*Biomedical Research 2016; 27 (1): 199-206* *ISSN 0970-938X*
*www.biomedres.info*

# Antioxidant activities of alkali-soluble polysaccharides from medicinal mushroom *Cordyceps taii* and its chemical characteristics.

**Dai-Min Xiao[1,2], Song Yu[1], Jian-Hui Xiao[1*]**

[1]Center for Translational Medicine of Guizhou Province, Affiliated Hospital of Zunyi Medical University, Zunyi 563000, PR China.

[2]Department of Medical Laboratory, Affiliated Hospital of Zunyi Medical University, Zunyi 563000, PR China.

## Abstract

***Cordyceps taii*, a folk medicine native to south China, is a promising source for the development of healthy foods and new drugs due to its diverse pharmacological properties including antioxidative stress effect. The antioxidant properties of alkali-soluble polysaccharide fractions from *C. taii* were systematically investigated, such as the scavenging abilities on DPPH (DPPH•), hydroxyl (•OH) and superoxide anion (•$O^{2-}$) free radicals, the reducing power and the $Fe^{2+}$chelating ability. Among these assays, the refined polysaccharide fraction (ARP) presented more excellent scavenging abilities towards •OH and •$O^{2-}$ with EC50 values of 2.72 ± 0.80 and 10.46 ± 1.97 mg/ml compared with the crude polysaccharide fraction (ACP). While the ACP showed slightly stronger scavenging activity on DPPH•, the reducing ability than the ARP. In comparison with the positive control, the ARP had more significantly scavenging •$O^{2-}$ activity, and had a moderate reducing ability and scavenging other free radical activities in a dose-dependent manner. A pure polysaccharide fraction from the ARP was found to be mainly composed of glucose, galactose and mannose (molar ratios=1.14: 1.00: 1.66) with series α-(1, 4) glucosidic bond, and the mannose were assigned as the backbone of the polysaccharide structure accordingly. Taken together, the alkali-soluble polysaccharide is an important antioxidant component in *C. taii*, and exerts the antioxidant properties by scavenging free radical.**

**Keywords:** *Cordyceps taii*, Alkali-soluble polysaccharide, Free radical scavenger, Antioxidant activity, Chemical characteristics.

*Accepted Dec 10, 2015*

## List of Abbreviations

ACP: Alkali-Solube Crude Polysaccharide; ARP: Alkali-Solube Refined Polysaccharide; APP: Alkali-Soluble Pure Polysaccharide; ROS: Reactive Oxygen Species; RNS: Reactive Nitrogen Species; TU: Thiourea; BHA: Butyl Hydroxyanisole; BHT: Butylated Hydroxytoluene; EDTA: Ethylenediamine Tetraacetic Acid; DPPH•: 1,1-Diphenyl-2-picrylhydrazyl free radical, •OH: Hydroxyl Free Radical; •O2-: Superoxide Anion Free Radical; $Fe^{2+}$: Ferrous Ions; EC50: 50% Effective Concentration; GC-MS: Gas Chromatography-Mass Spectrometry; UV: Ultraviolet; FT-IR: Fourier Transform Infrared Spectroscopy

## Introduction

Free radicals, generally known as reactive oxygen and nitrogen species (ROS/RNS), can generate oxidative stress to damage all cell structures of the living organisms at high concentrations, and play a major pathological part in the development of human chronic and degenerative diseases such as cancer, autoimmune disorders, cirrhosis, arthritis, aging, cardiovascular and neurodegenerative diseases [1-3]. Antioxidants, therefore, play important roles in preventing these ailments induced by ROS/RNS, and free radicals and antioxidants have become commonly used terms for these disease mechanisms in the past twenty years. Also, antioxidants therapy is receiving much attention around the world in clinical, as well as the research fields. As we known, some synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) were widely used in the food and drug industry. Recent studies showed that these synthetic antioxidants were dangerous towards human health such as liver damage and carcinogens, and have been forbidden to use in the food and pharmaceutical industries worldwide [4, 5]. Thereby it has resulted in an increasing interest in naturally occurring alternatives in recent years. Among natural antioxidantive agents, enzymatic antioxidants are mostly inactivated in food and drug processing, non-enzymatic antioxidant agents have received a great deal of attention accordingly.

*Cordyceps*, a well-known and valued traditional Chinese medicinal macrofungus, is an attractive source for the

development of natural antioxidants. Previous studies showed many *Cordyceps* fungi such as *C. sinensis, C. militaris, C. jiangxiensis* exerted potent antioxidant activities, and polysaccharides were recognized as major active ingredients with antioxidant activity in *Cordyceps* fungi [5-7]. Cordyceps taii is a folk medicine native to south China [8]. Our group has demonstrated that *C. taii* had a wide variety of pharmacological effects due to its diverse natural active compounds [9-12]. The water-soluble polysaccharide of *C. taii* was found to display potent immunomodulatory, and antitumor activities [8, 13]. Furthermore, within our recent screening program for antioxidative substances from *Cordyceps* fungi, the water-soluble polysaccharide of *C. taii* was found to exert potent antioxidant and immunoenhancing activities in the aging mouse model, and was a promising source of natural antioxidants [14]. To our knowledge, there have been no reports on the antioxidant activity of the alkali-soluble polysaccharides of *C. taii*. Therefore, the antioxidative potential in vitro and chemistry characteristics of alkali-soluble polysaccharides of *C. taii* were developed in the present study.

