African Journal of Biotechnology Vol. 7 (22), pp. 4004-4009, 19 November, 2008
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2008 Academic Journals

Full Length Research Paper

A study on *Cordyceps militaris* polysaccharide purification, composition and activity analysis

Hui Yan*, Dongjie Zhu, Dabao Xu, Jie Wu and Xuyun Bian

School of Biological and Environmental Engineering, Jiangsu University of Science and Technology, Zhenjiang, Jiangsu 212018, People's Republic of China.

Accepted 17 October, 2008

In this study, raw *Cordyceps militaris* polysaccharide (CPS) was extracted with ultrasonic- assisted extraction method. Proteins in the raw CPS were removed with the enzymatic and Sevag methods. After dialysis, an extraction rate of 0.82% was achieved. Two components, namely CPS-1 and CPS-2, were obtained from DEAE-52 ionic exchange chromatography eluted with NaCl solution at a gradient from 0 to 0.6 mol•L^{-1}. The ultraviolet scanning demonstrated that CPS-1 did not couple with protein and nucleic acid, but CPS-2 was coupled with a few proteins and nucleic acids. After product derivatization using acid hydrolysis, gas chromatography analysis revealed that these two CPSs were both composed of mannose and galactose, with a mole ratio of 0.96:1 and 1.04:1, respectively. The two CPSs can reverse ethanol-induced injury in primary cultured rat hepatocytes. Its effect appeared dose-dependent, having better effect when higher dose was applied. Its effect is probably due to its anti-oxidation function.

Key words: *Cordyceps militaris* polysaccharide, gas chromatography, hepatocyte injury, anti-oxidation.

INTRODUCTION

Cordyceps sinensis is a valuable traditional Chinese medicine which has many pharmacological effects, such as enhancing immunity, tumor inhibition and anti-inflammation (Liu et al., 1996; Wang et al., 1995). It is a natural parasitism which demands very special environmental condition for growing. Therefore, artificial cultivation is difficult. In recent years, it has been very scarce due to over-harvesting. *C. militaris* is also called northern *C. sinensis*. Because *C. militaris* belongs to the same genus with *C. sinensis*, it has long been recognized as a desirable alternative for natural *C. sinensis* (Xu, 1997).

Polysaccharides, as signal molecules in cell recognition and regulatory processes, are becoming the new frontier of life science research (Zhang, 1999). *C. militaris* polysaccharide (CPS), as one of the main active component from *C. militaris*, has anti-liver fibrosis, immune regulation, anti-tumor, lowering blood glucose, and other favorable effects (Liu et al., 1997; Bin et al., 2003; Wen et al., 2000).

In recent years, a number of strains have been selected to carry out artificial cultivation for large-scale production of *C. militaris*. Studies on its polysaccharide component will help clarify mechanisms for its medical effects, which, therefore is conducive for developing *C. militaris* resources.

MATERIALS AND METHODS

C. militaris

C. militaris cultivated with rice medium was obtained from silkworm physiological lab, Institute of sericulture, Chinese Academy of Agricultural Sciences (Zhenjing, Jiangsu). They were dried at 60°C, ground, sieved and stored for later use.

Reagents

Trypsin was purchased from Shanghai Runji Chemical Reagents Limited. Papain was supplied by Beijing Aobo Biotechnology Limited Liability Company. DEAE-52 was purchased from Beijing Bai'ao Biotechnology Limited Company. D-xylose was obtained from China Huixing Biochemical Reagent Co. Ltd. D-mannose, glucose, D-galactose and acetic anhydride were purchased from Guoyao Chemical Reagents Group Ltd. L-(+) - rhamnose and D-(+) - arabinose were obtained from Biobasicinc Corporation. Inositol

*Corresponding author. E-Mail: yanh1006@163.com.

was purchased from Solarbio Corporation. Hydroxylamine hydrochloride was purchased from Shanghai Zhanyu Chemical Co. Ltd. Pyridine was purchased from Tianjin Damao Chemistry Reagent Factory.

