

# Characterization of Alkali Extracting Polysaccharides from Cultured *Cordyceps militaris*

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## Research Article

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### ABSTRACT

Two kinds of new polysaccharides (A-WSCBP50I and A-WISCBP50I) were extracted with sodium hydroxide and isolated by gel filtration chromatography approach from the fruiting bodies of *Cordyceps militaris*. Both of them mainly contained  $\alpha$ -type glycosidic linkages according to their own IR and <sup>1</sup>H NMR spectra. Furthermore, A-WSCBP50I had a molecular weight of 8.17 kD and was primarily composed of glucose, mannose and arabinose with the content of 38.67%, 27.22% and 21.96%, respectively. The other polysaccharide A-WISCBP50I was mainly composed of glucose (69.06%) and mannose (27.80%). In addition, the crude polysaccharide A-WSCBP exhibited scavenging capacity against DPPH radical *in vitro* with the IC<sub>50</sub> of 0.068 mg/mL. A-WISCBP could scavenge superoxide radical produced in NBT-riboflavin system in dose dependent *in vitro* with the IC<sub>50</sub> of 1.608 mg/mL.

## INTRODUCTION

Polysaccharides from many sources, catching more and more attention because of their multiple pharmacological activities [1-4], can be extracted by hot water, alkali, and enzyme and so on. In traditional viewpoint, polysaccharides are soluble in water for their hydroxyl and the solubility is the base of its bioactivities while insoluble polysaccharides have low or no bioactivities. The predominant extracting method is hot water extraction to date because of low productivity and restricted research technology of other method. However, the method has some defects, including high extraction temperature and long time to operate, which probably lead to degradation of polysaccharides. Moreover, a great deal of residue, possibly containing active components, is discarded after hot water extraction. Alkaline extraction is another commonly used method to extract polysaccharides and the products usually have bioactivity [5-8]. Nevertheless, most of the researchers focus on polysaccharides extracted by hot water. As a result, there are fewer researches about polysaccharides extracted by alkali as well as their corresponding biological activities, and those about water insoluble polysaccharides are even more less.

Polysaccharides from *Cordyceps militaris*, a forceful candidate substituting for the famous Chinese herb *Cordyceps sinensis*, possess many pharmacological activities [9-11], including immunostimulatory [12-14], antioxidant [12,14,15] and anti-tumor activities [14]. Structures of some of the polysaccharides have been elucidated, but almost none of alkali extracted polysaccharides except few reports [16,17].

We reported one polysaccharide extracted by hot water with strong antioxidant activities *in vitro* [18]. In the current study, we explored the structural characteristics and potential antioxidant activity of two kinds of new polysaccharides extracted by alkali from *Cordyceps militaris*.

## MATERIALS AND METHODS

### Materials and Chemicals

The cultured *C. militaris* C19 fruiting bodies, which were obtained from Shenzhen Pengyuan biotechnology company

(Shenzhen, China), were ground and sifted for polysaccharide extraction. Several kinds of chemicals were purchased from Sigma Co. Ltd, including riboflavin, nitroblue tetrazolium (NBT), Methionine (Met) and 1, 1-diphenyl-2-picrylhydrazyl (DPPH), while Sephadex G-100 from GE Healthcare Life Sciences.

### Extraction of Polysaccharides by Alkali

After polysaccharides were extracted from the fruiting bodies by hot water thoroughly, the residue was mixed with 0.7 mol/L sodium hydroxide (NaOH) at a ratio of 1:8 (W: V) to extract polysaccharide, extracting 0.5 h for three times. Then the combined extracts were neutralized to pH 6.7 with acetic acid and centrifuged (10000 rpm, 10 min). Next, the supernatant was collected as the alkali extracted water soluble polysaccharides (A-WSCBP), while the precipitate as the alkali extracted water insoluble polysaccharides (A-WISCBP) and dissolved with NaOH (0.5 mol/L) for later use. Both of them was precipitated again with 95% alcohol and successively defatted with acetone and diethyl ether for 30 min. A-WSCBP was deproteinized as described with Sevag method<sup>[18]</sup>. The precipitate of A-WISCBP was dissolved with NaOH, mixed with Sevag dose, stirred 1 h and centrifuged (11000 rpm, 20 min) to remove protein, repeating 3 times. The contents of protein and polysaccharides were analyzed using Bradford method and the phenol-sulfuric acid method<sup>[19]</sup>.

