

## Enzymatic Modification and Antioxidant Activity of *Russula alutacea* Polysaccharides

Baijuan Wang<sup>a</sup>, Mingzhong Jiang<sup>b</sup>, Wen Peng<sup>a</sup>, Qingsong Yang<sup>c</sup>, Xiaoyun Zhang<sup>a</sup>, Yunxian Yang<sup>a</sup>, Yan Zhao<sup>\*a</sup>

<sup>a</sup>Yunnan Agricultural University, Kunming 650201, China

<sup>b</sup>Yunnan Police Officer Academy, Kunming 650223, China

<sup>c</sup>School of Ethnic Medicine, Yunnan Minzu University, Kunming 650500, China  
 zhaoyankm@126.com

This paper aims to explore the effect of enzymatic modification on the antioxidant activity of *Russula alutacea* (*R. alutacea*) polysaccharides. To this end, the polysaccharides were extracted by water and precipitated by alcohol, and subjected to enzymatic modification. Then, the hydroxyl radicals, superoxide anions, DPPH radicals scavenging rates and reducing power were used to determine and compare the properties of the modified and unmodified *R. alutacea* polysaccharides. The results show that the polysaccharide content of the sample was 1.92%; the ability to scavenge hydroxyl radicals, superoxide anions and DPPH radicals increased with the concentration; the modified polysaccharides had higher scavenging rates than the unmodified polysaccharides, but the rates were similar at the same concentration; the free radicals scavenging rate of the modified polysaccharides was slower than that of ascorbic acid; in terms of the reducing power, the modified polysaccharides was stronger than unmodified polysaccharides, but far weaker than ascorbic acid.

### 1. Introduction

*Russula alutacea* (*R. alutacea*), a species of the *Russula* genus under the family of Russulaceae family, forms an ectomycorrhizal symbiosis with trees (Wang, 2004). Known for its edible value and medicinal importance, *R. alutacea* can nourish yin, promote blood circulation, refresh the heart, relieve the internal heat, lower cholesterol, protect the liver, and guard against cancer (Yunnan Forest Compilation Committee, 2004).

The fruit body of *R. alutacea* is generally very large. *R. alutacea* mainly grows in summer and autumn, when there are relatively concentrated rainfall and high humidity (Gan et al., 2005). At present, about 90 species are recorded in China, and 317 reported across the world. In foreign countries, the fungus is mainly distributed in the US, Japan, South Korea and Russia. Domestically, the main producing areas include Liaoning, Jiangsu, Yunnan, Guangxi, Anhui, Fujian, Hebei and other provinces (Wang et al., 2004). Yunnan is the leading producer of *R. alutacea* in China, thanks to the large area of subtropical humid climate region in the province (Guo et al., 2013).

Polysaccharides are polymeric carbohydrate molecules composed of long chains of monosaccharide units bound together by glycosidic linkages (e.g.  $\alpha$ -1, 4-glycoside bond;  $\beta$ -1, 4-glycoside bond and  $\alpha$ -1, 6-glycoside bond) and on hydrolysis give the constituent monosaccharides or oligosaccharides.

Previous studies have shown that polysaccharides have a strong biological activity, such as immune regulation, lipid reduction, and prevention of cancer, aging and radiation (Zhang et al., 2004; Tian, 2000). Since researchers first set foot in the field of polysaccharides, 300 kinds of natural polysaccharide compounds have been discovered, including but not limited to fungus polysaccharide, plant polysaccharide (Zhou, 2004), animal polysaccharide, algal polysaccharide and bacterial polysaccharide.

In recent years, the molecular modification of polysaccharides has become increasingly popular. The polysaccharide compounds are structurally modified by chemical, physical and biological means, aiming to alter their physical and chemical properties or create derivatives with new biological functions. By far, enzymatic modification is the best method for molecular modification of polysaccharides, due to its specificity, mild reaction conditions, good selectivity and easy process control.

The enzymatic modification can be further divided into enzymatic degradation, enzymatic synthesis and other kinds of modification. The enzymatic degradation often uses polysaccharide-degrading enzyme as the tool enzyme. The enzyme mainly breaks down the glycosidic linkages, and forms double bonds at the resulting non-reducing end (Zhou et al., 2007). It is widely applied to enhance the rheological properties of polysaccharides, regulate protoplast formation, process food, treat adjuvant drugs, and produce oligosaccharides (Michaud et al., 2003; Tong et al., 2012). Dextran, pectin polysaccharides, xylan and xyloglucan are common substrates of enzymatic modification.