## Materials and Methods

### *Chemicals and reagents*

All chemicals used were of analytical grade. 1, 1-Diphenyl-2-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT), and butyl hydroxyanisole (BHA) from Sigma Inc. (St. Louis, MO, USA). Ethylenediamine tetraacetic acid (EDTA) and dimethyl sulphoxide (DMSO) from Invitrogen Inc. (Carlsbad, CA, USA). DEAE-cellulose-52 gel from Merck (Germany), and Sephadex G-100 from Amersham Biosciences (Pharmacia, Sweden). Trichloroacetic acid (TCA), ferrozine, potassium ferricyanide, ferrous chloride and ascorbic acid from Advanced Technology and Industrial Co. Ltd (Hongkong, China). Ortho-phenanthroline (1, 10-phenanthroline) from Damao Chemical Reagents Factory (Tianjin, China), and thiourea (TU) from Kexing biotechnology Co. Ltd. (Shanghai, China).

### *Strain, media and mycelia preparation*

The voucher specimen of *C. taii* GYYA 0601 was deposited at the Laboratory of Microbial Resource and Drug Discovery, Center of Translational Medicine of Guizhou Province, Affiliated Hospital of Zunyi Medical University, China. *C. taii* was maintained on a slant seed medium (2.5% w/v sucrose, 0.3% soybean, 0.5% yeast extract, 0.2% peptone, 0.3% wheat bran, 0.1% $KH_2PO_4$, 0.05% $MgSO_4 \cdot 7H_2O$, 1.8% agar, and 1 L of distilled water; initial pH=6.0). The stock culture was incubated at 28ºC for 15 d, and then stored at 4ºC in a refrigerator before use. The mycelia of *C. taii* were prepared as previously described [14]. Subsequently, the mycelia were lyophilized and grinded (60 mesh to 100 mesh) for later experiments.

### *Isolation of alkali-soluble polysaccharides*

Dry power of cultured *C. taii* mycelia (g) was repeatedly defatted with petroleum ether at room temperature. The residue was got by centrifugation (5000 × g for 10 min), dried at room temperature, extracted thrice with distilled water in a ratio of material to water 1:10 (m/v) at 95°C for 2 h, and then centrifuged at 5000 × g for 10 min. Subsequently, the solid residue was extracted with 0.5M NaOH solution at 60°C for 2 h, the material/extraction ratio is 1:10 (m/v). After vacuum filtration, the solid residue was extracted again six times under the same condition. The combined filtrates were completely neutralized by 0.5M HCl solution, and then condensed to one-fifth of their total volume using a rotary evaporator under reduced pressure at 50°C to 55°C. The filtrates were then precipitated at 4°C for 12 h with ethanol to an 80% final concentration. The precipitate was washed thrice with 85% ethanol and acetone then lyophilized in a vacuum, yielding the alkali-soluble crude polysaccharide of *C. taii* (ACP, 135 g). The ACP was stored at 4°C before use. Using the modified phenol-sulfuric acid method [15], the ratio of ACP fresh weight to dry cell weight (w/w) was 13.5%, and polysaccharide content of ACP was 56.1% (w/w). The ACP was re-dissolved in distilled water to prepare the alkali-soluble refined polysaccharide (ARP, 18.5 g) as previously described [14,16]. By the modified phenol-sulfuric acid method [15], the yield of ARP was determined to be 1.85% (w/w) of the dry cell weight, and its polysaccharide content was 95.7% (w/w).

The ARP was successively purified by ion-exchange and gel filtration column chromatography methods to get alkali-soluble pure polysaccharide (APP) fractions. The ARP sample (1.0 g) was dissolved, filtered, and loaded to a column (2.4 cm × 50 cm) of DEAE-cellulose-52. The column was gradiently eluted with NaCl aqueous solution (0.01 M to 0.15 M), followed by 0.3 M and 0.5M NaOH. Flow rate 2.5 ml/min, 15 ml/tube. This process was monitored by the modified phenol–sulfuric acid method [15]. Consequently, three subfractions (ARP-Fr1 to Fr3) were obtained after DEAE-cellulose chromatography separation. ARP-Fr2 (398 mg) eluted out of ion-exchange column with 0.05 M NaCl aqueous solution was further purified to yield the APP fraction by Sephadex G-100 gel filtration column chromatography (1.6 cm × 60 cm) with distilled water elution. The resulting APP fraction was used in the subsequent chemical analyses.

### *Analyses of Chemical properties*

Ultraviolet (UV) spectroscopy of the APP fraction was detected within 200 nm to 400 nm using a Lambda UV spectrometer (PerkinElmer, USA). The infrared (IR) spectra of the APP was determinated within 4000 $cm^{-1}$ to 400 $cm^{-1}$ using a Varian 1000 Fourier-transform Infrared spectroscopy (FT-IR) instrument (Varian, USA) with KBr pellets. The monosaccharide composition analysis of APP was performed using a gas chromatography- mass spectrometry (GC/MS) method as previously described by our group [14].

### *Assay of DPPH• scavenging*

The modified method as described by Xiao et al. [14] was applied in this study to assess the scavenging lipid-soluble DPPH• ability of the alkali-soluble polysaccharide fractions of

*C. taii*. Two milliliter of pH 6.86 phosphate buffer, 2 ml of 0.5 mM fresh DPPH in ethanol solution, and 0.5 ml of the samples tested at different concentrations were added to a glass tube in turn. Subsequently, the reaction mixture was shaken vigorously, and left to stand at room temperature for 30 min in the dark. The reduction of the DPPH• was measured by monitoring the absorbance at 520 nm. The radical scavenging activity was calculated as a percentage of DPPH discoloration by the following equation: scavenging ability (%)=[1–(As–Asb)/Ab]×100, where As, Asb, and Ab are the absorbances at 520 nm of the reaction mixture of the sample or antioxidant, sample blank, and blank, respectively. Here antioxidant thiourea (TU) was used as the positive control, and the sample solution without DPPH was used as a sample blank. Ultrapure water was used as the blank control without samples or antioxidant. All the tests were performed in triplicate.