Equipment

AvantiJ-25 refrigerated centrifuge was of Beckman Coulter Experimental System Limited. UV-9100 spectrophotometer was of Beijing Rayleigh Analysis Equipment Company. FD-1 freeze-drying machine was from Beijing Kang Bo Medical Equipment Limited Experiment Company. DHL-computer constant current pump and DBS-160 computer automatic collection system were from Shanghai Huxi Analysis Instrument Factory. UV-2450 UV-Vis was of Shimadzu Corporation. Agilent 6820 GC was from Agilent Corporation. 125-1017 DB-1-type column with length 15 m, ID 0.53 mm and Film 0.5 was purchased from Agilent.

Polysaccharide extraction

C. militaris was packed with filter paper and placed in Soxhlet extractor, degreased through backflow with petroleum ether overnight, and then dried. 20 g *C. militaris* polysaccharide was extracted with ultrasonic-assisted method based on the result of orthogonal test. Extraction was done three times under the following conditions: temperature 80°C, the ratio of material to liquid 1:45, ultrasonic power 140 W, extraction time 80 min. After centrifugation, the supernatant was collected.

Two methods, enzymatic and Sevag, had been used to remove proteins in the supernatant. 1/40 volume 3% trypsin solution (3 g trypsin in 100 ml 0.07% anhydrous calcium chloride) was added into the supernatant on the condition of pH 8.0 and 65°C for 1.5 h. After that, 1/40 volume 2.5% papain was added at pH7.0 and 50°C for 1 h. Chloroform, 1/5 volume of solution, was added followed by addition of butyl alcohol with 1/5 volume of chloroform. After thermal agitation for 20 min and centrifugation at 9000 $r \cdot min^{-1}$ for 10 min, the aqueous phase was acquired with a tap funnel. Ethanol was added with final concentration of 90% and then left for overnight. Polysaccharide precipitation was collected and washed repeatedly with acetone and ethanol. After dilution to appropriate concentration, the polysaccharide solution was subjected to dialysis with tap water for two days and with distilled water for one day, and later freeze-dried.

Ion-exchange chromatography

100 mg dry polysaccharide was dissolved in 10 ml double distilled water, subjected into DEAE- cellulose column (35 × 300 mm), eluted with NaCl solution at a gradient from 0 to 0.6 $mol \cdot L^{-1}$ and flow speed of 1.0 $ml \cdot min^{-1}$. The eluate was collected by partial collector with 5 ml in each tube. Track was detected by sulfuric acid-phenol method. The eluates was merged from collection tubes based upon main peaks. After dialysis, condensation and freeze-drying, two solid state CPS named CPS-1 and CPS-2 were acquired.

CPS ultraviolet spectroscopy

CPS was dissolved in double distilled water to the concentration of 0.1 $mg \cdot ml^{-1}$, and scanned with UV spectrophotometer in wavelengths from 600 to 200 nm.

Polysaccharide component analysis

Gas chromatography was commonly used in the analysis of polysaccharide composition including the type and mole ratio of monosaccharide (Wang et al., 2001). In this experiment, derivation of acid hydrolytic product from CPS was carried out with sugar nitrile acetate method. The obtained derivative was analyzed by gas chromatography to identify its monosaccharide components.

10 mg CPS-1 and CPS-2 were placed in 5 ml ampoules respectively, and 3 ml of 4.0 $mol \cdot L^{-1}$ trifluoroacetic acid was added and oscillated to dissolve. Ampoules were filled with nitrogen and sealed; these were stored in oven at 115°C for 12 h. The acid solution was placed in water bath at 65°C, nitrogen-dried, dissolved in 1 ml chloroform, and then dried. This was repeated twice to remove trifluoroacetic acid. 8 mg hydroxylamine hydrochloride and 10 mg inositol were added as internal standard. The sample was dissolved in 0.5 ml pyridine to carry on reaction with shaking at 90°C for 30 min. After reaction completion, it was cooled to room temperature. 0.5 ml of acetic anhydride was added to carry on acetylation reaction at 90°C for 30 min. Derivation process of single monosaccharide and mixture monosaccharide was operated with the same method aforementioned.