Based on the preliminary experiment, this paper selects the  $\alpha$ -amylase is selected to modify the molecules of *R. alutacea* polysaccharides and compare the antioxidant activities of the polysaccharides. The purpose is to provide reference for the development and production of drugs made of *R. alutacea* polysaccharides.

## 2. Materials and Methods

### 2.1 Materials

Fresh *R. alutacea* fruit bodies were purchased from a local market in Jinggu County, Yunnan Province. The fresh bodies were dried and crushed with small plant sample grinder (DFT-100, Beijing Kesijia Technology Co., Ltd.), and then the powder was sieved with an 80-mesh screen.

The following reagents were purchased from Chongqing Chuandong Chemical (Group) Co., Ltd., all of which belong to the analytical grade (Wang et al., 2009; Weng et al., 2008; Sun et al., 2006): glucose, butyl alcohol, petroleum ether, ethyl alcohol absolute, phenol, sulfuric acid, activated carbon,  $\alpha$ -amylase, sodium hydroxide, hydrogen peroxide, ferrous sulphate, salicylic acid, sodium dihydrogen phosphate and dibasic sodium phosphate.

### 2.2 Methods

#### (1) Extraction of *R. alutacea* polysaccharides

20.0g *R. alutacea* powder was diluted in 400mL distilled water, and let sit for 2h at 80°C (3 times) and centrifuged. The supernatant was obtained and added with 100% (v/v) ethanol in a volume three times as much as the supernatant. The mixture was stored for 12h at 4°C. Then, the precipitation was centrifuged for 3min at 12,000r/min and dried in a constant temperature oven at 65°C. The dried precipitation was dissolved with 100mL distilled water in a water bath (60~70°C), and added with 1% (w/v) activated carbon and the precipitation discarded after centrifugation.

The Sewage method was employed to remove proteins from the solution: the solution was centrifuged after a 30min-long standing at room temperature; then, the supernatant was added with ethanol in a volume four times as much as the supernatant, and let sit for 4h at 4°C. Then, the mixture was centrifuged at 12,000r/min, and the precipitation was collected as *R. alutacea* samples.

#### (2) Determination of polysaccharide contents

The polysaccharide content was measured by the phenol-sulfuric acid method using glucose as standard (Blois, 2002). The polysaccharide content in *R. alutacea* extraction was represented by OD490 absorbance value, i.e. the wavelength of maximum absorption. Then, the polysaccharide extraction rate was defined as:

$$\text{Polysaccharide extraction rate (\%)} = \left( \frac{C \times V \times M_1}{1000} \right) / m \times 100$$

where C is the mass concentration of *R. alutacea* polysaccharides in the extracting solution (mg/ml); V is the constant volume (mL);  $M_1$  is the weight of the extracted polysaccharides (g); m is the weight of the sample (g).

### 2.3 Enzymatic modification of *R. alutacea* polysaccharides (Jia et al., 2013)

The *R. alutacea* polysaccharides was prepared into a solution (mass concentration: 0.5g/100mL) with distilled water, and added with 259.5U/g pepsin. The enzymatic hydrolysis was carried out with  $\alpha$ -amylase at the pH of 5.8 for 15min at 48.5 °C. Next, the enzyme was deactivated by boiling for 10min in a water bath. After cooling, the solution was centrifuged for 10min at 3,000r/min. The supernatant fluid was collected and freeze dried to obtain the modified *R. alutacea* polysaccharides.

#### (1) Specific viscosity analysis (Song et al., 2014)

The specific viscosity of the polysaccharides was measured by an Ubbelohde viscometer in the water bath at 40 °C. First, the time for 20mL distilled water to flow through the viscometer was counted on a stopwatch and recorded as  $t_0$ ; without changing the conditions, the time for 20mL 3.0mg/mL polysaccharide solution to flow through the viscometer was counted and recorded as t; then, the specific viscosity of polysaccharides was calculated by the formula below:  $\eta_{sp} = (t - t_0) / t_0$

### 2.4 FT-IR analysis

For IR spectroscopy, polysaccharide powders were mixed with potassium bromide, grounded, and pressed into a 1mm pellet. The measurement range was set to 4,000~400 $\text{cm}^{-1}$ .