### *Assay of •OH scavenging*

The modified ortho-phenanthroline method was applied to detect the scavenging ability on •OH produced by Fenton reaction of the alkali-soluble polysaccharide fractions of *C. taii* [14]. In this study, phosphate buffer (4 ml, pH 7.4), orthophenanthroline in ethanol (1.5 ml, 5 mM) and $FeSO_4$ (1 ml, 7.5 mM) were immediately mixed. The samples (1 ml) at different concentrations, ultrapure water (1.5 ml), and hydrogen peroxide (1 ml, 1% v/v) were then added to the mixture in sequence. After incubating at 37°C for 60 min, the absorbance change of reaction mixture caused by the color change of iron-orthophenanthroline was measured at 510 nm. The antioxidant TU was used as the positive control, the ultrapure water in place of sample and antioxidant was used as damage control (the control of the •OH generation system) and the ultrapure water was used as the blank without sample, antioxidant and hydrogen peroxide. All the tests were performed in triplicate. The •OH scavenging activity was calculated using the following equation: Scavenging ability (%)=[(As-Ao)/(Ab-Ao)] × 100. Where, As, Ao and Ab are the absorbance at 510 nm of reaction mixture of the sample or antioxidants, damage control and blank, respectively.

### *Assay of $\bullet O^{2-}$ scavenging*

2,4-Iodiphenyl-3,4-nitrophenyl-5-phenyltetrazolium chloride (NBT) method was employed to measure the scavenging $\bullet O^{2-}$ activity of the alkali-soluble polysaccharide fractions of *C. taii*. Here an antioxidant kit was used as previously reported by Xiao et al. [5]. The $\bullet O^{2-}$ generated by the xanthine - xanthine oxidase system reacted with the NBT to form amethyst formazan. The absorbance of formazan in the reaction system measured at 550 nm was used to reflect indirectly the presence of the $\bullet O^{2-}$. Here the production of formazan is inversely related to the $\bullet O^{2-}$ scavenging ability of the samples tested. Therefore, the final results were expressed as the inhibition degree of formazan production. BHA was used as the positive control and ultrapure water was used in the place of samples or antioxidant as blank. All the tests were performed in triplicate. The percentage inhibition of the $\bullet O^{2-}$ was calculated as (1-As/Ab) × 100. Where As, and Ab are the absorbance of the reaction mixture of the sample or antioxidant, and blank at 550 nm, respectively.

### *Assay of reducing ability*

The modified Prussian blue method as described by Xiao et al. [5] was employed to assess the $Fe^{3+}$ to $Fe^{2+}$ transformation ability for the total antioxidant potential of the alkali-soluble polysaccharide fractions of *C. taii*. The samples (1 ml) at different concentration, phosphate buffer (2.5 ml, pH 6.6), and $K_3Fe(CN)_6$ (2.5 ml, 1%, m/v) were mixed in a glass tube, and then incubated at 50°C for 20 min. The mixture was added into TCA (2.5 ml, 10% m/v), followed by centrifugation at 1000 × g for 10 min. The supernatant (2.5 ml) was diluted with isometric ultrapure water, and was then reacted for 10 min by the addition of 0.1% fresh ferric chloride (2.5 ml). Finally, the reaction mixture was cooled to room temperature, and the absorbance was measured at 700 nm. The antioxidant BHA was used as the positive control. The reference solution was prepared as stated earlier and was used as the blank, but contained ultrapure water instead of the samples or BHA. The total antioxidant ability was expressed as the absorbance value at 700 nm, and higher absorbance value of the reaction mixture indicates greater antioxidant activity. The reducing ability was calculated as follows: $A=A_s-A_b$, Where, Ab is the absorbance of the blank, and As is the absorbance of the polysaccharide fractions of the sample or antioxidants at 700 nm. Three replicates were carried out for all tests

### *Assay of $Fe^{2+}$ chelating activity*

The chelating activity of the alkali-soluble polysaccharide fractions of *C. taii* on ferrous ion was measured as reported by Xiao et al. [5]. Samples (0.5 ml) at different concentration, ultrapure water (1.8 ml), $FeCl_2$ (0.05 ml, 2 mM) and ferrozine (0.1 ml, 5 mM) were mixed in a glass tube. Here ferrozine reacted with the divalent iron to form stable magenta complex that was very soluble in water. After 20 min at room temperature, the absorbance of the $Fe^{2+}$- ferrozine complex in the reaction system was measured at 562 nm. EDTA was used as positive control, and ultrapure water was used in place of the sample or EDTA as blank. Three replicates were carried out for all tests The chelating activity of the polysaccharide fraction on Fe2+ was calculated as chelating rate (%)=(Ab-As)/Ab × 100. Where, Ab is the absorbance of the blank without sample or EDTA, and As is the absorbance in the presence of the sample or EDTA.

