# Preparation and antioxidant activity of *Athyrium multidentatum* (Doll.) Ching polysaccharide derivatives

**Sheng JW**

*Department of Pharmacy and Biological Sciences, Weifang Medical University, Weifang, PR China*
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**ABSTRACT**

*Polysaccharides extracted from Athyrium multidentatum (Doll.) Ching rhizome (AMCP) were chemically modified by introducing different functional groups. The acetylated, sulphated and phosphorylated derivatives were synthesized successfully, and characterized by IR spectra. The antioxidant activities of polysaccharide derivatives were explored employing various established in vitro systems. Acetylated derivative was prepared using N-bromosuccinimide (NBS) as catalyst. Polyphosphoric acid method was applied in phosphorylation reaction. The phosphorylated derivative exhibited the greatest antioxidant activity among all derivatives, and possessed much stronger reducing power than AMCP. Available data suggested that the substituted groups of AMCP played important roles on the antioxidant activity, and the mechanism on the antioxidant activity of acetyl, sulphonyl and phosphoryl group was indicated.*

**Key words**: *Athyrium multidentatum* (Doll.) Ching, Antioxidant activity, Derivatives, Polysaccharides.
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## INTRODUCTION

It is well known that the medicines derived from natural sources are very popular in food, cosmetic and pharmaceutical industries for their safeties, novel structures and unique mechanism of actions. Natural polysaccharides are long, linear or highly branched carbohydrate molecules that possess various biological activities, including immunoregulation, antivirus, anti-tumor and antioxidant activity [1-3]. The biological activity of polysaccharide is closely correlated with several structural parameters such as the molecular weight, substituted groups and position, type of sugar, and glycosidic branching. Many studies have demonstrated that chemical modification of polysaccharides provide a probability to improve their biological activities. Wang et al. found phosphorylated fucoidan showed stronger hydroxyl radical and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity and reducing power [4]. The chemically sulfated polysaccharide of *Grifola frondosa* mycelia could accelerate the antitumor activity of cyclophosphamide and improve the immunocompetence damaged by cyclophosphamide [5]. The acetylated fucoidan exhibited stronger activity on scavenging DPPH radical and reducing power than fucoidan [6].

*Athyrium multidentatum* (Doll.) Ching (AMC) is a fern species in the Athyriaceae family, which is native to northeast of China. It grows mainly in montane

Abbreviations: AMC, *Athyrium multidentatum* (Doll.) Ching; AMCP, polysaccharides extracted from *Athyrium multidentatum* (Doll.) Ching rhizome; APA, acetylated polysaccharides; APS, sulphated polysaccharides; APP, phosphorylated polysaccharides; FA, formamide; ROS, reactive oxygen species; TCA, trichloroacetic acid; DPPH, 1,1-diphenyl-2-picrylhydrazyl.

wetland regions throughout Changbai Mountain area of Jilin Province. AMC is very popular for its nutrition and medicinal value. According to the natives, the plant is used in herbal medicine for health-promoting and therapeutic

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effects, such as tranquilization, lowering blood pressure and diuresis et al.. Our preliminary study had shown that polysaccharides extracted from AMC were mainly made of mannose (Man), rhamnose (Rha), glucose (Glc), galactose (Gal) and arabinose (Ara) in a molar ratio of 1.0:1.6:5.2:2.1:0.2, and possessed significant antioxidant activity [7, 8]. However, the correlation of acetylated, sulfated and phosphorylated derivatives of AMCP vs. antioxidation yet remained unclear.

In this study, three AMCP derivatives were obtained and their in vitro antioxidant activities were investigated, including hydroxyl, superoxide and DPPH radical scavenging activity, and reducing power. The in vitro antioxidant activity and the relationship between substitute groups and antioxidant activity were reported.

## EXPERIMENTAL SECTION

**Materials**: *Athyrium multidentatum* (Doll.) Ching rhizome was collected from Changbai Mountain area of China in September 2012, and stored in plastic bags for use. N-bromobutanimide (NBS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), and pyrogallol were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals and reagents were of analytical grade, and were used without further purification.

