the absorbance A_3 and A_4 were determined. The absorbance A_3 was determined after the mixture was incubated at 37°C for another 10 min. The absorbance A_4 was determined after distilled water (equal volume with sample) was added into the mixture. The hydroxyl radical inhibition percentage was calculated according to the given formula:

$$\text{Scavenging effect (\%)} = [A_1 - A_2 - (A_3 - A_4)] \times 100 / A_1$$

Superoxide radical scavenging assay

Measurement of superoxide radical scavenging activity was based on the method of Cao et al. (2008) with slight modification. Superoxide radicals were generated in the pyrogallol autoxidantion system containing 4.5 ml Tris-HCl buffer (50 mM, pH 8.2), 0.3 ml pyrogallol (3 mM), and varying concentrations of samples (11.90 to 95.24 µg/ml); the mixture prepared earlier was incubated at room temperature for 20 min. The absorbance A_1 at 319 nm was read every 30 s for 5 min against the blank. The speed of pyrogallol autoxidantion V_1 within 5 min can be calculated. In the control, sample was substituted with Tris-HCl buffer, the absorbance A_0 was measured, and the speed of pyrogallol autoxidantion V_0 within 5 min can be calculated following the same method. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. The capability of scavenging on

Table 1. Chemical composition (%, dry weight) of PR (polysaccharide from rhizome) and PA (polysaccharide from aerial part) isolated from AMC (*Athyrium Multidentatum* (Doll) Ching.).

Sample	Total sugar	Sulfate	MW[a] (kDa)		
			MW_1	MW_2	MW_3
PR	81.04	3.45	1774.39	43.35	4.52
PA	84.72	4.68	2872.51	23.36	–

[a]MW, molecular weight, was measured by high performance size exclusion chromatography.

superoxide radical was calculated using the following equation:

Scavenging effect (%) = $(V_0 - V_1) \times 100 / V_0$

Statistical analysis

The data presented are means ± SD of three determinations and followed by Student's *t* test. Differences were considered to be statistically significant if $P < 0.05$.

RESULTS AND DISCUSSION

Chemical analysis

The chemical properties were summarized in Table 1. As shown in Table 1, the sulfate content in PR and PA were 3.45 and 4.68%, respectively. PR and PA consisted of different polysaccharide fractions. PR consisted of three polysaccharides fractions and their molecular weights were respectively 1774.39, 43.35 and 4.52 kDa. PA consisted of two polysaccharides fractions and their molecular weights were 2872.51 and 23.36 kDa, respectively. Differences between polysaccharides from rhizome and aerial part have been observed in our previous study (Liu et al., 2010). The chemical properties may have great influence on antioxidant activities, which have been proved in this research.

Reducing power assay

In the reducing power assay, the presence of reductants (antioxidants) in the tested samples would result in reducing Fe^{3+}/ferricyanide complex to the ferrous form (Fe^{2+}) (Zhao et al., 2006.), and a greenish reaction mixture with precipitant was observed; the color and precipitant would turn thicker depending on the reducing power of antioxidant samples. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. Higher absorbance value means stronger reducing power of samples. As shown in Figure 1, the reducing power of PA was stronger than that of PR, and the reducing power of PA and PR was concentration dependent. With the concentration increased from 5.0 to 40.0 µg/ml, the absorbtion of PA was increased from 0.138 to 0.548; however, the absorbtion of PR was only increased from 0.068 to 0.348. The reducing power of our tested samples was lower than that of Vc. The relation of reducing power to sulfate content was significant. The reducing properties were generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom (Chung et al., 2002). Reductones also react with certain precursors of peroxide, thus preventing peroxide formation. Our data showed that reducing power of different polysaccharide fractions probably play a role in the antioxidation observed.

Hydroxyl radical scavenging assay

Hydroxyl radical is the most reactive free radical and can be formed from superoxide anion and hydrogen peroxide in the presence of metal ions, such as copper or iron (Macdonald et al., 2003). The hydroxyl radicals, generated by the Fenton reaction in the system, were scavenged by PR and PA. In the hydroxyl radical scavenging assay, the reaction solution was colorless at first, and then changed into light yellow and blue-violet with the addition of H_2O_2 and salicylate. Figure 2 depicts the hydroxyl radical effects of tested samples. The scavenging activity of PR was slightly stronger than that of PA at a concentration of 5.0 to 40.0 µg/ml. The greatest inhibition percentage of PR and PA was 9.28 and 7.14% at concentrations of 5.0 and 30.0 µg/ml, respectively. The similar inhibition percentage of PR and PA was exhibited at concentrations from 10.0 to 30.0 µg/ml. However, the scavenging abilities of PR and PA were weaker than that of Vc. For hydroxyl radical, there are two types of antioxidation mechanism; one suppresses the generation of the hydroxyl radical, and the other scavenges the hydroxyl radicals generated. In the former, the antioxidant activity may ligate to the metal ions which react with H_2O_2