### *Statistical analysis*

The experimental results were processed by SPSS 13.0 (SPSS Inc.). The data were analyzed by one-way analysis of variance (ANOVA), and were expressed as mean ± standard deviation of triplicate determinations. Dunnett's t-test was used to compare the differences between the treated groups and control groups and differences were regarded as significant at $p<0.05$.

## Results

### *Scavenging the DPPH• ability of alkali-soluble polysaccharide fractions*

As shown in Figure 1A, two different polysaccharide fractions from the cultured *C. taii* showed moderate activities against the DPPH radicals at most of the tested doses, and their scavenging abilities were increased with increased doses ranging from 0.5 mg/ml to 8.0 mg/ml in a dose-dependent manner. Among the tested fractions, the ACP fraction showed stronger activity than ARP fraction at the same doses. However, the scavenging activities of the positive control TU were stronger than that of the polysaccharide samples at all the tested doses.

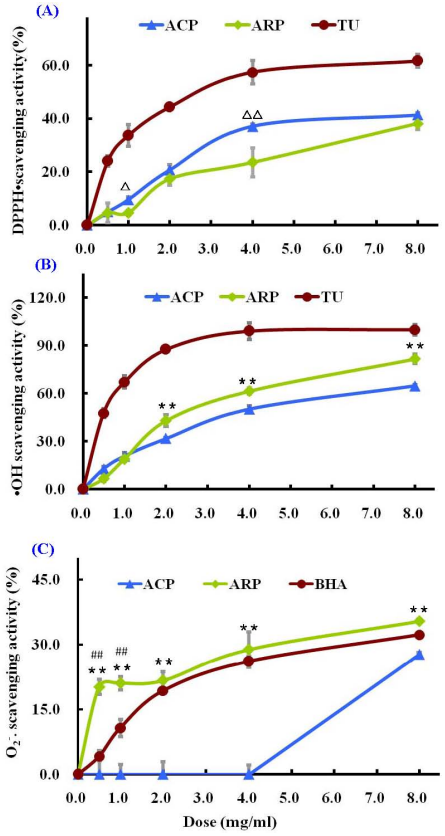


***Figure 1:*** *Effects of alkali-soluble polysaccharides from C. taii against different free radicals such as DPPH• (A), •OH(B), and $O^{2-}$•(C) (□x ± sd. ACP, alkali-solube crude polysaccharide; ARP, alkali-solube refined polysaccharide; TU, thiourea; BHA, butyl hydroxyanisole; P<0.05, P<0.01 vs ARP; P<0.01 vs ACP; ## P<0.01 vs positive control.*

### *Scavenging the •OH ability of alkali-soluble polysaccharide fractions*

The scavenging effects of two different polysaccharide fractions on hydroxyl radicals were shown in Figure 1B. Both ACP and ARP all exhibited potent scavenging abilities towards hydroxyl radicals in a dose-dependent manner at all the tested doses. Especially ARP had more significant scavenging activity against the hydroxyl radicals than ACP at doses ranging from 2.0 mg/ml to 8.0 mg/ml. The scavenging rate of ARP reached 81.73 ± 1.89% at a high dose of 8.0 mg/ml. However, their scavenging abilities to hydroxyl radicals were lower than that of the radical scavenger TU. TU at a dose of 4 mg/ml showed a dramatic inhibition of hydroxyl radical (99.09% ± 5.26%).

### *Scavenging the O2-• ability of alkali-soluble polysaccharide fractions*

The superoxide anion radicals scavenging activities of the different polysaccharide fractions from the cultured *C. taii* were given in Figure 1C. The ACP fraction at doses ranging from 0.5 mg/ml to 8.0 mg/ml showed no activities against the superoxide anion radicals. However, the ARP fraction could more significantly scavenge the superoxide anion radicals in comparison with the radical scavenger BHA at all the tested doses, and also appeared a dose-dependence response.

### *Effect of alkali-soluble polysaccharide fractions on the reducing power*

The $Fe^{3+}$-$Fe^{2+}$ transformation assay is a standardized antioxidant capacity method. In this assay, the presence of reductant in the antioxidant sample can cause the reduction of the $Fe^{3+}$/ferricyanide complex to the $Fe^{2+}$/ferrous form, so the reducing power of the antioxidant sample can be monitored by measuring the formation of Prussian blue at a wavelength of 700 nm.

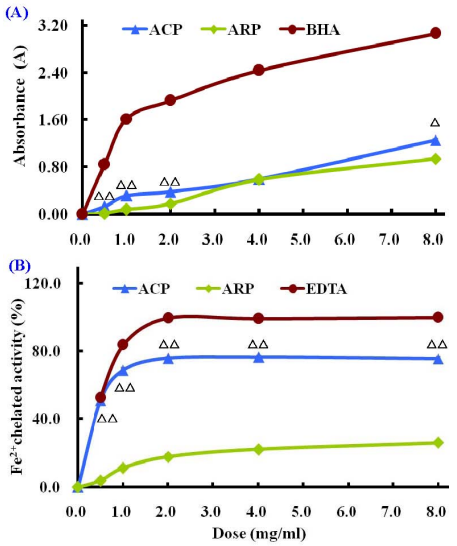


***Figure 2:*** *Effects of alkali-soluble polysaccharides from C. taii on reducing power **(A)** and $Fe^{2+}$ chelating activity **(B)** (□x ± sd). ACP, alkali-solube crude polysaccharide; ARP, alkali-solube refined polysaccharide; EDTA, ethylenediamine tetraacetic acid; BHA, butyl hydroxyanisole; P<0.05, P<0.01 vs ARP.*

Compared with the positive control BHA, the reducing powers of different polysaccharide fractions from *C. taii* presented moderate abilities as shown in Figure 2A. Also, the reduction potential of ACP and ARP fractions exhibited a dose-dependent manner within the concentration range of 0.5 mg/ml to 8.0 mg/ml.