## 2.5 Analysis of antioxidant activities (Qu et al., 2012)

### (1) Determination of reducing power (Zhou et al., 2014)

1mL unmodified and 1mL modified polysaccharide test solutions were taken at different concentrations. 2.5mL (pH 6.6) 0.2mol/L phosphate solution was prepared as the buffer solution. For each test solution, 2.5mL 1g/100mL  $K_3[Fe(CN)_6]$  solution was added. The mixed solution was stirred and placed in the water bath for 20min at 50°C. Then, 2.5mL 1g/100mL trichloroacetic acid solution was added into the mixed solution and centrifuged. After that, 2.5mL supernatant was relocated to another test tube, added with 2.5mL distilled water and 0.5mL 0.1g/100mL  $FeCl_3$  solution. After mixing, the absorbance was measured at the wavelength of 700nm.

### (2) Analysis of superoxide anions scavenging ability

The superoxide anions scavenging ability was evaluated by the pyrogallol autoxidation method (Qu et al., 2011). 1mL unmodified and 1mL modified polysaccharide test solutions were taken at different concentrations. For each test solution, 1.0mL 45mmol/L pyrogallol solution and 4.5mL (pH8.2) Tris-HCl solution were added. The mixed solution was stirred and placed in the water bath for 10min at 25 °C. Then, 1.5mL 1mol/L HCl was added to terminate the reaction. The absorbance was measured at the wavelength of 325nm with ascorbic acid as positive control. The scavenging rate of superoxide anions was calculated according to the following formula:

The scavenging rate of  $O_2^-$  (%) =  $[1 - (A_s - A_b) / A_c] \times 100$

where  $A_s$  is the absorbance of the test solution at different concentrations;  $A_b$  is the absorbance of the control group, in which the pyrogallol solution was replaced by 1mL distilled water;  $A_c$  is the absorbance of the negative control group, in which the sample was replaced by 1mL distilled water.

### (3) Analysis of DPPH radicals scavenging rate

2mL unmodified and 2mL modified polysaccharide test solutions were taken at different concentrations. For each test solution, 2mL 0.1mmol/L DPPH ethanol solution was added. The mixed solution was stirred and let sit for 30min in dark condition. The absorbance was measured at the wavelength of 517nm. The scavenging rate of DPPH radicals was calculated according to the following formula:

The scavenging rate of DPPH radicals (%) =  $[1 - A_s - A_b] / A_c \times 100\%$

Where  $A_s$  is the absorbance of the test solution at different concentrations;  $A_b$  is the absorbance of ethanol solution at different concentrations;  $A_c$  is the absorbance of DPPH ethanol solution.

### 2.5.4 Analysis of hydroxyl radicals scavenging rate

1mL unmodified and 1mL modified polysaccharide test solutions were taken at different concentrations. For each test solution, 2.0mL 1.8 mmol/L  $Fe_2SO_4$  solution and 1.5mL salicylic acid-ethanol solution were added. The mixed solution reacted with 1.0mL 0.3%  $H_2O_2$  the water bath for 30min at 37°C. The absorbance was measured at the wavelength of 510nm with ascorbic acid as positive control. The scavenging rate of hydroxyl radicals was calculated according to the following formula:

The scavenging rate of hydroxyl radicals (%) =  $[1 - (A_s - A_b) / A_c] \times 100\%$

where  $A_s$  is the absorbance of the test solution at different concentrations;  $A_b$  is the absorbance of the test solution;  $A_c$  is the absorbance measured without the test solution.

## 3. Results and Analysis

### 3.1 Determination of polysaccharides content

A certain amount of sample backup solution was extracted to measure the absorbance at the wavelength of 490nm, and the polysaccharide content in the sample was calculated by the regression equation. The results show that the polysaccharide content was 1.92%.

### 3.2 Effect of *R. alutacea* polysaccharides on the specific viscosity of enzymatic modification

Specific viscosity is an important indicator of the molecular weight of polysaccharides. Under a constant polysaccharide concentration, the specific viscosity is positively correlated with molecular weight. According to Table 1, the specific viscosity of *R. alutacea* decreased significantly after enzymatic modification, indicating a decline in the molecular weight of the polysaccharide through the modification.

Table 1: Effect of enzymatic modification on specific viscosity of polysaccharide extract from *R. alutacea* ( $\bar{x} \pm s$ ,  $n=3$ )

Name	Modified polysaccharides	Un-modified polysaccharides
Hsp	0.772±0.005	0.251±0.006

### 3.3 IR spectrum analysis

Figures 1 and 2 display the IR spectrum of the unmodified and the modified *R. alutacea* polysaccharides, respectively. As shown in the figures, there are stretching vibration at 1,500~1,100 $\text{cm}^{-1}$  and 900~500 $\text{cm}^{-1}$  resulted from the enzymatic modification of *R. alutacea* polysaccharides.