N_2 was used as carrier gas, and H_2 as burning gas. Sample injection volume was 1 µl on condition of nitrogen flow of 50 $ml \cdot min^{-1}$ at split ratio of 50:1. Temperature of sample mouth and FID detector were 250 and 210°C, respectively. Programmed temperature was first maintained 160°C for 30 min, rose to 190°C at the rate of 15°C$\cdot min^{-1}$ and maintained for 20 min, then it rose to 230°C at the rate of 20°C$\cdot min^{-1}$ and maintained for 20 min.

CPS on rat hepatocyte injury

A rat was anesthesiaed by intraperitoneal administration of 1% sodium pentobarbital at a dose of 0.1 $mg \cdot g^{-1}$ weight, and intraperitoneal injection of heparin 0.2 $U \cdot g^{-1}$ weight for anticoagulant. After disinfection of the skin, the abdomen was cut with abdomen median incision. After the portal vein was revealed, an 18th blunt needle was inserted into it at 2 cm away from hepatic portal, and the needle was connected to a constant current pump. Liver superior vena cava and the inferior vena cava were clipped, and the 16th catheter was inserted in the liver inferior vena to release blood. 500 ml hanks solution without calcium and magnesium (containing 1 $mmol \cdot L^{-1}$EDTA) was perfused first at a speed of 30 $ml \cdot min^{-1}$, and then 100 ml 0.25% trypsin at speed of 10 $ml \cdot min^{-1}$.

The liver was removed and placed into a plate containing 4°C Hanks liquid. Liver capsule and blood vessels were removed. Then, liver tissue was torn bluntly. The sample was filtered with multi-layer gauze and 200 mesh stainless steel filter to prepare cell suspension. Cell suspension was centrifuged at 700 $r \cdot min^{-1}$ at 4°C for 2 min. After suction and discarding culture solution, cells were washed with a little of D-Hanks. This washing was repeated three times (centrifugation speed of the 2nd and 3rd was 600 and 500 $r \cdot min^{-1}$ respectively) to remove trypsin and non-hepatocyte. The rate of living cells dyed by 0.4 percent trypan blue was counted on cell count plate. Purified hepatocytes were diluted into 1×10^6 $cell \cdot ml^{-1}$ suspension with synthetic culture solution (90 $ml \cdot L^{-1}$ DMEM, 10 $ml \cdot L^{-1}$ newborn calf serum, $l0^{-7}$ $mol \cdot L^{-1}$dexamethasone, 10^{-8} $mol \cdot L^{-1}$insulin injection, 100 $\mu g \cdot L^{-1}$ penicillin, and 100 $\mu g \cdot L^{-1}$ streptomycin). 1 ml cell suspension per well was added to 24 well culture, and 0.1 ml was added into each well of a 96 culture plate, both with three duplicate wells. Cell culture plates were placed in incubator with 5% CO_2 at 37°C. 12 h later, hepatocytes can be seen growing at bottom of the wells.

After 12 h cultivation, culture solution was discarded and replaced with new medium. CPS-1 and CPS-2 was added respectively to different final concentrations of 0.1, 0.2, 0.4 and 0.8 $mg \cdot ml^{-1}$, and cultured for 6 h. Then, ethanol was added into each well at a final concentration of 100 $mmol \cdot L^{-1}$, and the culture was continued for 12 h. MDA contents and the GSH-PX activity of supernatant in 24 well were determined. For the 96 well culture plate, culture solution was

disposed, and 10 µl of 5 mg•ml^{-1} MTT was added in darkness, and the culture was continued for 4 h. 100 µl DMSO was added to each well and fully mixed. 10 min latter, the absorbance value was measured with UV-9100 spectrophotometer at wavelength of 570 nm.

RESULTS AND DISCUSSION

CPS

Gray yellow CPS of 0.1641 g was obtained from 20 g *C. militaris*. The yield rate was 0.82%. It was less than Li Shi Ying's results (Shi et al., 2006). The relative low yield may come from the utilization of enzymatic and Sevag methods for removing proteins and dialysis for removing small molecules; both of which led to less amount but much purer polysaccharide.

Ion-exchange chromatography purification of CPS

In this study, two CPS peaks were observed in ionic exchange chromatograph which is consistent with previous reports (Guanghao, 2007). DEAE-52 elution curve of CPS is shown in Figure 1. Two CPS components were obtained through merging eluates based upon peaks on elution curve and freeze-dring. CPS-1 was white and CPS-2 was yellowish, with quantities of 12.3 and 39.2 mg, respectively.