**Preparation of crude polysaccharides:** About 1200 g of crushed rhizome of AMC was refluxed in 6000 mL methanol for 3 h. After the methanol solution was removed, the rhizome residues were air-dried and refluxed with 9600 mL distilled water for 3 h. The aqueous extract solution was collected and evaporated to about 100 mL under reduced pressure, then precipitated with anhydrous ethanol. The resultant precipitate was washed with anhydrous ethanol, and dried at 60°C. Polysaccharides extracted from AMC rhizome (AMCP) was obtained and stored in desiccator for use (mean yield 4.45%).

**Phosphorylation of AMCP**: 2 g of AMCP was dissolved in 100 mL of formamide (FA) and stirred at room temperature for 30 min to be fully dispersed and dissolved, then tributylamine (10 mL) and polyphosphoric acid (5 g) were added to the clear solution. The mixture was stirred for 24 h at room temperature, and precipitated with 500mL anhydrous ethanol. The resultant precipitate was separated from the alcohol solution by filtration through the filter paper, and dissolved in 40 mL distilled water. The pH of the solution was adjusted to 7 with NaOH (1 mol/L), and the neutral solution was dialyzed against distilled water for 24 h using 3500 Da Mw cutoff dialysis membranes. The dialysis solution was evaporated at 50°C in vacuo, and lyophilized to give phosphorylated polysaccharides (APP).

**Sulphation of AMCP**: The sulphation reagent ($SO_3$-FA) was obtained by dropping 10 mL of chlorosulphonic acid ($HClSO_3$) into 60 mL of FA under stirring and cooling in an ice bath. Dry AMCP (2 g) was added to 60 mL FA, and the mixture was stirred at room temperature for 30 min and 80°C for another 30 min. 25 mL of $SO_3$-FA reagent was added drop by drop in 1 h. After stirred at 80°C for 4 h, the whole mixture was cooled to room temperature, neutralised with 1 mol/L NaOH solution, and precipitated with cold anhydrous ethanol. The precipitate was filtered off and washed three times with anhydrous ethanol, and then dissolved in 100 mL distilled water. The solution was dialyzed against tap water for 48 h and distilled water for 24 h using 3500 Da Mw cutoff dialysis membranes. The resultant was concentrated and lyophilized to give sulphated polysaccharides (APS).

**Acetylation of AMCP**: 1 g of AMCP was dispersed in 50 mL FA and stirred at 80°C for 30 min, the mixture was added of 25 mL acetic anhydride and 1% NBS (25 mg NBS dissolved in 25 mL acetic anhydride), then reacted at 80°C for 6 h. The reaction was terminated by 20 mL distilled water. The mixture was cooled to room temperature and precipitated with anhydrous ethanol. The precipitate was filtered off and washed three times with anhydrous ethanol, and dissolved in 100 mL distilled water. The solution was neutralised with 1 mol/L NaOH solution and dialyzed against tap water for 48 h and distilled water for 24 h using 3500 Da Mw cutoff dialysis membranes. The resultant was concentrated and lyophilized to give acetylated polysaccharides (APA).

**Analytical methods:** Total sugar content was determined according to the method of Du et al. [9], using glucose as standard. Sulfate content was analyzed by the barium chloride–gelatin method [10]. The total phosphate was determined by the ascorbic acid method [11]. The introduced acetyl group onto AMCP was estimated using acecoline as standard [12]. Infrared spectra were recorded from polysaccharide powders in KBr pellet on a Fourier-transform infrared spectrophotometer (AVATAR360, USA) in the frequency range 4000–500 cm$^{-1}$.

**Superoxide radical scavenging activity**: Superoxide radical scavenging activity was examined with the method of pyrogallol autoxidantion [13]. 1 mL of different samples (40–200 μg/mL) was incubated with 50 mmol/L Tris–HCl (4 mL, pH 8.2) at 37°C for 10 min, and 50 mmol/L pyrogallol (0.5 mL) pre-incubated at 37°C was added. The reaction proceeded exactly for 6 min at room temperature, then terminated by 0.5 mL HCl (8 mol/L). The absorbance was read at 320 nm against the blank. In the control, sample was substituted with Tris–HCl buffer. The

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capability of scavenging to superoxide radical was calculated by the following equation:

$$\text{Scavenging effect (\%)} = (1 - A_{\text{sample 320}}/A_{\text{control 320}}) \times 100$$