### *Effect of alkali-soluble polysaccharide fractions on the $Fe^{2+}$ chelating activity*

The $Fe^{2+}$ chelating activities of the different polysaccharide fractions from the cultured *C. taii* are shown in Figure 2B. Similar to the above results of antioxidant activity, the chelating abilities of the polysaccharide fractions ACP and ARP exhibited in dose-dependent manner at all the tested doses of 0.5 mg/ml to 8.0 mg/ml, in particular ACP exerted a high $Fe^{2+}$ chelating potential of greater than 70% at doses up to 1.0 mg/ml. Furthermore, like the positive control EDTA, the chelating activities of ACP sharply increased with increased dose from 0 mg/ml to 1mg/ml, and reached a plateau at a dose of 2.0 mg/ml. However, here ARP only gave a markedly lower chelating activity at all the tested doses compared with EDTA.

### *50% effective concentration ($EC_{50}$) values for antioxidant properties of alkali-soluble polysaccharide fractions*

The antioxidant properties were normalized and expressed as $EC_{50}$ value for the comparison among different samples, and the $EC_{50}$ values of ACP and ARP were summarized in Table 1. In terms of the $EC_{50}$ values, there existed certain differences of the antioxidant properties between ACP and ARP. For example, the scavenging abilities against •OH and $O^{2-}$• of ARP fraction presented about 2-fold stronger than that of ACP fraction.

***Table 1:*** *$EC_{50}$ values for antioxidant property of alkali-solube polysaccharide fractions from C. taii Note: ACP, alkali-solube crude polysaccharide; ARP, alkali-solube refined polysaccharide; EDTA, ethylenediamine tetraacetic acid; TU, thiourea; BHA, butyl hydroxyanisole ; $^{a}P<0.01$ vs ACP; $^{b}P<0.01$ vs ARP ; $^{c}P<0.01$ vs positive control.*

| Sample | $EC_{50}$ value (mg/ml) | | | | |
|---|---|---|---|---|---|
| | Reducing power activity | Scavenging DPPH radical | Scavenging hydroxyl radical | Scavenging superoxide anion radical | $Fe^{2+}$chelating ability |
| ACP | 2.84 ± 0.35 | 9.80 ± 0.66 | 4.78 ± 0.83 | 51.37 ± 1.45 | 0.52 ± 0.43$^{b}$ |
| ARP | 3.23 ± 0.47 | 13.60 ± 1.95 | 2.72 ± 0.80a | 10.46 ± 1.97a$^{,c}$ | 48.08 ± 0.33 |
| EDTA | - | - | - | - | 0.47 ± 0.29$^{b}$ |
| TU | - | 3.03 ± 1.86a$^{,b}$ | 1.12 ± 1.79 | - | - |
| BHA | 0.28 ± 0.06a$^{,b}$ | - | - | 19.98 ± 0.73a | - |

However, the reducing power and scavenging DPPH• capacity of ARP were slightly lower than that of ACP. Interestingly, ACP displayed potent $Fe^{2+}$ chelating activities with the low $EC_{50}$ value at 0.52 ± 0.43 mg/ml, which was approximately *similar* to the most frequently chelating agent EDTA.

### *Chemical properties and composition of ARP from C. taii*

As described above, the polysaccharide fraction of cultured *C. taii* displayed a potential source for the development of natural antioxidants. Therefore, it is necessary to further elucidate its chemical properties. Here ARP is a fine alkali-soluble polysaccharide fraction from ACP through depigmentation, deproteinization, dialyzation, and ethanol precipitation. After fractionation on DEAE-cellulose-52, ARP-Fr1 (278 mg), ARP-Fr2 (398 mg), and ARP-Fr3 (80 mg) were isolated by 0.01 M, 0.05 M and 0.1M NaCl elution, respectively (Figure 3A). Subsequently, the ARP-Fr2 fraction was further purified by gel filtration chromatography on a Sephadex G-100 column with distilled water elution. As a result, a major APP fraction was acquired. The homogeneity of APP fraction was then demonstrated by the following analysis. The APP fraction was eluted again with distilled water on the Sephadex G-100 gel column, and could only yield a single peak according to the elution profile as shown in Figure 3B. Furthermore, APP, as a subfraction of ARP-Fr2, didn't like the ACP and ARP fractions, and appeared no specific absorption at 260 nm and/or 280 nm by the scanning analysis of full wavelength UV (Figure 4A). It implied that the APP fraction had no proteins and nucleic acids accordingly. As shown in Figure 4B, the IR absorption spectrum of APP presented main absorption bands at 3420, 3158, 1637, 1401, 1223, 1043, and 809 $cm^{-1}$. The largest absorption band at 3420 and 3158 $cm^{-1}$ exhibited broad and intense stretching, which were assigned to hydroxyl groups （O-H or N-H and C-H）. The band between 950 and 1222 $cm^{-1}$ were mostly attributed to C–O–C and C–O–H linkages. The stretching peak at 1042 $cm^{-1}$ was suggestive of a C–O bond. The band at 1637 $cm^{-1}$ and 1401 $cm^{-1}$ can be attributed to water bound to the polysaccharide molecules [17]. The small absorption band at 809 $cm^{-1}$ suggested that APP contained α-type glycosidic linkages in its structure [14]. Further investigation showed that APP was composed of only glucose, mannose, and galactose by the GC-MS analysis (Figure 5), and theirs molar ratios were 1.14: 1.66: 1.0 with serials α-(1,4) glycosidic bond by iodine reaction. In addition, mannose may be the backbone of the structure of APP on the basis of their molar ratios.