Ultraviolet absorption chart of polysaccharide

CPS-1 had no absorption at wavelength 280 and 260 nm in ultraviolet absorption chart as shown in Figure 2, indicating that no nucleic acid and protein was combined with CPS-1. However, a weak absorption of CPS-2 was in 260 and 280 nm, which demonstrated that it coupled with a small amounts of nucleic acid and protein.

CPS gas chromatography analysis

Standard monosaccharide derivative was analyzed by gas chromatography. As shown in Figure 3, mixtures of monosaccharide and inositol were separated completely under this GC parameters used. The peaks emerged in the order of rhamnose, arabinose, xylose, mannose, glucose, galactose and inositol.

Figures 4 and 5 showed gas chromatography of CPS-1 and CPS-2, respectively. On the basis of the standard single-sugar retention time, CPS-1 and CPS-2 were all only composed by mannose and galactose. It was calculated on correction factor of single-sugar relative to the standard target. According to peak area of the monosaccharide and internal standard inositol, mole ratio of monosaccharide composition were acquired on basis

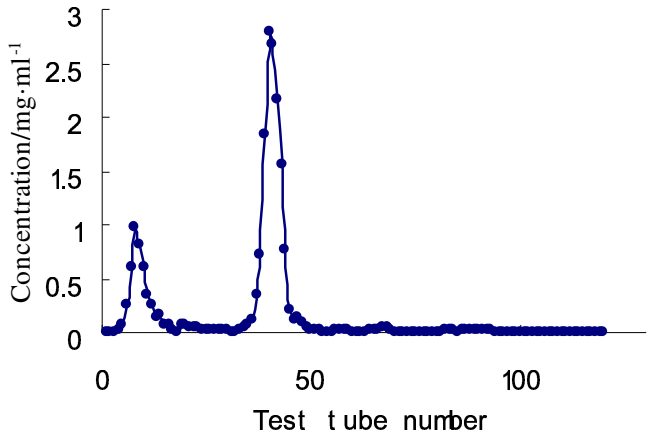

Figure 1. CPS ion-exchange chromatography.

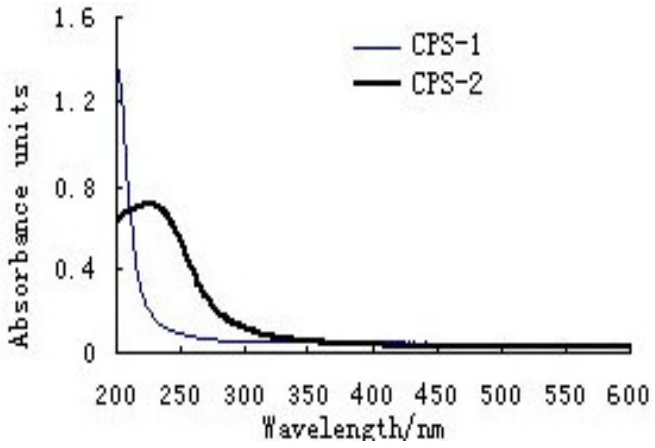

Figure 2. Ultraviolet absorption chart of CPS components.

of monosaccharide and the internal standard chromatograph peak area as well as each monosaccharide correction factor. The mole ratio of mannose to the galactose in CPS-1 was 0.96:1, and that in CPS-2 was 1.04:1.

Wu Guanghao and his colleagues (Wu and Wang, 2007) have reported that CPS extracted by them was composed of mannose, glucose and galactose, at a mole ratio of 20.5:71.7:7.8. Yu and his group (Yu et al., 2004) extracted 3 types of CPS, named CPS1, CPS2 and CPS3, from *C. militaris*. CPS-1 was composed of rhamnose, xylose, mannose, glucose and galactose with mole ratio of 1:6.43:25.6:16.0:13.8. CPS2 was made of 3 monosaccharides, namely rhamnose, glucose and galactose, and the mole ratio iwas 1:4.46:2.43. CPS3 only contained one monosaccharide, glucose. Ohta et al. (Ohta et al., 2007) studied polysaccharide from *C. militaris*, and discovered that it was composed of galactose, arabinose, xylose, rhamnose and galacturonic