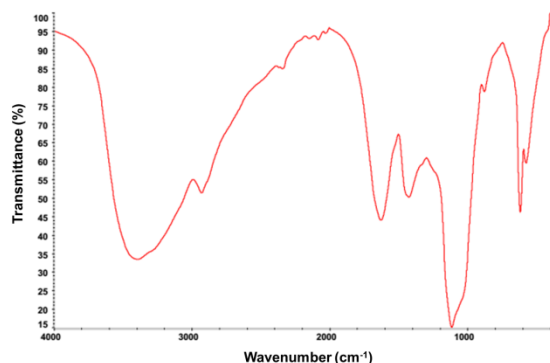


Figure 1: Infrared spectrum of unmodified polysaccharides

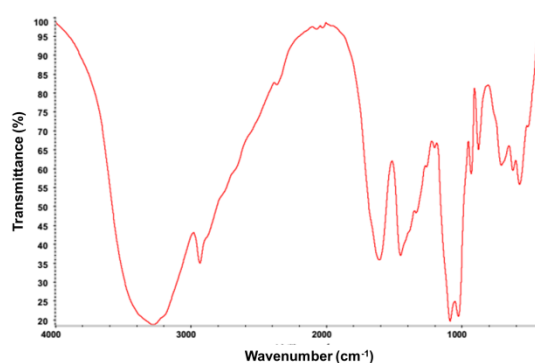


Figure 2: Infrared spectrum of modified polysaccharides

### 3.4 Determination of antioxidant capacity

#### (1) Effect of enzyme modification on scavenging hydroxyl radicals

It can be seen from Figure 3a that unmodified polysaccharides, modified polysaccharides and ascorbic acid all had a certain scavenging effect on hydroxyl radicals, and that the scavenging effect increased with the concentration of the solutions. Specifically, the modified polysaccharides outperformed the unmodified polysaccharides in scavenging rate, but the lead gradually diminished with the increase in the concentration of the two solutions. Moreover, the modified polysaccharides shared a similar scavenging rate of hydroxyl radicals with the ascorbic acid at the low concentration of 0.4mg/mL, but lagged behind the latter with the increase of the concentration.

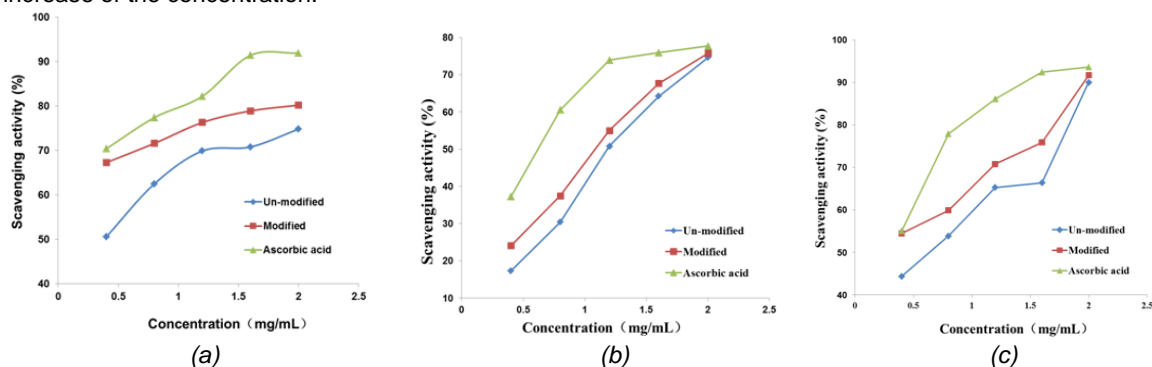


Figure 3: (a) Effect of enzymatic modification on scavenging hydroxyl radicals. (b) Effect of enzyme modification on scavenging superoxide anions. (c) Effect of enzyme modification on scavenging DPPH radicals

#### (2) Effect of enzyme modification on scavenging superoxide anions

It can be seen from Figure 3b that unmodified polysaccharides, modified polysaccharides and ascorbic acid all had a certain scavenging effect on superoxide anions, and that the scavenging effect increased with the concentration of the solutions. According to the regression equation, the  $EC_{50}$  values of ascorbic acid, unmodified polysaccharides and modified polysaccharides are 0.573mg/mL, 1.26mg/mL and 1.14mg/mL, respectively. In terms of superoxide anions scavenging rate, the three solutions were ranked as ascorbic acid > modified polysaccharides > unmodified polysaccharides. Specifically, the modified polysaccharides outperformed the unmodified polysaccharides in scavenging rate, but the lead gradually diminished as the concentration of the two solutions reached 2.0mg/mL. Moreover, the ascorbic acid had a faster scavenging rate of superoxide anions than the modified polysaccharides, but the lead gradually shrunk as the concentration of the two solutions reached 2.0mg/mL.

