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Structure and anti-tumor activity of a high-molecular-weight polysaccharide from cultured mycelium of *Cordyceps gunnii*

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ABSTRACT

Cordyceps gunnii (berk.) Berk (*C. Gunnii*) is well known as a Chinese rare caterpillar fungus and has similar pharmacological activity with *C. sinensis*. In this work, a high-molecular-weight polysaccharide (CPS) was isolated and purified from the mycelia of *C. gunnii*. The total sugar content of CPS was amounted to 92.84%. The result of HPLC indicated that CPS was a homogeneous polysaccharide. The estimated average molecular weight of CPS was 3.72×10^6 Da. The specific rotation of CPS was recorded $[\alpha]_D^{25} = +134.2°$. Its characteristic was determined by chemical analysis, gas chromatography, IR spectroscopy and NMR data. The results showed that CPS was mainly composed of glucose, and a small amount of rhamnose, arabinose, xylose, mannose and galactose with a molar ratio of Rha:Ara:Xyl:Man:Glu:Gal = 3.0:2.6:1.0:1.3:106.0:2.8. The main chain of CPS was majorly composed of α-(1 → 4) glucose. The tumor inhibition ratio on K562 cell by CPS was 56.65%.

1. Introduction

Cordyceps, one of the famous traditional Chinese medicines, has been used as health food for a long time in China. Recently, owing to its anti-tumor activity, anti-inflammatory activity, anti-aging effect and improving immunity effect, *Cordyceps* has attracted much attention (Wang, Yu, & Yuan, 2004). *Cordyceps gunnii (berk.)* Berk (*C. gunnii*), is also well known as the Chinese rare caterpillar fungus and has similar pharmacological activity with *C. sinensis*. The anamorph of *Paecilomyces gunnii* of *C. gunnii* has been isolated, verified and identified (Liang, 1985). Many important secondary metabolic products were found in *C. gunnii* mycelia including cordycepin, cordycepic acid, polysaccharide and anti-ultraviolet radiation constituents (Huang, Liang, & Liu, 1992). Polysaccharides have been reported to account for the anti-tumor, anti-inflammatory, antioxidant, steroidogenic, hypolipidemic and immunomodulatory effects. Many polysaccharides and polysaccharide–protein complexes, isolated from fungi, have attracted much attention recently in the biochemical and medical areas due to their anti-cancer effects. At present, although many studies on polysaccharides from entomogenous fungi such as *C. sinensis* (Leung, Zhao, Ho, & Wu, 2009; Liu, Zhong, Zhu, & Zhu, 2008; Liu, Leung, & Wu, 2008; Wu, Sun, & Pan, 2006; Zhang, Li, Qiu, Chen, & Zheng, 2008) and *C. militaris* (Hou et al., 2008; Kim et al., 2008; Wang, Wei, & Zhang, 2003; Wu, Hu, Pan, Zhou, & Zhou, 2007; Yu et al., 2007) have been reported, the anti-tumor activities of the polysaccharides from mycelium of *C. gunnii* have been scarcely studied.

In our previous work, a low-molecular-weight polysaccharide was isolated and purified from the mycelia of *C. gunnii* (Liu, Zhong, et al., 2008; Liu, Leung, et al., 2008; Zhu, Si, et al., 2011). In this paper, a novel high-molecular-weight polysaccharide (CPS) was described. The structure of CPS was characterized and its anti-tumor activity was confirmed by using human K562 cells.

2. Materials and methods

2.1. Materials

The *C. gunnii* mycelium and K562 cell were obtained from the Key Laboratory of Food Nutrition and Safety, Ministry of Education, College of Food Science and Biotechnology, Tianjin University of Science and Technology, Tianjin, China.

The standard monosaccharides (D-glucose, D-xylose, D-galactose, L-rhamnose, D-mannose, and D-arabinose), DEAE-Sephadex A-25 and Sephadex G-100 were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

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2.2. Extraction and purification of polysaccharides

The *C. gunnii* mycelium was extracted three times by distilled water at 80 °C for 2 h. The supernatant was mixed with 1 volume of EtOH to obtain the crude polysaccharide (Liu, Zhong, et al., 2008; Liu, Leung, et al., 2008; Zhu, Si, et al., 2011). The crude polysaccharide was subjected to the Sevag method three times in order to remove the protein. The obtained polysaccharide was then decolorized by AB-8 resin and purified by DEAE-Sephadex A-25 and Sephadex G-100 (30 cm × 3 cm) with distilled water. Each fraction showed only one main peak, and it was collected and freeze-dried. Each fraction was determined by using a HPLC (Agilent-1200) equipped with a TSKgel G4000 PWxl column (7.8 mm × 300 mm, column temperature 30 °C) and Refractive Index Detector (RID, detecting temperature 40 °C). The purified polysaccharide was named CPS.