### Isolation, Purification and Purity Evaluation of the Polysaccharides

A-WSCBP50 was obtained from A-WSCBP by fractional precipitation with absolute ethanol to the final concentration of 50%. It was dissolved with adequate water, centrifuged (10000 rpm, 10 min), and the supernatant was purified by Sephadex G-100 gel filtration chromatography (2 cm × 60 cm column) with the flow rate of 0.4 mL/min. For the other polysaccharide A-WISCBP, the dialyzed polysaccharide solution was added absolute alcohol to concentration of 50%, precipitated at 4 °C overnight and centrifuged (10000 rpm, 10 min) to obtain the precipitation, name as A-WISCBP50. A-WISCBP50 was dissolved with 0.5 mol/L NaOH and ejected into a Sephadex G-100 chromatography column (2 × 60 cm) balanced with 0.1 mol/L Na<sub>2</sub>HPO<sub>4</sub> with flow rate of 0.5 mL/min.

Then, the homogeneity of A-WSCBP50I and A-WISCBP50I were determined using gel permeation chromatography (GPC) and ultraviolet spectrum scanned at 190-700 nm, respectively.

### Physicochemical Properties of the Polysaccharides

The molecular weight of A-WSCBP50I was determined using GPC and the monosaccharide composition of A-WSCBP50I and A-WISCBP50I using GC-MS as described in previous report<sup>[18]</sup>.

Their structural characteristics were analyzed by IR spectrum with KBr pellet method in the range of 4000–400 cm<sup>-1</sup> and by <sup>1</sup>H NMR spectrum with D<sub>2</sub>O as the solvent (some of 0.5 mol/L NaOH was added to improve solubility of A-WISCBP50I) according to the previous report<sup>[18]</sup>.

### Free Radical Scavenging Assay

DPPH scavenging assay was performed in 3.0 mL of DPPH ethanol system according to our previous report<sup>[18]</sup>. The calculation formula was expressed as:

Scavenging rate (%) = [(Ac-As)/Ac] × 100% (formula 1), in which Ac and As standing for the absorbance of the control and the sample, respectively.

The scavenging ability of A-WISCBP on superoxide radical was analyzed according to the reference<sup>[18]</sup>, with 0.5 mol/L NaOH taking place of the PBS in the reactive system.

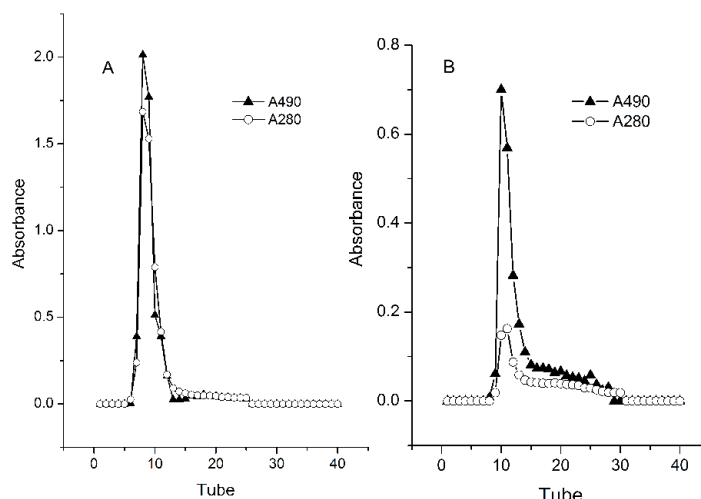
## RESULTS AND DISCUSSION

### Isolation, Purification and Purity

The deproteinization rate of A-WISCBP50 was 60.63% with the polysaccharides loss rate of 32.34%. Both of the crude polysaccharide gave one polysaccharide peak overlapping with a protein peak when they were fractionated on Sephadex G-100 column (**Figure 1**). The main part of the polysaccharide peak was collected, concentrated and lyophilized, named as A-WSCBP50I and A-WISCBP50I, respectively. The purity of the former was high according to its GPC (**Table 1**), eluted as four peaks (with the relative content of 0.37%, 0.46%, 0.23% and 98.94%, respectively). The latter, A-WISCBP50I, displayed the characteristic absorption peak of polysaccharides in the UV spectrum for the maximum absorption peak of 224 nm (**Figure 2**). There was a little of coloring matter because of a very weak absorption peak at 454 nm, but there was no nucleic acid since no absorption peak appeared at 260 nm.