### (3) Effect of enzyme modification on scavenging DPPH radicals

It can be seen from Figure 3c that unmodified polysaccharides, modified polysaccharides and ascorbic acid all had a certain scavenging effect on DPPH radicals, and that the scavenging effect increased with the concentration of the solutions. According to the regression equation, the EC<sub>50</sub> values of unmodified polysaccharides and modified polysaccharides are 66mg/mL and 0.29mg/mL, respectively. The results show an improvement in the DPPH radicals scavenging ability of *R. alutacea* polysaccharides was improved by enzymatic modification. The improvement is most likely caused by the hydrolyzation of the  $\alpha$ -1, 4-glycoside bond by  $\alpha$ -amylase (HUANG Hui-hua, *et al*, 2002): the  $\alpha$ -amylase degrades the (1 $\rightarrow$ 4)- $\alpha$ -D-glucose polymer in the branch of *R. alutacea* polysaccharides, exposing some bioactive groups in the polysaccharides. Furthermore, the molecular weight had a major impact on the biological activity of polysaccharides. Within a certain range, the molecular weight is negatively correlated with the biological activity (SUN Yongxu, *et al*, 2009). In addition, the modified polysaccharides at a low concentration of 0.4mg/mL had almost the same scavenging rate with ascorbic acid at a high concentration of 2.0mg/mL. In other words, the modified polysaccharides were equivalent to ascorbic acid in terms of DPPH radicals scavenging rate at low or high concentrations.

### (4) Determination of reducing power

Reducing power is one of the evaluation indices of antioxidant activity. A material with reducing power can stabilize free radicals by interrupting the lipid peroxidation (LPO) of the chain reaction. As shown in Table 2, the mass concentration of unmodified polysaccharides, modified polysaccharides and ascorbic acid was positively correlated with the reducing power. It is clear that the modified polysaccharides had a much greater reducing power than the unmodified polysaccharides. This is because the molecular weight of *R. alutacea* polysaccharides decreased through the enzymatic modification by  $\alpha$ -amylase. With a small molecular weight, the modified polysaccharides generally had a loose structure, and weak hydrogen bonds. The resulting exposure of active groups provided lots of electrons, which enhanced the reduction power. Moreover, the reducing power of ascorbic acid was far greater than that of modified and unmodified polysaccharides.

Table 2: Effect of enzymatic modification on reducing power of polysaccharide extract from *R. alutacea*

No.	Concentration (mg/ml)	Absorbance
Un-modified polysaccharides	0.4	0.176
	0.8	0.352
	1.2	0.583
	1.6	0.675
	2.0	0.823
Modified polysaccharides	0.4	0.233
	0.8	0.448
	1.2	0.621
	1.6	0.794
	2.0	0.892
Ascorbic acid	0.04	0.297
	0.08	0.621
	0.12	0.944
	0.16	1.340
	0.2	1.600

## 4. Conclusion

This paper aims to explore the effect of enzymatic modification on the antioxidant activity of *Russula alutacea* (*R. alutacea*) polysaccharides. To this end, the polysaccharides were extracted by water and precipitated by alcohol, and subjected to enzymatic modification. Then, the hydroxyl radicals, superoxide anions, DPPH radicals scavenging rates and reducing power were used to determine and compare the properties of the modified and unmodified *R. alutacea* polysaccharides. The results show that the polysaccharide content of the sample was 1.92%; the ability to scavenge hydroxyl radicals, superoxide anions and DPPH radicals increased with the concentration; the modified polysaccharides had higher scavenging rates than the unmodified polysaccharides, but the rates were similar at the same concentration; the free radicals scavenging rate of the modified polysaccharides was slower than that of ascorbic acid; in terms of the reducing power, the modified polysaccharides was stronger than unmodified polysaccharides, but far weaker than ascorbic acid.