2.3. Determination of molecular weight by HPLC

The molecular weights of CPS were determined by using a HPLC, which was described above. A sample solution (20 μL) was injected and run with purified water at 0.6 mL/min as mobile phase. The standard curve was established using T-series Dextran as the standards (T-10, T-40, T-70, T-500 and T-2000) (Zhu, Liu, et al., 2011).

2.4. IR analysis

1 mg of CPS was mixed with 150 mg of dried KBr, and pressed into disk for the analysis. The IR spectrum was recorded in the range of 400–4000 cm^{-1} on a Fourier transformed IR spectrophotometer (VECTOR-22).

2.5. NMR spectroscopy

^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker spectrometer (600 MHz) at a probe temperature of 298 K. Prior to analysis, sample was exchanged twice with D_2O upon freeze-drying.

2.6. Monosaccharide analysis

Dried CPS sample (10.0 mg) was hydrolyzed for 6 h at 100 °C with 4 mL of TFA (2 M). The soluble fraction was evaporated to dryness under stream of nitrogen. The product was acetylated with Ac_2O-Pyridine (1:1, v/v) at 100 °C for 1 h. The sample was ready for GC analysis. D-Glucose, D-xylose, D-galactose, L-rhamnose, D-mannose, and D-arabinose were also derivatized as standard.

2.7. Periodate oxidation and smith degradation analysis

10.0 mg of CPS sample was dissolved in 0.015 M sodium metaperiodate (25 mL) and kept in the dark, with the absorption at 223 nm monitored every 8 h. The reaction was completed after 56 h and ethylene glycol (0.2 mL) was added to the solution to decompose the excess of the reagent. Consumption of $NaIO_4$ was measured by a spectrophotometric method (Dixon & Lipkin, 1954) and the production of formic acid was determined by titration with 0.01 M NaOH. The reaction mixture was dialyzed against distilled water, and the nondialysate was reduced with $NaBH_4$ (25 mg) for 12 h. The pH was adjusted to 5.0, the solution was dialyzed, and the nondialysate was lyophilized, and then hydrolyzed with 2 M TFA at 110 °C for 4 h. The hydrolysate was analyzed by GC.

2.8. Methylation analysis

The methylation analysis was performed based on the Ciucanu method (Ciucanu & Kerek, 1984). The sample was treated with 4 mL of 90% formic acid for 6 h at 100 °C, then the residue was hydrolyzed using 3 mL of 2 M TFA for 4 h at 110 °C. After removal of formic acid, the hydrolysate was concentrated to dryness. The methylated monosaccharides were converted into their corresponding alditol acetates by reduction with $NaBH_4$ at room temperature for 6 h. The reduced polysaccharide was acetylated with acetic anhydride, and dissolved in chloroform and ready for GC–MS analysis.

2.9. Cell lines

The K562 cell line was maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 100 mg/L streptomycin, and 100 mU/L penicillin at 37 °C in a humidified atmosphere of 5% CO_2.

2.10. In vitro anti-tumor activity against K562 cell

The anti tumor activity assays of K562 tumor cells was evaluated *in vitro* using MTT assay. The K562 cells were seeded at a concentration of 1×10^5 cell/mL in a volume of 0.1 mL in 96-well plates. Cells were incubated with the CPS samples at concentrations of 25, 50, 100, 200 and 400 μg/mL. After 20 h, 44 h, 68 h, each well was added 20 μL of 5 mg/mL of MTT and incubated for another 4 h. Then the culture media were removed, 150 μL of DMSO was added to each well. Absorbance at 490 nm was detected by microplate ELISA reader. The inhibition ratio of K562 cell proliferation was calculated as follows:

$$\Phi = \frac{(\mathrm{ODa} - \mathrm{ODb})}{\mathrm{ODa}} \times 100\%$$

where ODa is the absorbance value of negative control group, and ODb is that of sample group (Liu, Song, Yang, Liu, & Zhang, 2007; Liu, Lin, Gao, Ye, & Xi, 2007).