**Hydroxyl radical scavenging assay:** The scavenging activity of hydroxyl radical was assayed according to an improved method of Cao et al. [14]. Briefly, the reaction mixture, containing 1 mmol/L hydrogen peroxide (1 mL) and 2 mmol/L ferrous sulfate (1 mL), was incubated with 6 mmol/L salicylate (1 mL) at 37°C for 15 min. Afterwards, 1 mL of different concentration of samples (40–200 µg/mL) was added to the mixture and allowed to stand at room temperature for 5 min. Hydroxyl radical was measured by monitoring absorbance at 520 nm. In the control, distilled water was substituted for samples. Lower absorbance of reaction mixture indicated higher free-radical scavenging capability. The hydroxyl radical scavenging activity was calculated according to the following formula:

$$\text{Scavenging effect (\%)} = (1 - A_{\text{sample 520}}/A_{\text{control 520}}) \times 100$$

**DPPH free-radical scavenging activity**: The scavenging activity of DPPH free-radical was determined by Shimada et al. [15]. Briefly, a 0.14 mmol/L solution of DPPH in anhydrous ethanol was prepared, and to 2 mL of this solution was added 2 mL of sample solution in 50% ethanol at different concentrations (10–50 µg/mL). The mixture was shaken vigorously and reacted for 5 min at room temperature. Then the absorbance was measured at 517 nm. In the control, 95% ethanol was substituted for samples. The DPPH radical scavenging effect was calculated using the following equation:

$$\text{Scavenging effect (\%)} = (1 - A_{\text{sample 517}}/A_{\text{control 517}}) \times 100$$

**Reducing power assay:** The reducing power was evaluated as the method of Yamaguchi et al. [16]. Briefly, 0.5 mL of different concentration of samples (40–200 µg/mL) in phosphate buffer (0.2 mol/L, pH 6.8) was mixed with 2 mL of potassium ferricyanide (1%, w/v), and incubated at 50°C for 20 min. Then 1 mL of TCA (10%, w/v) was added to terminate the reaction. Finally, the solution was mixed with 0.5 mL of ferric chloride (0.1%, w/v) and left stillness for 10 min. The absorbance was measured at 700 nm. Increased absorbance of reaction mixture indicated increased reducing power.

**Statistical analysis:** All the data are shown in means ± S.D. ($n = 3$) within significance $p < 0.05$ after passing Duncan's multiple-range test, and processed with Excel and Statistica (2003).

## RESULTS AND DISCUSSION

**Chemical analysis:** Three AMCP derivatives were synthesized successfully. The chemical properties of AMCP and its derivatives were summarized in Table 1. As shown in Table 1, the phosphorylated derivative possessed the highest total sugar content and molecular weight among all samples. The acetylate and sulfate contents in APA and APS were estimated to be 12.96% and 2.54%, respectively. All the contents of introduced groups were higher than that of AMCP. However, the total sugar content and molecular weight of APA (11.95%, 6943 Da) were much lower than that of AMCP (48.30%, 22767 Da), we suppose AMCP was degraded at 80°C under the acid condition. The presence of acetyl, sulphonyl and phosphoryl groups was ascertained by IR spectroscopy (Fig. 1). APS and APP showed strong abroad bands, ascribed to their sulfate and phosphate groups, at 1262.53 and 1002.88, 1297.15 and 1112.62 cm$^{-1}$, respectively. Acetylated derivative was prepared by introducing acetyl group onto the AMCP. The peaks at 1744.09 cm$^{-1}$ was assigned to the characteristic absorbance of C=O (ester) stretching vibration. The signals at 1234.97 and 1034.13 cm$^{-1}$ were attributed to the stretching vibration of C–O (ester). The derivative reaction was complexed, some vice reaction happened which led to a low sulphate and phosphate content of APS and APP. The reaction conditions should be optimized to prepare derivatives with higher content of substituent groups.