### Molecular Weight

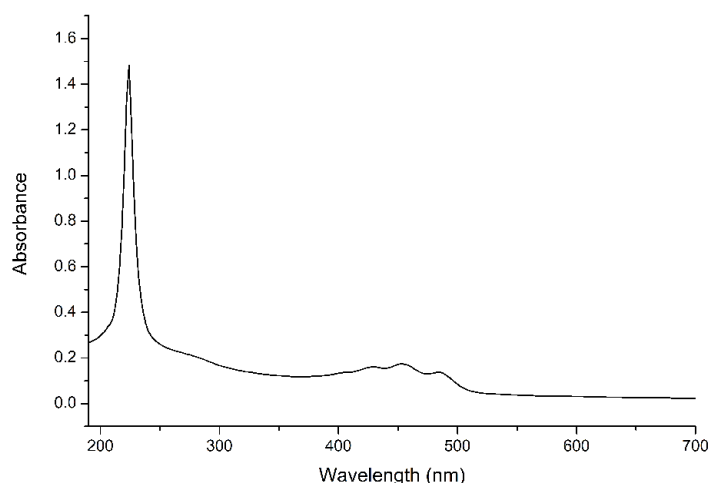
The molecular weight of the main component of A-WSCBP50 I was 8.17 kD according to GPC (**Table 1**), which meant that it mainly contained low molecular weight component.



**Figure 1.** Figure of Sephadex G-100 column chromatography of A-WSCBP50 and A-WISCBP50; A: Figure of Sephadex G-100 column chromatography of A WSCBP50; B: Figure of Sephadex G-100 column chromatography of A-WISCBP50.

**Table 1.** Result of molecular weight of A-WSCBP50 I.

Retention time(min)	Relative mass fraction (%)	M <sub>n</sub> (Daltons)	M <sub>p</sub> (Daltons)	M <sub>w</sub> (Daltons)	D M <sub>w</sub> / M <sub>n</sub>
6.984	0.37	149 580	309 590	216 110	1.44
9.290	0.46	28 930	29 337	28 965	1.00
10.901	0.23	20 736	21 137	20 823	1.00
12.236	98.94	7 593	8 389	8 174	1.08
Retention time(min)	Relative mass fraction (%)	M <sub>n</sub> (Daltons)	M <sub>p</sub> (Daltons)	M <sub>w</sub> (Daltons)	D M <sub>w</sub> / M <sub>n</sub>
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10.901	0.23	20 736	21 137	20 823	1.00
12.236	98.94	7 593	8 389	8 174	1.08