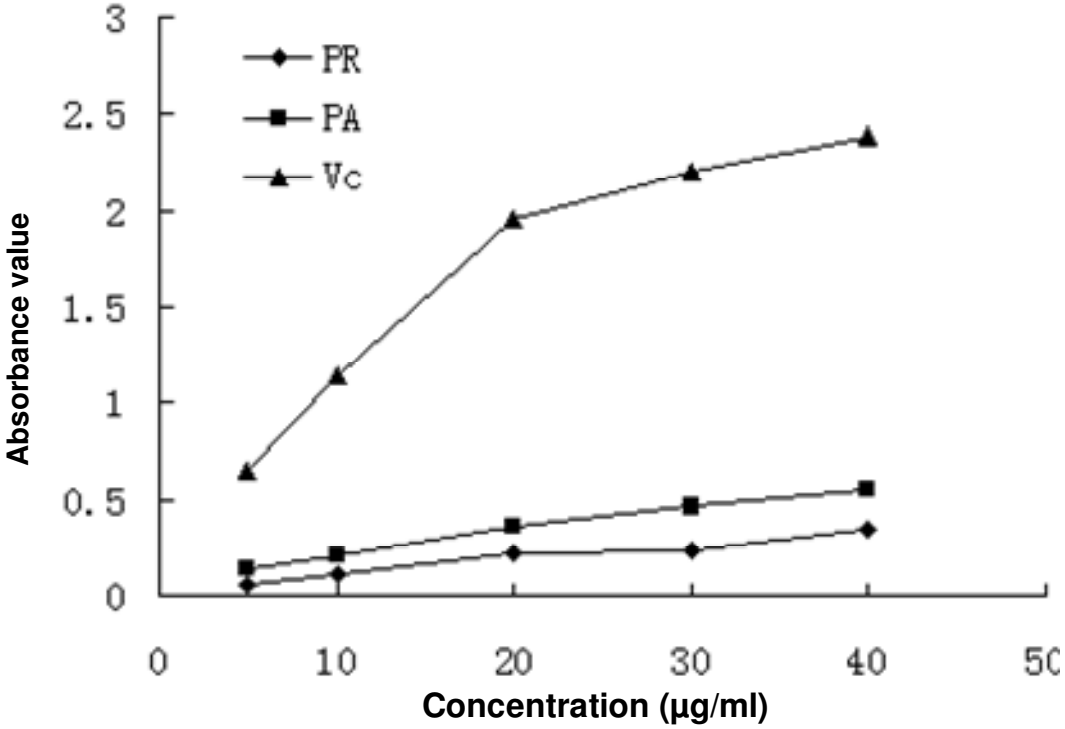

Figure 1. Reducing power of polysaccharide from rhizome (PR), polysaccharide from aerial part (PA), and vitamin C (Vc). Values are means ± SD (n = 3).

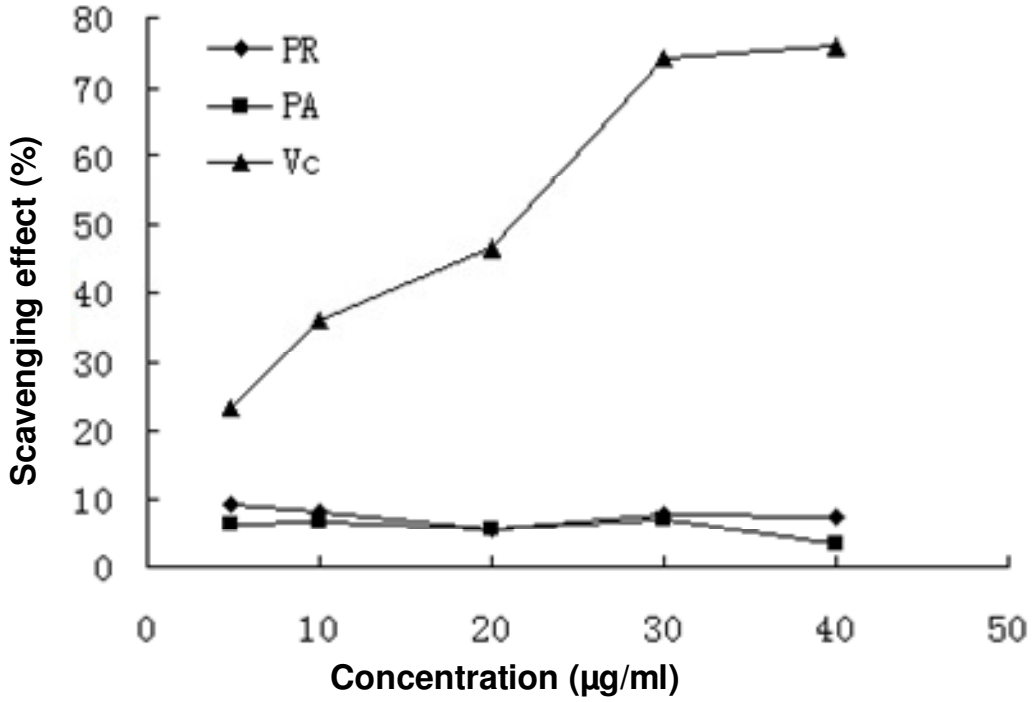

Figure 2. Hydroxyl radical scavenging activity of polysaccharide from rhizome (PR), polysaccharide from aerial part (PA), and vitamin C (Vc). Values are means ± SD (n = 3).