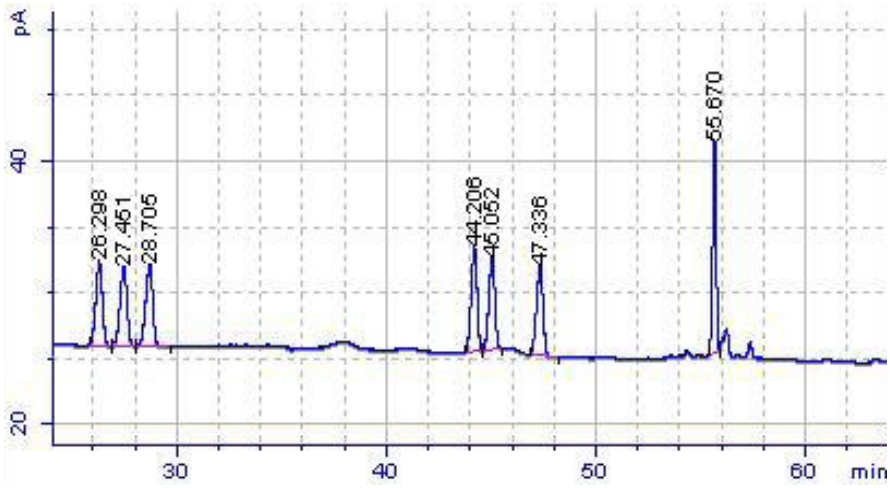

Figure 3. GC chromatogram of standard monosaccharides. From left to right: rhamnose, arabinose, xylose, mannose, glucose, galactose and inositol.

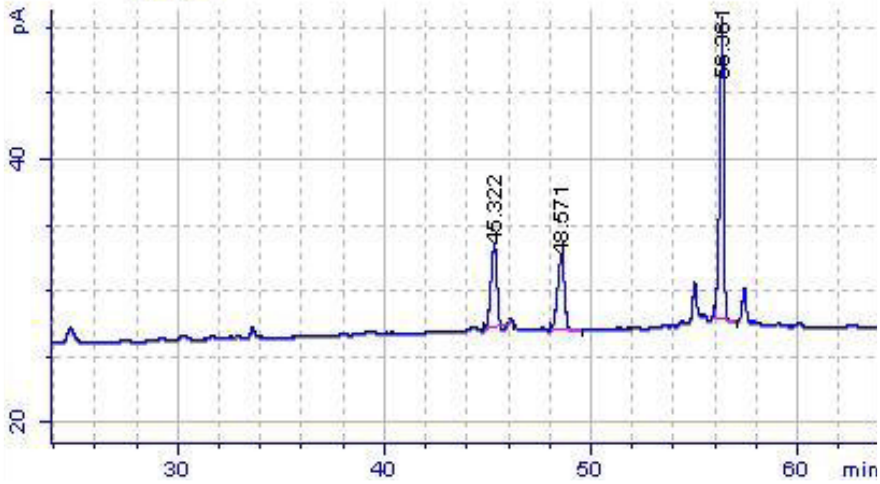

Figure 4. GC chromatogram of CPS-1.