**Figure 2.** Ultraviolet and visible spectrum of A-WISCBP50I.

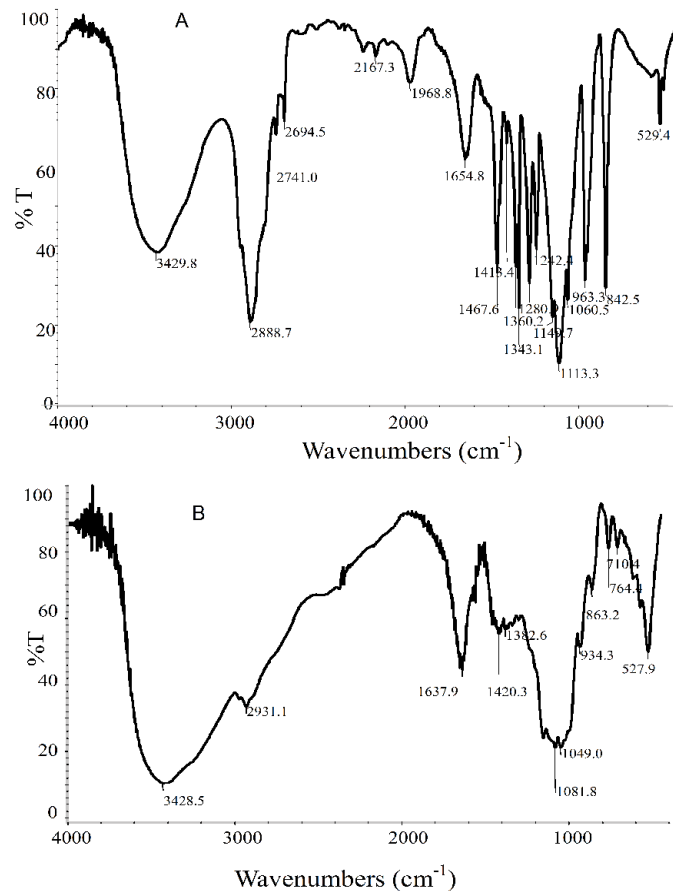
**Chemical Composition**

According to the GC-MS, A-WSCBP50I mainly contained Glc (38.67 %), Man (27.22 %) and Ara (21.96%), with little Gal (6.90%), Rib (3.13%), Rha (1.38%) and Xyl (0.75%), which was remarkably different from that of W-CBP50 II at monosaccharide content [18]. A-WISCBP50 I was mainly composed of Glc and Man with the content of 69.06% and 27.80%, respectively. In addition, it contained little Ara (1.81%), Gal (0.24%), Rib (0.43%), Rha (0.39 %) and Xyl (0.28%).

**IR and <sup>1</sup>H NMR Spectra**

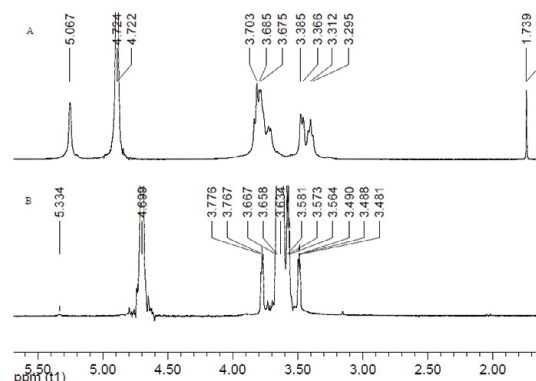
The IR spectra of A-WSCBP50 I and A-WISCBP50 I are shown in **Figure 3** and both of them exhibit common characteristics of polysaccharides at 4000-400 cm<sup>-1</sup>. The absorption band centered at 842 cm<sup>-1</sup> of A-WSCBP50 I was indicative of α- anomeric configuration [20]. A-WSCBP50 I had similar features to that of W-CBP50 II with some change of characteristic peaks intensity and peak position, possessing three more absorption peaks range from 1654-2888 cm<sup>-1</sup> (**Figure 3A**). Nevertheless, A-WISCBP50I has obvious difference from that of water soluble polysaccharides (**Figure 3B**).

The configuration of its compositive monosaccharide could not be estimated according to its IR spectrum, for there was neither absorption peak near  $891 \pm 7 \text{ cm}^{-1}$  nor that near  $844 \pm 8 \text{ cm}^{-1}$ .



**Figure 3.** IR spectra of A-WSCBP50 I and A-WISCBP50I: A: IR spectrum of A-WSCBP50 I; B: IR spectrum of A-WISCBP50I.

The anomeric signals at 5.33/5.07 ppm in the  $^1\text{H}$  NMR spectra of A-WSCBP50 I and A-WISCBP50I indicated that both of them contained  $\alpha$ -type glycosidic linkages (**Figure 4**). The strong signal at 4.70/4.72 ppm represented solvent resonance or that of  $\beta$ -type glycosidic linkages simultaneously, which suggested that both  $\alpha$  and  $\beta$ -type glycosidic linkages might exist in A-WSCBP50I and A-WISCBP50I according to their  $^1\text{H}$  NMR spectra.



**Figure 4.**  $^1\text{H}$  NMR spectra of A-WSCBP50I and A-WISCBP50I; A:  $^1\text{H}$  NMR spectrum of A-WSCBP50I; B:  $^1\text{H}$  NMR spectrum of A-WISCBP50I.

Thus, it could be inferred that A-WSCBP50I mainly contained pyranose and  $\alpha$ -type glycosidic linkages according to IR and  $^1\text{H}$  NMR spectra. A-WISCBP50I mainly contained  $\alpha$ -D-pyranose and little furanose, and maybe both  $\alpha$  and  $\beta$  glycosidic bonds according to GC-MS, IR and  $^1\text{H}$  NMR.