**Table 1：Chemical composition (%, dry weight) of AMCP and its derivatives isolated from *Athyrium multidentatum* (Doll.) Ching**

| Sample | Total sugar | Molecular weight (Da) | Phosphate | Acetyl | Sulfate |
|--------|-------------|-----------------------|-----------|--------|---------|
| **AMCP** | 48.30 | 22767 | nd | nd | 1.70 |
| **APP** | 64.80 | 33647 | 1.03 | nd | 1.27 |
| **APA** | 11.95 | 6943 | nd | 12.96 | 1.30 |
| **APS** | 45.98 | 30210 | nd | nd | 2.54 |

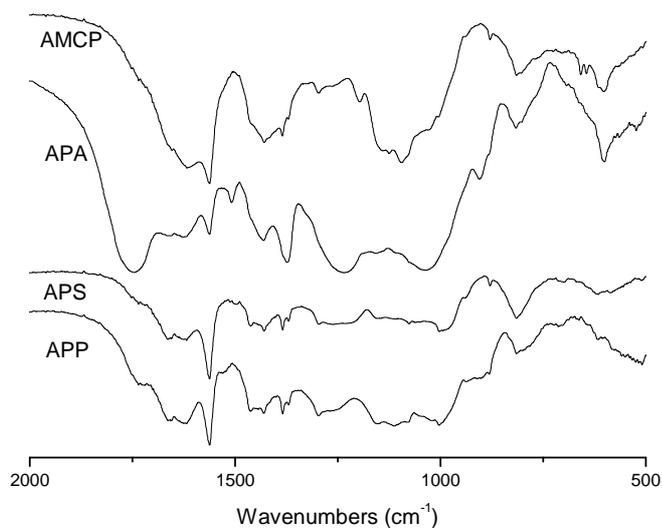*nd: not detectable below the limit at 0.001.*

**Figure. 1. FT-IR spectra of AMCP, APA, APS and APP**

**Superoxide radical scavenging activity**: Superoxide anion is the least active and harmful molecules among reactive oxygen species (ROS). However, it can manify the cellular damage by producing other kinds of free radicals and oxidizing agents, further induced pathological events such as arthritis and Alzheimer's disease [17]. The superoxide radical scavenging ability of all tested samples were shown in Fig. 2. AMCP and APP had scavenging abilities on superxoide radicals in a dose dependent manner (160–200 μg/mL). AMCP and its derivatives exhibited lower scavenging ability than fucoidan derivatives [18]. It might attribute to the structural difference between polysaccharides extracted from *Athyrium multidentatum* (Doll.) Ching and *Laminaria japonica*. Marine polysaccharides usually exhibit structural features such as sulfate and uronic acid groups, which distinguish them from polysaccharides of terrestrial plants, but are similar to mammalian glycosaminoglycans, such as heparin and chondroitin sulfate [19]. The presence of the sulphonyl group could improve the activity of scavenging radicals by increasing electron density at carbon atoms in the heterocyclic ringe. Although sulfate group was detected in AMCP and APS, the sulfate content of AMCP (1.70%) and APS (2.54%) were much lower than that of seaweed, such as *Laminaria japonica* (20.14%) and *Ulva pertusa* (19.5%) [18, 20]. However, it may not the only reason to influence the strength of the antioxidant activity. APA showed stronger scavenging capacity than AMCP from 160 to 200 μg/mL. We suppose that because the presence of acetyl group could change the polarity of the compound, which had effect on antioxidant ability. So the superxoide radical scavenging activity of acetylated derivative was stronger than natural polysaccharides from AMC.
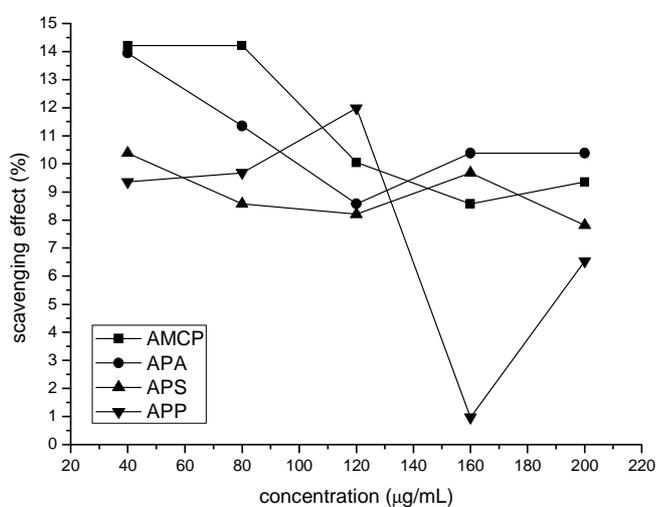