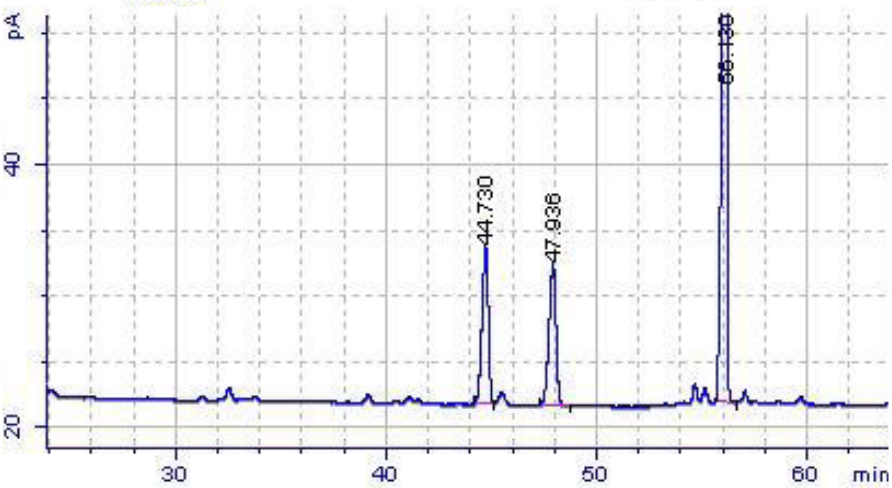

Figure 5. GC chromatogram of CPS-2.

Table 1. Effects of CPS-1 and CPS-2 on primary cultured rat hepatocyte injured by ethanol.

Group	Final concentration	MDA (nmol/10^6)	GSH-PX (unit of activity)	Cell activity (AU)
normal	-	2.96	302.54	0.272
Ethanol (mmol•L^{-1})	100	20.30	76.06	0.185
CPS-1 intervention(mg•ml^{-1})	0.10	7.99	280.56	0.204
	0.20	6.88	283.94	0.216
	0.40	6.48	292.39	0.220
	0.80	5.88	312.68	0.230
CPS-2 intervention(mg•ml^{-1})	0.10	12.26	290.70	0.181
	0.20	12.16	285.63	0.193
	0.40	12.11	289.01	0.212
	0.80	11.91	297.46	0.267

acid. The explanation of CPS comprising of different monosaccharide may lie in the fact that there were different *C. militaris* strains. Polysaccharide structure and activity are related closely (Takada et al., 2003; Nagaoka et al., 2000) and they should be studied simultaneouslyfor better understanding and development of *C. militaris* resources.

Effect of CPS on rat hepatocyte injury

A considerable amount of data has been documented indicating that hepatocyte injury induced by ethanol is as a result of damage by free radical-mediated lipid peroxidation. In liver metabolism, ethanol induces the formation of a large amount of free radical, which causes lipid peroxidation on cell membranes, resulting in cell membrane structural damage. Free radicals can inhibit synthesis of GSH, an anti-oxidant, and also reduce activity of GSH-PX (an antioxidant enzyme). So, hepatocyte metabolic disorder, abnormal liver function and fat-like vacuolar degeneration often occur, which lead to cell swelling and death (Kanbagli et al., 2002; Farbiszewski et al., 1992; Husain and Somani, 1997). Therefore, antioxidant enzymes play role in protecting hepatocyte and restoring integrity of structure and function by removal of free radicals and inhibition of lipid peroxidation.

In this study, after ethanol stimulation, hepatocyte was injured obviously, and a large number of cells became necrotic. GSH-PX activity decreased significantly. MDA as a product of lipid peroxidation was significantly higher than the control group. Cell activity also declined based upon MTT assay. After the intervention of CPS-1 and CPS-2, hepatocyte and GSH-PX activity increased, and MDA levels decreased. This reveals that the two components can reverse ethanol-induced hepatocyte damage. Moreover, its effect appeared dose-dependent. The higher the dose was, the better the curative effect becomes (Table 1).

Its protective effect may be that CPS can increase cell activity and enhance the expression of enzymatic system relating to antioxidant, such as GSH-PX, while production of MDA was reduced accordingly. Therefore, they have the ability of stabilizing the hepatocyte membrane structure and reducing cell necrosis.

CPS-1 and CPS-2 had little difference in enhancing the GSH-PX and cell activity. However, they had significant differences in decreasing MDA, which reflected that CPS-1 has stronger anti-oxidation activity than CPS-2. The reason may lie in the fact that CPS-2 is coupled with some nucleic acids and proteins which are not in CPS-1. However, further study is necessary to reveal this mechanism.

Conclusions

The extraction rate of CPS is 0.82% using ultrasound-assisted extraction, protein removal with Sevag and enzymatic method, and dialysis. After the polysaccharide was subjected to ion-exchanged DEAE-52 chromatography, two polysaccharide components, CPS-1 and CPS-2, were obtained. Gas chromatography analysis of CPS-1 and CPS-2 acid hydrolysis derivatives revealed that the two components were both composed of two kinds of monosaccharide, mannose and the galactose. The molar ratio was 0.96:1 and 1.04:1, respectively. CPS-1 and CPS-2 can reverse ethanol-induced hepatocyte damage in a dose-dependent manner; the higher the dose the better the effect. The effect may come from its anti-oxidation function.