2.11. Statistical analysis

Data were expressed as means ± SD. Data in all the bioassays were statistically evaluated by analysis of variance and $P < 0.05$ was considered significant.

3. Results and discussion

3.1. Structural analysis

The yield of crude polysaccharide from *C. gunnii* mycelium was 12.96%. The crude polysaccharides were purified by AB-8 resin, DEAE-A25 and Sephadex G-100, each showing a main peak (Fig. 1), as detected by the phenol–sulfuric acid assay and HPLC (Fig. 2). The total carbohydrate content was determined based on the phenol–sulfuric acid method as D-glucose equivalents, and the total sugar contents of CPS was 92.84%. The result of HPLC (Fig. 2D) indicated that CPS was a homogeneous polysaccharide. The estimated average molecular weight of CPS was 3.72×10^6 Da. The specific rotation of CPS was recorded $[\alpha]_D^{25} = +134.2°$.

The IR spectrum of CPS (Fig. 3) exhibited a strong band at 3385 cm^{-1}, attributing to the hydroxyl stretching vibration. The band at 2925 cm^{-1} was due to C—H stretching vibration and the band at 1417 cm^{-1} was assigned to C—H bending vibration. The three bands above are characteristic absorption peaks of polysaccharide. The three bands at 1024–1154 cm^{-1} indicated pyranose. The band at 850 cm^{-1} showed α-polysaccharides (Barker, Bourne, Stacey, & Whiffen, 1954).

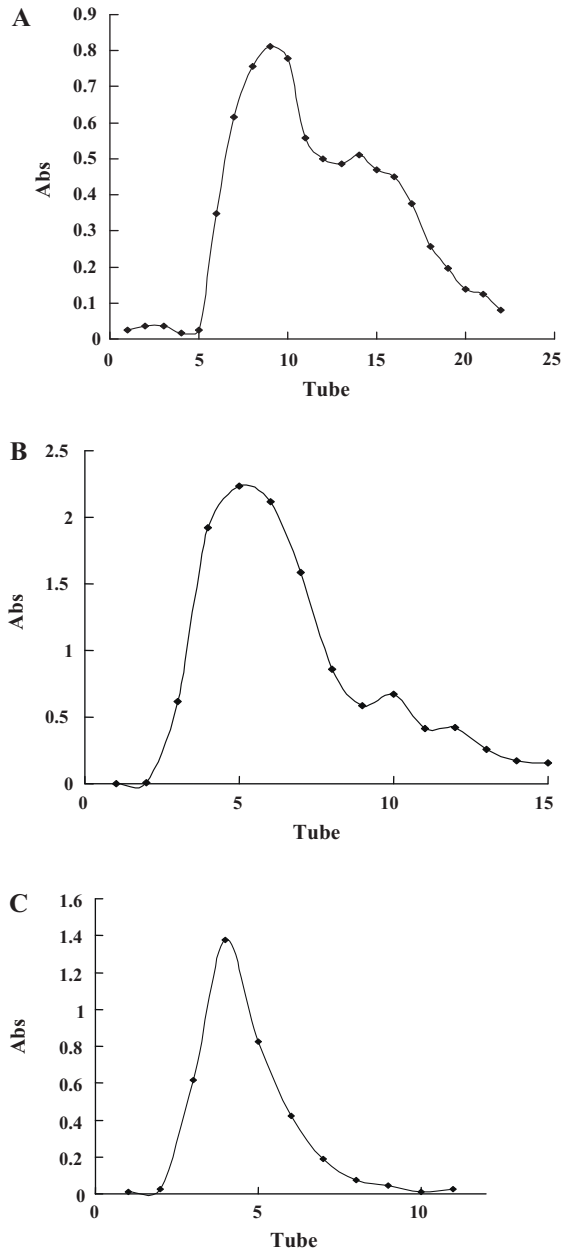

Fig. 1. Elution profiles of CPS on AB-8 (A), CPS on DEAE-Sephadex A-25 (B), and CPS on Sephadex G-100 (C).