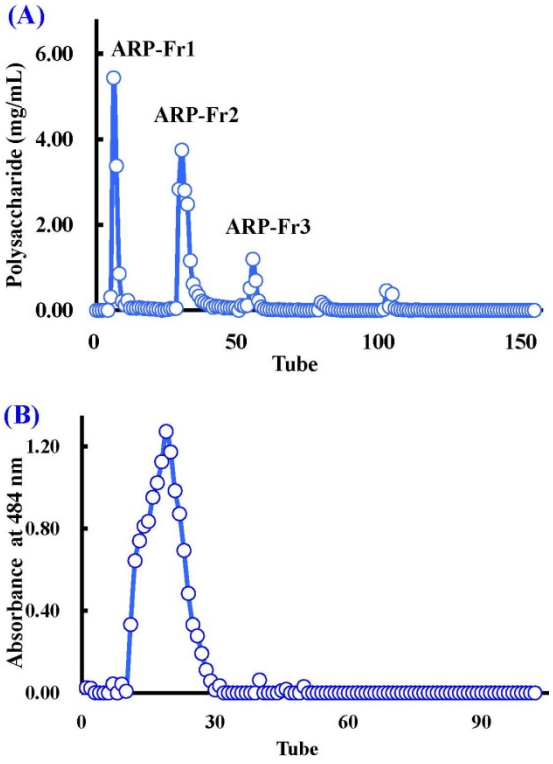


***Figure 3:*** *Separation and purification of the alkali-souble refined polysaccharide from C. taii by chromatography column.* ***(A)*** *Profile of ARP on DEAE-cellulose-52 column by eluting with 0.01M, 0.05M and 0.1M NaCl water solution* ***(B)*** *Profile of ARP-Fr2 on Sephadex G-100 gel column by eluting with distilled water.*

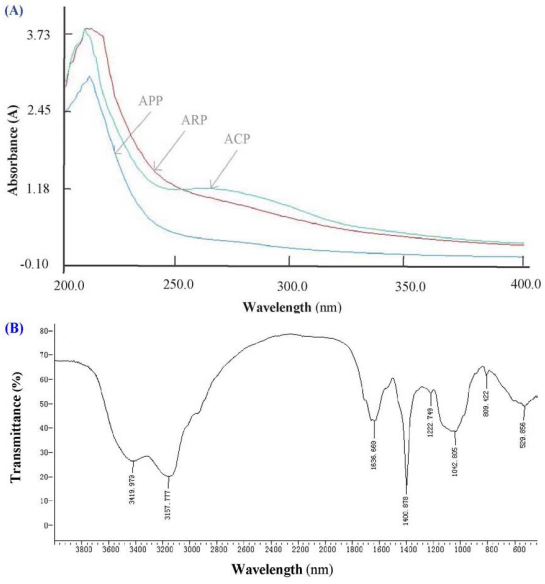


***Figure 4:*** *Typical spectrogram of the alkali-souble polysaccharide from C. taii.* ***(A)*** *UV spectrograms of different polysaccharide fractions such as ACP, ARP and APP;* ***(B)*** *IR spectrogram of APP fraction.*

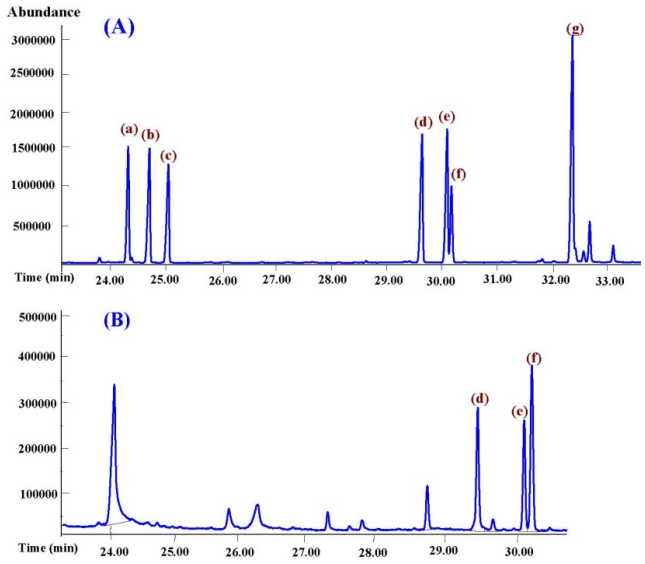


***Figure 5:*** *GC-MS profiles of the monosaccharide standards and hydrolysate of APP from C. taii.* ***(A)*** *Standards:* ***(a)*** *rhamnose****(b)*** *arabinose****(c)*** *xylose****(d)*** *glucose****(e)*** *galactose****(f)*** *mannose****(g)*** *sorbose;* ***(B)*** *Sample.*