**Figure. 2. Scavenging effect on superoxide radicals by AMCP and its derivatives (APA, APS and APP). Values are means ± S.D. (*n* = 3)**

**Hydroxyl radical scavenging assay:** The inhibition abilities of AMCP and its derivatives on hydroxyl radical were shown in Fig. 3. The phosphorylated derivative had the strongest scavenging ability against hydroxyl radical among all samples, and the activity increased with concentration increased. However, the acetylated derivatives showed the weakest scavenging activities at test concentrations ranging from 40 to 200 μg/mL. Wang et al. reported phosphorylated fucoidan derivatives had stronger scavenging ability against hydroxyl radicals than fucoidan [4], which was in accordance with our results. 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 [21]. Hence, the molecules that could chelate iron, and render them inactive of poorly active fenton reaction might have scavenging ability on hydroxyl radical. The phosphate group had high nucleophilic characteristic and could chelate with metal ion, so the scavenging ability of phosphorylated derivative on hydroxyl radical was much stronger than AMCP. However, acetyl group did not have nucleophilic characteristic, so it showed weak scavenging ability on hydroxyl radical.
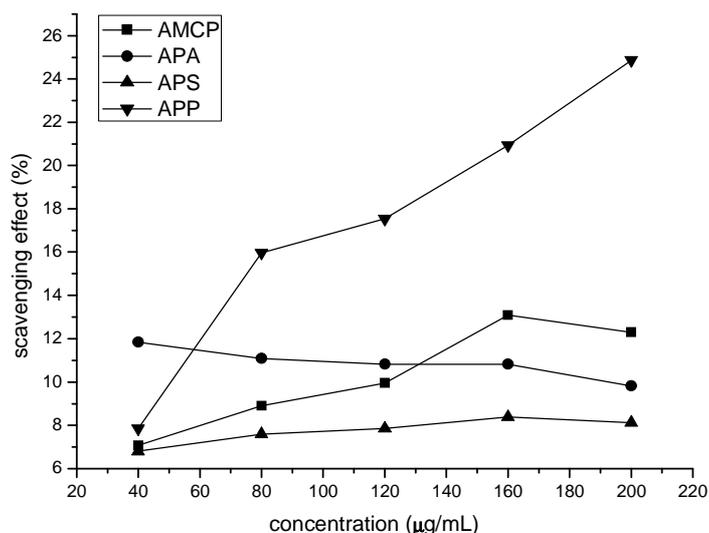


**Figure. 3. Scavenging effect on hydroxyl radicals of AMCP and its derivatives (APA, APS and APP). Values are means ± S.D. ($n = 3$)**
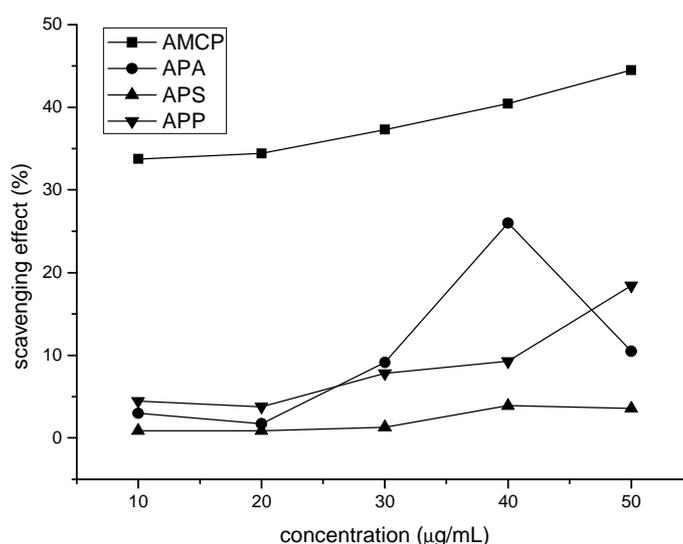


**Figure. 4. Scavenging effect of AMCP and its derivatives (APA, APS and APP) on DPPH radicals. Values are means ± S.D. ($n = 3$)**