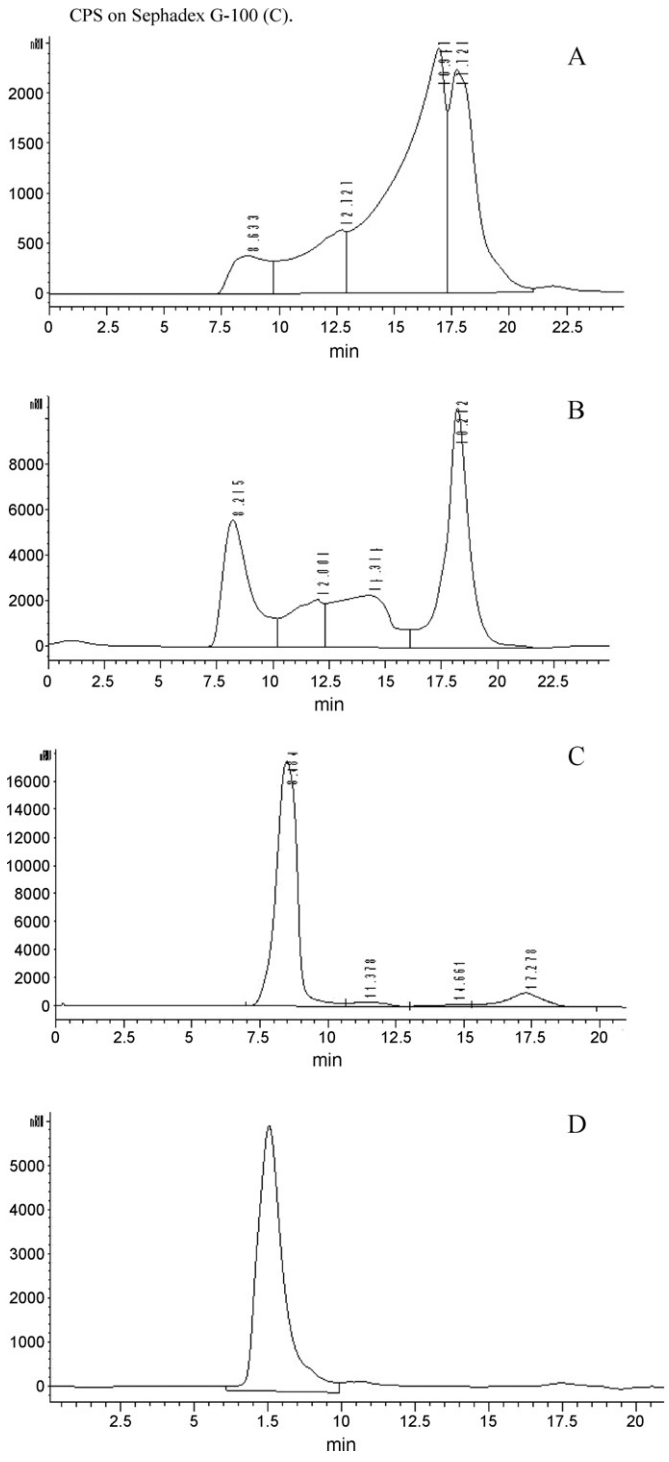

Fig. 2. HPLC profiles of crude polysaccharide (A), purified by AB-8 (B), purified by DEAE-A25 (C), CPS (D). The results supported the conclusion that CPS was homogeneous polysaccharides.

The ^{1}H NMR spectrum (Fig. 4a) of CPS showed one anomeric H at δ 5.29 which indicated that the CPS was mainly composed of one type of sugar. In addition, the ^{13}C NMR spectrum (Fig. 4b) also showed one anomeric C at δ 99.76, which confirmed that the CPS was mainly composed of one type of sugar.

The Monosaccharide analysis showed that the CPS mainly contained glucose (Fig. 5a), Table 1 gave the monosaccharides composition and the molar ratio of CPS, Rha:Ara:Xyl:Man:Glu:Gal = 3.0:2.6:1.0:1.3:106.0:2.8.

The results of periodate oxidation demonstrated that the consumption of $NaIO_4$ was 0.153 mmol and no formic acid for CPS.

Table 1
Monosaccharides composition and molar ratio of CPS.