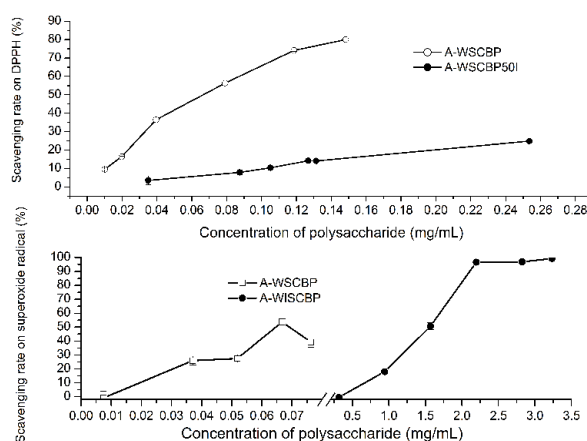
Although A-WSCBP50I had some similar aspect to that of W-CBP50II we had reported previously, they were two kinds of different polysaccharide. First, A-WSCBP50I was mainly composed of glucose, mannose and arabinose, while W-CBP50II of glucose, mannose and galactose. Second, there were different signal region in their  $^1\text{H}$  NMR spectra. Furthermore, there was notably difference between them in their corresponding IR spectrum. The intensity of  $2888 \text{ cm}^{-1}$  of A-WSCBP50I was higher than that of W-CBP50II. Moreover, the intensity of fingerprint region of the former was higher than that of the latter, which meant that

there was much difference in structure between them. Third, the scavenging ability of A-WSCBP50 I on DPPH free radical was significantly low than that of W-CBP50II.

In addition, A-WSCBP50I was also different from the polysaccharide CBP-1 which was extracted by alkaline from the cultured *C. militaris* at composition, molecular weight and glycosidic linkages [16]. As was mentioned above, A-WSCBP50I mainly contained Glc, Man and Ara with little Gal, Rib, Rha and Xyl, while CBP-1 was composed of Man, Gal and Glc. Meantime, CBP-1 contained both  $\alpha$  and  $\beta$  glycosidic linkages for its 891 and 840  $\text{cm}^{-1}$  absorption bands. A-WSCBP50I was different from CBP-1 because of its  $\alpha$  conformation with peak at 842  $\text{cm}^{-1}$  in IR spectrum. The reason may be that we used different methods in extraction, isolation and purification.

### Antioxidant Activities of the Polysaccharides

A-WSCBP could scavenge DPPH in a concentration dependent manner with the IC50 of 0.068 (Figure 5A). A-WSCBP50I had far weaker scavenging ability on the same free radical, with the scavenging rate of 24.92% when its concentration of 0.254 mg/mL. The possible reason was that there was loss of some active components during the purification progress. Moreover, the antioxidant activity of A-WSCBP50I was far lower than that of W-CBP 50II. It probably because that sodium hydroxide solution used as the extraction solvent partly decomposed polysaccharides causing structure degradation and activity loss. Another evidence for the suggestion was that IC50 of CBP-1 (an alkaline extracting polysaccharide) and P70-1 (a water extracting polysaccharide) was 0.638 and 0.548 mg/mL, respectively. However, the antioxidant activity of A-WSCBP50I itself was not low which meant that it had potential potency for application.



**Figure 5.** Radical scavenging effect of A-WSCBP, A-WSCBP50I and A-WISCBP. A: Scavenging effect of A-WSCBP and A-WSCBP50I on DPPH radical; B: Scavenging effect of A-WSCBP and A-WISCBP on superoxide radical.

Both A-WSCBP and A-WISCBP could scavenge superoxide radical (Figure 5B). The former exhibited scavenging activity within low concentration range, with the scavenging rate of 53.67% when concentration of 0.067 mg/mL. The latter polysaccharide, A-WISCBP, exhibited scavenging activity on dose dependence within higher concentration range, with IC50 of 1.608 mg/mL. The scavenging rate was 99.20% when the concentration of A-WISCBP was 3.233 mg/mL, which meant that the polysaccharide had considerable antioxidant activity *in vitro*. The reason for the difference of scavenging activities between A-WSCBP and A-WISCBP maybe relate their structural properties but need more study in the future.