REFERENCES

Bin W, Li SY, Yu RM, Li B (2003). Studies on the Anti-inflammatory and Immunomodulating Actions of the Polysaccharide in the Tissue Culture of Cordyceps militaris. Lishizhen Med. Med. Res. 14(1): 1-2.
Farbiszewski R, Radecka A, Chwiecko M, Holownia A (1992). The effect

of heparegen on antioxidant enzyme activities in ethanol-induced liver injury in rats. Alcohol, 9(5):403-407.
Husain K, Somani SM (1997). Interaction of exercise training and chronic ethanol ingestion on hepatic and plasma antioxidant system in rat. J. Appl. Toxicol. 17(3): 189-194.
Kanbagli O, Balkan J, Aykaç-Toker G, Uysal M (2002). Hepatic mitochondrial prooxidant and antioxidant status in ethanol-induced liver injury in rats. Biol. Pharm. Bull. 25(11): 1482-1484.
Liu J, Yang S, Yang X, Chen Z, Li J (1997). Anticarcinogenic effect and hormonal effect of Cordyceps militaris. Zhongguo Zhong Yao Za Zhi. 22(2): 111-113.
Liu P, Zhu J, Huang Y, Liu C (1996). Influence of Cordyceps sinensis (Berk.) Sacc. and rat serum containing same medicine on IL-1, IFN and TNF produced by rat Kupffer cells. Zhongguo Zhong Yao Za Zhi. 21(6): 367-369, 384.
Nagaoka M, Shibata H, Kimura-Takagi I, Hashimoto S, Aiyama R, Ueyama S, Yokokura T (2000). Anti-ulcer effects and biological activities of polysaccharides from marine algae. Biofactors, 12(1-4): 267-274
Ohta Y, Lee JB, Hayashi K, Fujita A, Park DK, Hayashi T (2007). *In vivo* anti-influenza virus activity of an immunomodulatory acidic polysaccharide isolated from Cordyceps militaris grown on germinated soybeans. J. Agric. Food Chem. 55(25): 10194-10199.
Shi Y, Yu WM, Miu ST, Zhou YX (2006). Microwave-assisted extraction of polysaccharides from Silkworm *Cordyceps militaris*. Guangdong Agric. Sci. 11: 41-42.
Takada T, Katagiri T, Ifuku M, Morimura N, Kobayashi M, Hasegawa K, Ogamo A, Kamijo R (2003). Sulfated polysaccharides enhance the biological activities of bone morphogenetic proteins. J. Biol. Chem. 278(44): 43229-43235.
Wang J, Wang Q, Xiang WS (2001). Application of chromatoraphy to the analysis of carbohydrates. Chin J. Anal. Chem. 29(2): 222-227.
Wang ZX, Wang XM, Wang TZ (1995). Current status of pharmacological study on *Cordyceps sinensis* and *Cordyceps hyphae*. Zhongguo Zhong Xi Yi Jie He Za Zhi. 15(4): 255-256.
Wen B, Rong M, Bai XF (2000). Studies on Polysaccharide in the Tissue Cultures of *Cordyceps Militaris*. J. Shenyang Pharm. Univ. 17(5): 361-364.
Wu GH, Wang M (2007). Separation, Purification and the Immunoregulation Effect of Polysaccharides in Cultured Cordyceps militaris. Chin. J. Nat. Med. 5(1): 73-76.
Xu JT (1997). Medicinal fungus in China. Beijing: Beijing Medical University, Peking Union Medical College Joint Press, p. 394.
Yu R, Wang L, Zhang H, Zhou C, Zhao Y (2004). Isolation, purification and identification of polysaccharides from cultured *Cordyceps militaris*. Fitoterapia, 75(7-8): 662-666.
Zhang SZ (1999). Glycobiology in the new life science forward. Chem. Life. 19(3): 103-106.