Name	Rha	Ara	Xyl	Man	Glu	Gal
Molar ratio	3.0	2.6	1.0	1.3	106.0	2.8

The GC analysis of the Smith degradation of the periodate-oxidized CPS showed that it mainly contained erythritol and little glycerol and arabinose (Fig. 5b). The molar ratio of glycerol, erythritol and arabinose was 2.29:15.66:1.00. The periodate oxidation and smith degradation analysis indicated that the linear chain of CPS was mainly composed of (1 → 4) glucose, small amount of (1 → 2) linked monosaccharides, (1 → 3) arabinose and no (1 → 6) linked saccharides were detected.

The fully methylated CPS was hydrolyzed with acid, converted into alditol acetates, and analyzed by GC–MS (Table 2). GC–MS was

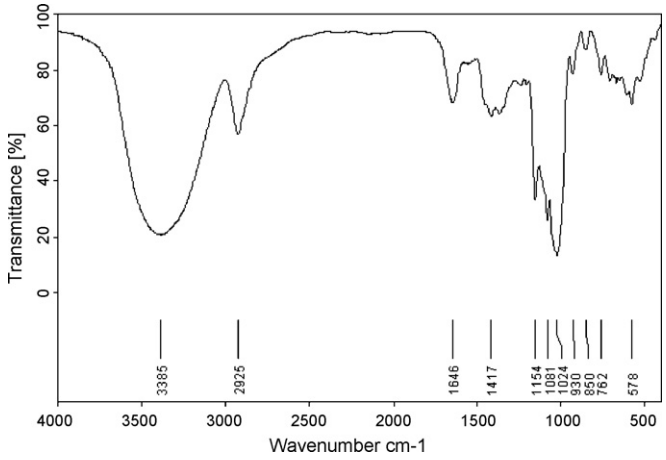

Fig. 3. IR spectrum of CPS.

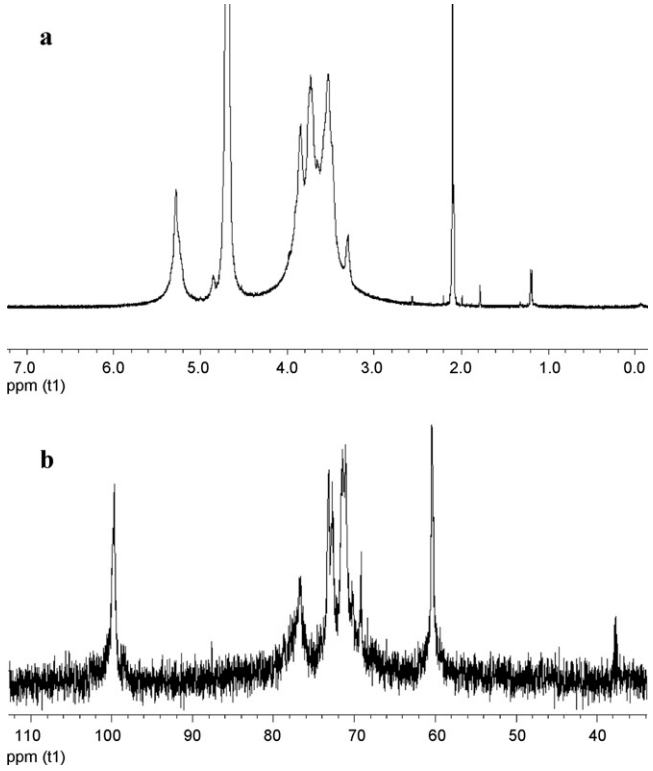

Fig. 4. ^{1}H NMR spectra (a) and ^{13}C NMR spectra (b) of CPS.

performed to indicate the presence of five components, namely 2,3,6-tri-*o*-methyl-glucose, 6-*o*-methy-glucose, 2,4-di-*o*-methyl-arabinose, 3,4-di-*o*-methyl-ribose and 1,4,6-tri-*o*-methy-mannose.

Table 2
Results of the methylation analysis of CPS.