## Discussion

Many previous studies only described the antioxidant activites of crude polysaccharide and/or aqueous extract of *Cordyceps* species [18-20]. Therefore, it was essential to further reveal the antioxidant ingredients for the development of natural antioxidant agents. In this present study, we demonstrated that both the ACP and the ARP of cultured *C. taii* could exert moderate antioxidant potentials. However, there was a significant difference between both samples towards different antioxidant assays. For example, the antioxidant properties of ACP in the range of 0.5-8 mg/ml, including the DPPH• scavenging ability, the reducing power, and $Fe^{2+}$ chealting ability, were superior to that of the ARP. Surprisingly, here the ACP exhibited a potent chealting activity with an approximately EC50 value (0.52 mg/ml) compared with the most frequently chelating agent EDTA (0.47 mg/ml). While the ARP displayed more outstanding scavenging potencies on •OH and $•O^{2-}$ than the ACP in this study. As described in materials and methods section, the ARP was consisted of more than 95% polysaccharide, and 56% polysaccharide for the ACP only. Thus polysaccharides were major active ingredients against •OH and $•O^{2-}$. Furthermore, many investigations reported that the purified polysaccharides from other *Cordyceps* species mainly exhibited potent scavenging free radicals such hydroxyl radical [21-23]. Partial antioxidant properties of the ACP, especially in the chealting activity, had more significant activities compared with the ARP, the non-polysaccharide unknown antioxidant compounds were interesting for further investigation accordingly. In comparison with the previous investigation by our group 14, there had approximately antioxidant potentials between the alkali- and water-souble crude polysaccharide fractions in cultured *C. taii*. However, there exist distinguished differences among their refined polysaccharides, of which the antioxidative abilities such as

scavenging DPPH•, and •OH, and reducing power of the alkali-souble refined polysaccharide were least 2-fold than that of the water-souble refined polysaccharide. In addition, the water-souble crude polysaccharide possessed stronger antioxidative abilities than its refined polysaccharide at all the tested parameters, including scavenging abilities on DPPH•, •OH and $•O^{2-}$, $Fe^{2+}$ chelating and reducing power [14]. As above, the water-souble crude polysaccharide attenuated its antioxidant abilities after purification, the antioxidant components of the water-souble crude polysaccharide should involve in non-polysaccharide antioxidant ingredients such as polyphenols, flavonoids, and protein. For example, the water-souble crude polysaccharides of *Cordyceps sinensis* and *Cordyceps militaris* contained polyphenolic and flavonoid compounds, which may in part be responsible for their antioxidant activities [19]. Therefore, these findings suggested that the alkali-souble polysaccharide was major antioxidant active fraction among different polysaccharides of *C. taii*, and its antioxidant protective effect was a result of its free radicals scavenging ability. In addition, the scavenging DPPH• abilities of ACP and ARP with EC values of 9.8 mg/ml, and 13.6 mg/ml were significantly inferior to that of the positive control TU (EC50=3.03 mg/ml) in this study. Likewise the aqueous polysaccharide of *C. taii* and *C. jiangxiensis* also showed weak sensitivities towards to DPPH• [5, 14]. These antioxidant substances, therefore, presented a possible selective difference against free radical. For this reason, the DPPH is a stable hydrophobic free radical that possibly interferes with the scavenging abilities of water-souble antioxidants. Previous study also demonstrated that the water extracts of both *C. militaris* and *C. sinensis* displayed more sensitive to free radicals in hydrophilic system than in hydrophobic system [19].

In the further chemical composition analysis of the polysaccharide, the APP fraction from the ARP fraction of *C. taii* was identified as a heteropolysaccharide fraction composed of glucose, galactose and mannose. The result was in agreement with the water-souble polysaccharide of *C. taii*. However, the molar ratios of monosaccharide compositions, and other chemical properties were different, e.g. glucose was its backbone [14]. As a result, their antioxidant abilities presented also evident differences as the above described. Actually, many polysaccharides with antioxidant activity from *Cordyceps* fungi were composed of glucose, galactose and mannose. For instance, Li et al. reported that a polysaccharide being composed of glucose, mannose, and galactose in *C. sinensis* showed antioxidant activity [24]. Yu et al. demonstrated that P70-1 polysaccharide fraction isolated from the water-souble crude polysaccharide of cultured *C. militaris* was also composed of glucose, mannose, and galactose, and possessed potent hydroxyl radical-scavenging activity with an EC50 value of 0.548 mg/ml [22]. Therefore, the heteropolysaccharides composed of glucose, mannose, and galactose in Cordyceps fungi are a potential source for the development of natural antioxidant agent.

## Conclusion

In summary, our results suggest that the alkali-souble polysaccharide has a moderate antioxidant potential compared with the synthetic antioxidant agents, and its antioxidant protective effect is mainly a result of its free radical scavenging ability. Therefore, it is a potential source for the development of natural antioxidant agents. Interestingly, the alkali-souble crude polysaccharide shows a surprising chelating activity due to some unidentified non-polysaccharide ingredients. The further work is still necessary to reveal additional chemicals and their structure-function relationship accordingly.

## Competing interests

The authors declare that they have no competing interests.

## Acknowledgments

The authors are grateful to financial supports from the National Natural Science Foundation of China (No. 81260278), Guizhou High-Level Innovative Talent Support Program (No. QKH-RC-20154028), and Program for Innovative Research Team in Guizhou Province (No. QKH-RCTD-20134035).