## CONCLUSION

The alkali extracting water soluble polysaccharide A-WSCBP50I was mainly composed of Glc, Man and Ara. The glycosidic linkage of A-WSCBP50 I was  $\alpha$ -type according to IR and  $^1\text{H}$  NMR spectra and the molecular weight of its component was 8.17 kDa. Both A-WSCBP and A-WSCBP50I could scavenge DPPH free radical and A-WSCBP also could scavenge superoxide radical, which meant that they had antioxidant activities *in vitro*.

The water insoluble polysaccharide A-WISCBP50I was a heteropolysaccharide and mainly composed of Glc and Man. The glycosidic linkage of it mainly was  $\alpha$  type; however, it may be contained  $\beta$  type glycosidic bonds. Besides the established appropriate analysis method, we also found that the water insoluble polysaccharide had strong scavenging ability on superoxide radical *in vitro*.

## REFERENCES

1. Stachowiak B and Regula J. Health-promoting potential of edible macromycetes under special consideration of polysaccharides: a review. *Eur Food Res Technol.* 2012;234:369-380.
2. Jin ML, et al. Biological activities and potential health benefit effects of polysaccharides isolated from *Lycium barbarum* L. *Int J Biol Macromol.* 2013;54:16-23.

3. Wang W, et al. The antiviral activities and mechanisms of marine polysaccharides: an overview. *Mar Drug*. 2012;10:2795-2816.
4. Zong A, et al. Anticancer polysaccharides from natural resources: a review of recent research. *Carbohydr Polym*. 2012;90:1395-1410.
5. Tong H, et al. Purification, chemical characterization and radical scavenging activities of alkali-extracted polysaccharide fractions isolated from the fruit bodies of *Tricholoma matsutake*. *World J Microb Biot*. 2013;29:775-780.
6. Wu GH, et al. In vitro antioxidant activities of the polysaccharides from *Pleurotus tuber-regium* (Fr.) Sing. *Food Chem*. 2014;148:351-356.
7. Zhang Z, et al. The immunological and antioxidant activities of polysaccharides extracted from *Enteromorpha linza*. *Int J Biol Macromol*. 2013;57:45-49.
8. Hu M, et al. Extraction of polysaccharides from *Fomes officinalis* Ames and their antitumor activity. *Exp Ther Med*. 2013;6:451-454.
9. Shrestha B, et al. The medicinal fungus *Cordyceps militaris*: research and development. *Mycol Prog*. 2012;11:599-614.
10. Das SK, et al. Medicinal uses of the mushroom *Cordyceps militaris*: current state and prospects. *Fitoterapia*. 2010;81:961-968.
11. Wang B, et al. Comparison of the hepatoprotective activity between cultured *Cordyceps militaris* and natural *Cordyceps sinensis*. *J Funct Foods*. 2012;4:489-495.
12. Wu F, et al. Comparison of the structural characterization and biological activity of acidic polysaccharides from *Cordyceps militaris* cultured with different media. *World J Microb Biot*. 2012;28:2029-2038.
13. Wang M, et al. *Cordyceps militaris* polysaccharides can improve the immune efficacy of Newcastle disease vaccine in chicken. *Int J Biol Macromol*. 2013;59:178-183.
14. Jing Y, et al. Elucidation and biological activities of a new polysaccharide from cultured *Cordyceps militaris*. *Carbohydr Polym*. 2014;102:288-296.
15. Liu Y, et al. Protective effect of *Cordyceps* polysaccharide on hydrogen peroxide-induced mitochondrial dysfunction in HL-7702 cells. *Mol Med Rep*. 2013;7:747-754.
16. Yu RM, et al. Structural elucidation and biological activity of a novel polysaccharide by alkaline extraction from cultured *Cordyceps militaris*. *Carbohydr Polym*. 2009;75:166-171.
17. Smiderle FR, et al. Isolation and chemical characterization of a glucogalactomannan of the medicinal mushroom *Cordyceps militaris*. *Carbohydr Polym*. 2013;97:74-80.
18. Chen X, et al. Structural analysis and antioxidant activities of polysaccharides from cultured *Cordyceps militaris*. *Int J Biol Macromol*. 2013;58:18-22.
19. M. Dubois, et al. Colorimetric method for determination of sugars and related substances. *Anal Chem*. 1956;28:350-356.
20. Niu Y, et al. Characterization of a novel polysaccharide from tetraploid *Gynostemma pentaphyllum* makino. *J Agric Food Chem*. 2013;61:4882-4889.