**DPPH free-radical scavenging activity:** DPPH is a commercially available radical, DPPH assay is considered a valid, accurate and economic method to evaluate radical scavenging activity of antioxidants, since the radical is stable and need not be generated. In the DPPH free-radical scavenging assay, DPPH can accept an electron or hydrogen radical from antioxidant to become a stable, diamagnetic molecule, the absorption vanishes as the electron

pairs off. The absorption change of DPPH can be easily monitored spectrophotometrically at 517 nm. As shown in Fig. 4, the scavenging abilities were related to the concentrations of AMCP and APP. APP exhibited the greatest scavenging ability among AMCP derivatives, which might be attributed to the phosphate group. The phosphate group is electron-rich, it can quench DPPH radical by providing an electron. Furthermore, AMCP showed the most excellent scavenging activity on DPPH compared with other samples. We suppose that because AMCP had strong hydrogen-donating ability. The more hydroxyl groups, the stronger hydrogen atom-donating capacity. The scavenging activity of BHA was 12.2% at 1 mg/mL [22], which was lower than AMCP and APP, it was evident that AMCP and its phosphate derivatives did exhibit strong proton/electron donating ability and could serve as free-radical inhibitors or scavengers, acting possibly as primary antioxidants.

**Reducing power assay:** Reducing power is a significant marker to measure the antioxidant capacity of a compound. The presence of the reductants can convert $Fe^{3+}$/ferric cyanide complex to ferrous form by contributing one electron with subsequent turning of yellow color reaction solution to green, which can be monitored at 700 nm. Fig. 5 depicted the reducing power of AMCP and its derivatives using the potassium ferricyanide reduction method. The reducing power of all samples was concentration dependent. The phosphated derivative possessed the highest reducing power. With the concentration increased from 40 to 200 μg/mL, the absorption was increased from $0.110 \pm 0.05$ to $0.361 \pm 0.03$, which was weaker than that of BHT and phosphorylated derivative of fucoidan [6, 23]. The reducing power of the samples was found in the following order: APP > AMCP > APS > APA. The reducing properties have been shown to exert antioxidant action by donating of a hydrogen atom to break the free radical chain [24]. APP with high donating hydrogen ability showed excellent reducing power, the same to the ability of scavenging DPPH radical. It was evident that reducing power of all tested samples, especially phosphorylated derivative, probably play a role in the antioxidation observed.
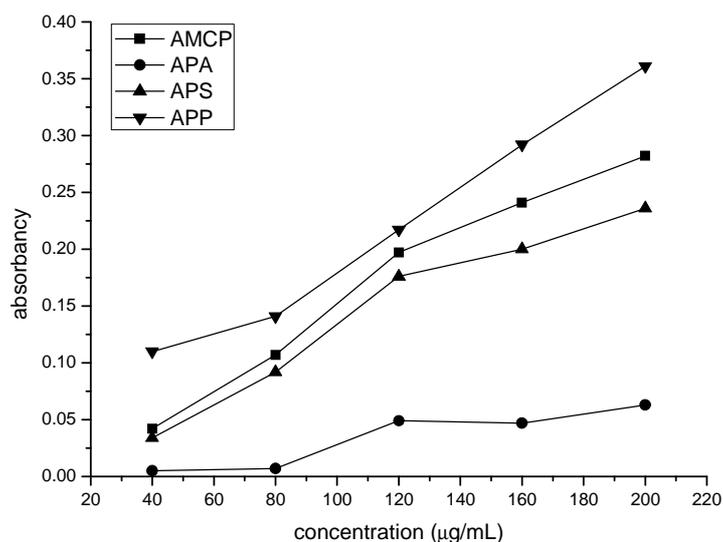


Figure. 5. Reducing power of AMCP and its derivatives (APA, APS and APP). Values are means ± S.D. (*n* = 3)

**CONCLUSION**

The results clearly demonstrated that acetylated, sulphated and phosphorylated derivatives of AMCP were synthesized successfully. The phosphorylated derivative exhibited the strongest antioxidant activity than other derivatives of AMCP in certain antioxidant systems in vitro. However, acetylated and sulphated derivatives showed poor antioxidant capability. Sulphonyl, acetyl and phosphoryl groups have different mechanism on influence the antioxidant activity of samples. The electron-rich phosphoryl group could donate more electrons or activate the hydrogen atoms of the anomeric carbon, and increase the antioxidant activity of AMCP. Therefore, further studies were necessary to investigate the structure–activity relationships of phosphorylated derivative of AMCP to find possible sources for novel antioxidants in food and pharmaceutical industry.

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