## References

1. Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol. 2007; 39: 44-84.
2. Pham-Huy LA, He H, Pham-Huy C. Free radicals, antioxidants in disease and health. Int J Biomed Sci. 2008; 4: 89-96.
3. Uttara B, Singh AV, Zamboni P, Mahajan RT. Oxidative stress and neurodegenerative diseases: A review of upstream and downstream antioxidant therapeutic options. Curr Neuropharmacol. 2009; 7: 65-74.
4. Xiao JH, Xiao DM, Sun ZH, Xiao Y, Zhong JJ. Antioxidative potential of polysaccharide fractions produced from traditional Chinese medicinal macrofungus Cordyceps jiangxiensis in vitro. Afr J Biotechnol. 2011; 10: 6607-6615.
5. Lobo V, Patil A, Phatak A, Chandra N. Free radicals, antioxidants and functional foods: Impact on human health. Pharmacogn Rev. 2010; 4: 118-126.
6. Leung PH, Zhao S, Ho KP, Wu JY. Chemical properties and antioxidant activity of exopolysaccharides from mycelial culture of Cordyceps sinensis fungus Cs-HK1. Food Chem. 2009; 114: 1251-1256.
7. Yu HM, Wang BS, Huang SC, Duh PD. Comparison of protective effects between cultured Cordyceps militaris and natural Cordyceps sinensis against oxidative damage. J Agric Food Chem. 2006; 54: 3132-3138.
8. Liang ZQ. Flora Fungorum Sinicorum, Cordyceps, Science Press, Beijing, China, 32 edition; 2007.
9. Xiao JH, Xiao DM, Sun ZH, Xiong Q, Liang ZQ, Zhong JJ. Chemical compositions and antimicrobial property of three

edible and medicinal Cordyceps species. J Food Agric Environ. 2009; 7: 91-100.

10. Liu RM, Zhang XJ, Liang GY, Yang YF, Zhong JJ, Xiao JHAntitumor and antimetastatic activities of chloroform extract of medicinal mushroom Cordyceps taii in mouse models. BMC Complem Altern Med. 2015; 15: 216.
11. Li XG, Pan WD, Luo HY, Liu RU, Xiao JH, Zhong JJ. New cytochalasins from medicinal marcofungus Cordyceps taii and their inhibitory activities against human cancer cells. Bioorg Med Chem Lett. 2015; 25: 1823-1826.
12. Liu RM, Zhang XJ, Li XF, Xiao JH, Zhong JJ. Simple and rapid method for simultaneous determination of eleven nucleosides and nucleobases in medicinal macrofungus Cordcyeps taii. Lat Am J Pharm. 2015; 34: 283-290.
13. Xiao JH, Xiao DM, Xiong Q, Liang ZQ, Zhong JJ. Nutritional requirements for the hyperproduction of bioactive exopolysaccharides by submerged fermentation of the edible medicinal fungus Cordyceps taii. Biochem Eng J. 2010; 49: 241-249.
14. Xiao JH, Xiao DM, Chen DX, Xiao Y, Liang ZQ, Zhong JJ. Polysaccharides from the medicinal mushroom Cordyceps taii show antioxidant and immunoenhancing activities in a D-galactose-induced aging mouse model. Evid-Based Compl Alt Med. 2012; 273435.
15. Xiao JH. A rapid method for the determination of polysaccharide in Cordyceps jiangxiensis. J Chinese Med Mat. 2008, 31: 689-692.
16. Xiao JH, Liang ZQ, Liu AY Chen DX, Xiao Y, Liu JW, Wan WH. Immunosuppressive activity of polysaccharides from Cordyceps gunnii mycelia in mice in vivo/vitro. J Food Agric Environ. 2004; 2: 69-73.
17. Yan JK, Li L, Wang ZM, Wu JY. Structural elucidation of an exopolysaccharide from mycelial fermentation of a Tolypocladium sp. fungus isolated from wild Cordyceps sinensis. Carbohydr Polym. 2010; 79: 125-130.
18. Zhan Y, Dong CH, Yao YJ. Antioxidant activities of aqueous extract from cultivated fruit-bodies of Cordyceps militaris (L.) Link in vitro. J Integr Plant Biol. 2006; 48: 1365-1370.
19. Yu HM, Wang BS, Huang SC, Duh PD. Comparison of protective effects between cultured Cordyceps militaris and natural Cordyceps sinensis against oxidative damage. J Agric Food Chem. 2006; 54: 3132-3138.
20. Wang M, Meng XY, Yang RL, Qin T, Wang XY, Zhang KY, Fei CZ, Li Y, Hu YL, Xue FQ. Cordyceps militaris polysaccharides can enhance the immunity and antioxidation activity in immunosuppressed mice. Carbohydr Polym. 2012; 89: 461-466.
21. Yu R, Yin Y, Yang W, Ma W, Yang L, Chen X, Zhang Z, Ye B, Song L. Structural elucidation and biological activity of a novel polysaccharide by alkaline extraction from cultured Cordyceps militaris. Carbohydrate Polymers. 2009; 75: 166-171.
22. Yu R, Yang W, Song L, Yan C, Zhang Z, Zhao Y. Structural characterization and antioxidant activity of a polysaccharide from the fruiting bodies of cultured Cordyceps militaris. Carbohydr Polym. 2007; 70: 430-436.
23. Wang Y, Wang M, Ling Y, Fan W, Wang Y, Yin H. Structural determination and antioxidant activity of a polysaccharide from the fruiting bodies of cultured Cordyceps sinensis. Am. J. Chin. Med. 2009; 37: 977-989.
24. Li SP, Zhao KJ, Ji ZN, Song ZH, Dong TT, Lo CK, Cheung JKH, Zhu SQ, Tsim KWK. A polysaccharide isolated from Cordyceps sinensis, a traditional Chinese medicine, protects PC12 cells against hydrogen peroxide-induced injury. Life Sci. 2003; 73: 2503-2513.