## Acknowledgments

This work was supported by Applied Basic Research Programs of Science and Technology Commission Foundation of Yunnan Province (No. 2015FB147), the Educational Commission of Yunnan Province, China (2014C070Y), National Natural Science Foundation of China (31460137, 61561054) and Yunnan Agricultural University Natural Science Foundation for Young Scientists Project (2015ZR15).

## Reference

- Blois M.S., 2002, Antioxidant determinations by the use of a stable free radical, *Nature*, 26, 1199-1200.
- Gan Y.K., Zhao L.F., Dai L., 2005, A Summary of The Uncultivated Red Mushroom, *Journal of Yulin teachers college (Natural Science)*, 26(3), 70-74.
- Guo Y.H., Zhang W.S., Luo X.K., 2013, Investigation of *Russula alutacea* Fr. and ecology in in southern Yunnan, *Edible fungi*, 1(3), 12-13+19.
- Huang H.H., Huang S.B., Liang H.H., 2002, Effects of tea polyphenols on the recovery of  $\alpha$  - amylase from *Aspergillus oryzae* and its characteristics, *Food Science And Technology*, 23(7), 30-34.
- Jia J.Q., Shen J., Chen L., 2013, Enzymatic Modification and Antioxidant Activity of Polysaccharides from *Cordyceps militaris* Fruit Bodies, *Food Science*, 34(1), 114-120.
- Michaud P., Da Cos T.A., Cour Tois B., 2003, Polysaccharidelyases: recent developmen ts as biotechnological tools, *Crit Re v Biotechnol*, 23(4), 233-266 1
- Qu C.L., Yu S.C., Luo L., 2012, Influences Of Carboxymethylation Modification On Antioxidant Activity Of Jujube Polysaccharides, *Journal of Henan University of Technology*, 33(6), 18-21.
- Qu J.Y., Ren D.M., 2011, Chemical Modification and in vitro Antioxidant Activity of Polysaccharides from *Cordyceps militaris*, *Food Science*, 32(15), 58-61.
- Song H.L., Zhu G.Q., Lu X.L., 2014, *Gentiana turkestanorum* (Gand) Holu total polyphenol content and antioxidant activity determination, *Global Traditional Chinese Medicine*, 7(2), 103-106.
- Sun Q.L., Lian G.Y.R., Lu J.L., 2006, A Review on the applicant ion of ultrasound to extraction of tea, *Journal of Tea*, 32(2), 79-82.
- Sun Y.X., Liang H.T., Cai G.Z., 2009, Sulfated modification of the water-soluble polysaccharides from *Polyporus albicans* mycelia and its potential biological activities, *International Journal of Biological Macromolecules*, 44(1), 14-17.
- Tian G. Y., 2000, Study and Applications of Natural Polysaccharides 1, *Shanghai Chemical*, 10, 29-31.
- Tong Y., Wu H.J., 2012, Research Progress on extraction methods of plant polysaccharides, *Information on Traditional Chinese Medicine*, 29(5), 108-110.
- Wang Q.Y., Shi M.B., 2004, Present and Future of *Russula* in China, *Edible Fungi of China*, 23(4), 10-12.
- Wang S.S., Zhang J., Wang J.Z., 2009, "Conventional water as a solvent ethanol shen law" to extract polysaccharide from sweet potato root neck, *Journal Of Shang Qiu Teachers College*, 25(3), 103-106.
- Wang X.H., 2004, Wild commercial mushroom manual of Yunnan province, KunMing: Yunnan Science and Technology Press.
- Weng L., Wen L., Yang F., 2008, Empirical study of different extraction methods influence on the antioxidation of polysaccharide in *cordyceps militaris*, *Food Science And Technology*, 33(11), 180-182.
- Yunnan Forest Compilation Committee, 2004, Yunnan forest, BeiJing: China Forestry Press.
- Zhang L.P., Zha A.H., 2004,. Function feature and comprehensive processing utility of oats, *Food & Machiney*, 20(2), 55-56.
- Zhou H.B., Zhao H., 2007, Enzymatic Modification of Polsaccharides: Recent Developments, *Progress in Pharmaceutical Sciences*, 31(8), 349-352.
- Zhou L., Shao J.H., Ren X.M., Wang F., Shi X.M., 2014, In vitro antioxidant properties of polysaccharides and its derivates from *Dioscorea Rhizoma*, *Food Science And Technology*, 39(2), 188-191.
- Zhou Y., 2004, Research Progress on antitumor effects of Polysaccharides, *Food & Machiney*, 28(3), 129-132.