Methylation positions	Linkages	Major mass fragments (*m*/*z*)
2,3,6-tri-*o*-methyl-glucose	1,4-Linked Glc	43, 45, 87, 99, 101, 113, 117, 233
6-*o*-methy-glucose	1,2,3,4-Linked Glc	43, 101, 117
2,4-di-*o*-methyl-arabinose	1,3-Linked Ara	43, 89, 101, 117, 131, 159, 173, 233
3,4-di-*o*-methyl-ribose	1,2-Linked Rib	43, 89, 101, 117, 189
1,4,6-tri-*o*-methy-mannose	1,2,3-Linked Man	43, 87, 101, 117

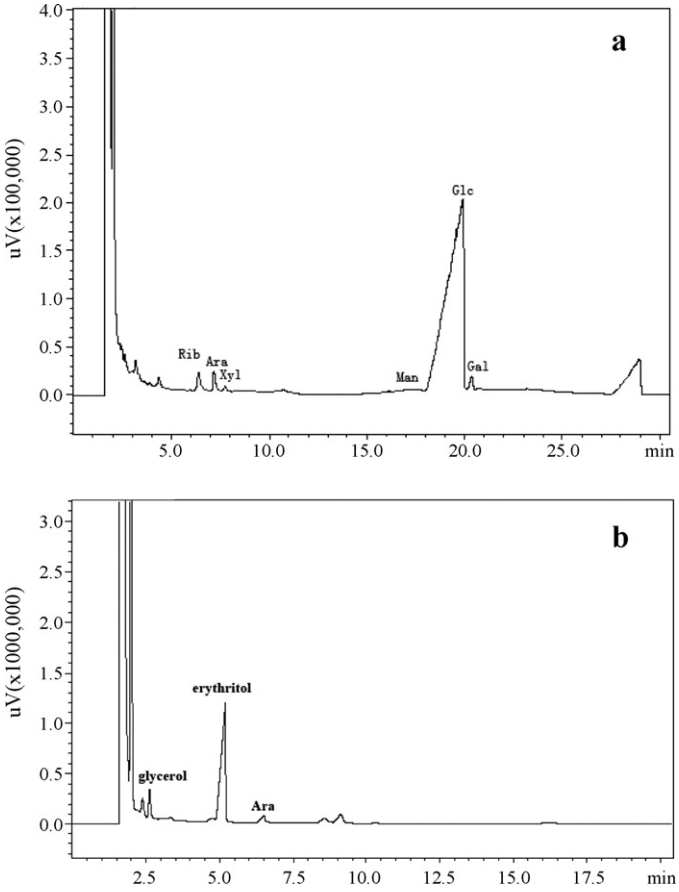

Fig. 5. GC profiles of monosaccharides of CPS (a) and GC analysis of the Smith degradation of the periodate-oxidized CPS (b).

3.2. Anti-tumor activity of CPS

In this work, K562 cells were cultured with different concentrations of CPS for different time. Fig. 6 demonstrated anti-tumor activities of CPS which were determined by inhibition ratio, when K562 cells were subjected to coculture with different concentrations of CPS. At 24 h coculture on K562, the CPS exhibited a relatively lower inhibition ratio, about <10% at the concentrations from 50 to 400 μg/mL. At 48 h, the activity had significant difference; the inhibition ratio reached 43.67% at 50 μg/mL of CPS. Between CPS concentration at 50 and 200 μg/mL, the inhibition ratios on K562 cells ranged from 43.67% to 32.97%. In addition, when CPS concentration increased from 200 to 400 μg/mL, the inhibition on K562 cells significantly enhanced from 32.97% to 56.65%. At 72 h, the inhibition ratio on K562 cell was reduced compared with the inhibition ratio at 48 h.

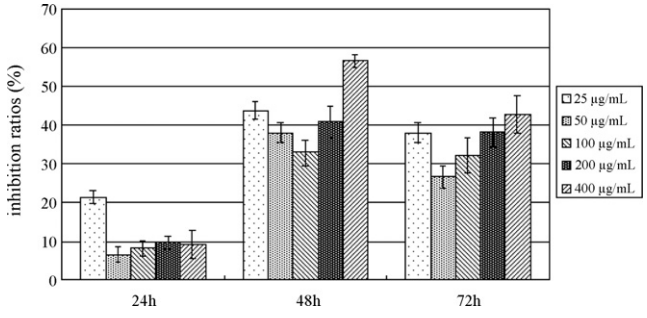

Fig. 6. The anti tumor activity of CPS.

4. Conclusions

The results of our study showed that the average molecular weight of the polysaccharide from *C. gunnii* mycelium was 3.72×10^7 Da. The CPS was D-glucan containing α-(1 → 4)-linked backbone. Preliminary biological tests suggested that CPS significantly inhibit the growth of K562 cell *in vitro*.

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