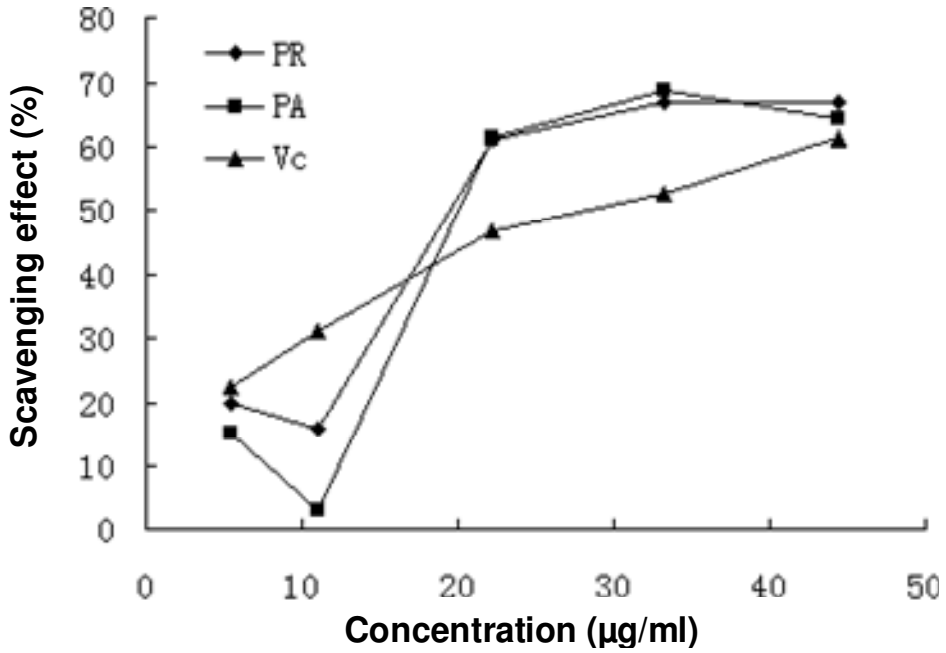

Figure 3. Superoxide radical scavenging activity of polysaccharide from rhizome (PR), polysaccharide from aerial part (PA), and vitamin C (Vc). Values are means ± SD (n = 3).

to give hydroxyl radicals (Halliwell et al., 1987; Shon et al., 2003). Hydroxyl radicals acted in superoxidation by hydrogen peroxide with metal ions, usually ferrous or cupper. The molecules that could chelate iron, and render them inactive of poorly active fenton reaction might have scavenging ability on hydroxyl radical (Macdonald et al., 2003). In this study, in another assay system, the iron chelating abilities of PR and PA were all investigated. All the samples exhibited poor chelating ability. The mechanism of these resultants on hydroxyl radical needs to be further researched by plural experimental methods.

Superoxide radical scavenging assay

Superoxide radical is an initial free radical and plays an important role in the formation of other reactive oxygen species such as hydrogen peroxide, hydroxyl radical, or singlet oxygen in living systems. In the superoxide radical scavenging assay, superoxide radicals were generated by pyrogallol autoxidantion system. Figure 3 depicts the scavenging effects of PR and PA on superoxide radicals. As shown in Figure 3, the inhibition effects of PR and PA on the autoxidation of pyrogallol were stronger than that of Vc at higher concentrations from 22.22 to 44.44 μg/ml. The maximum scavenging percentage of PR and PA was respectively 66.83 and 68.75% at a concentration of 33.33 μg/ml. PA exhibited stronger scavenging capacity than PR, which was proportional to the sulfate content in the samples. This demonstrated that the sulfate content affected their antioxidant activity; these results were in accordance with Zhang et al. (2003). Although superoxide was a weak oxidant, in most organisms, it could degrade continuously and form other active ROS, triggering peroxidation of lipids, and then induce pathological incidents such as arthritis and Alzheimer's disease (Zhu et al., 2004; Wade et al., 1987; Dahl and Richardson, 1978). However, superoxide anion is a reduced form of molecular oxygen created by receiving one electron, so it can scavenge more easily than hydroxyl and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. Other studies also indicated the tested samples had much stronger scavenging ability on superoxide radical than other radicals such as hydroxyl radical and reducing power (Qi et al., 2006). These results clearly suggested that the antioxidant activities of all samples were related to their abilities to scavenge superoxides.

In conclusion, the results clearly demonstrated that polysaccharides isolated from rhizome and aerial part of AMC were antioxidative and exhibited strong antioxidant activity on superoxide radicals. The reducing power of them was different; however, the scavenging activity on superoxide and hydroxyl radicals was similar. A positive correlation has been revealed between sulfate content and superoxide radical scavenging ability and reducing power. The results of chemical analysis and antioxidant

activity assays were applicable in the future experiments for preparation antioxidant activity of polysaccharide fractions from AMC.

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