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Characterization and anti-tumor activity of a polysaccharide from *Hedysarum polybotrys* Hand.-Mazz

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Abstract

A water-soluble polysaccharide named as HPS-1 was isolated from the roots of *Hedysarum polybotrys* Hand.-Mazz by hot water extraction, anion-exchange and gel-permeation chromatography and tested for its anti-tumor activity. Its structural characteristics were investigated by FTIR, HPLC, NMR spectroscopy, GLC–MS, methylation analysis, periodate oxidation and Smith degradation. Based on the data obtained, HPS-1 was found to be an α-(1 → 4)-D-glucan, with a single α-D-glucose at the C-6 position every nine residue, on average, along the main chain. The glucan has a weight-average molecular weight of about 9.4×10^4 Da. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay revealed that HPS-1 significantly inhibited the proliferation of human hepatocellular carcinoma HEP-G2 cells and human gastric cancer MGC-803 cells in vitro, indicating HPS-1 could have a possible cancer therapeutic potential.

Keywords: *Hedysarum polybotrys* Hand.-Mazz; D-Glucan; Structure; Anti-tumor

1. Introduction

In recent years, as more and more polysaccharides have been reported to exhibit a variety of biological activities, including anti-tumor (Wasser, 2002), immunostimulation (Wasser, 2002; Yamada, 1994), anti-oxidation (Li et al., 2003; Liu, Ooi, & Chang, 1997), etc., the nonstarch polysaccharides have emerged as an important class of bioactive natural products. In many oriental countries, several immunoceuticals composed of polysaccharides have been accepted such as lentinan, schizophyllan and krestin (Borchers, Stern, Hackman, Keen, & Gershwin, 1999; Liu, Ooi, & Fung, 1999).

Hedysarum polybotrys Hand.-Mazz (HP) has a long history of use in the treatment of various diseases in China. The previous study showed that the crude polysaccharide from HP displayed anti-cancer, anti-aging, anti-oxidation, hypoglycemic and immunological activities (Cui et al., 1998; Huang et al., 1992; Jin et al., 2004; Lan, Zhang, Cheng, Wang, & Xi, 1987; Song, Kobayashi, & Xiu, 2000; Wang, Ito, & Shimura, 1989; Zhou, Jin, Zhang, & Li, 2006). However, to our knowledge, few studies on the structural features and linkage composition of polysaccharide from HP have been undertaken. Therefore, the present paper was concerned with the isolation, chemical characterization and evaluation of the anti-tumor activity of a glucan from HP.

2. Materials and methods

2.1. Materials

The roots of *H. polybotrys* Hand.-Mazz (HP) are commercially available in Lanzhou, China and identified

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by Professor Zhao R.N. in the Department of Pharmacy, Lanzhou University (Lanzhou, China), by comparison with a voucher specimen collected in the herbarium in the Department of Pharmacy, Lanzhou University. The coarse powder of the roots was air-dried in the shade and stored in a sealed vessel prior to use.

T-series Dextran, DEAE-Sephadex A-25 and Sephadex G-200 were purchased from Amersham biosciences (Uppsala, Sweden). Trifluoroacetic acid (TFA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA). Roswell Park Memorial Institute (RPMI)-1640 medium, phosphate-buffered saline (PBS) and fetal bovine serum were purchased from Gibco (Grand Island, NY, USA). All other chemical reagents were of analytical reagent grade.

2.2. General methods

The specific rotation was determined at 20 ± 1 °C with an automatic polarimeter (Model WZZ-2B, China). UV–Vis absorption spectra were recorded with a Shimadzu MPS-2000 spectrophotometer. The FTIR spectra (KBr pellets) were recorded on a Nicolet 360 FTIR spectrophotometer. Elemental analysis (C, H and N) was conducted on an Elementar Vario EL III instrument. Total carbohydrate content was determined by the phenol–sulfuric acid method as D-glucose equivalents (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Uronic acid content was determined according to an *m*-hydroxydiphenyl colorimetric method in which neutral sugars do not interfere (Filisetti-Cozzi & Carpita, 1991). Protein was analyzed by the method of Bradford (1976).

2.3. Extraction and fractionation of polysaccharide

The powdered roots (4.0 kg) of HP were firstly refluxed with methanol to remove lipophilic compounds, and then successively boiled in distilled water for 4 h at 100 °C. After filtration to remove debris fragments, the filtrate was concentrated in a rotary evaporator. Protein was removed with the sevag method (Alam & Gupta, 1986). The crude polysaccharide fraction (26.8 g) was obtained through precipitation with 3 volumes of ethanol and desiccation in vacuo. The precipitate was redissolved in distilled water and applied to a DEAE-Sephadex A-25 column (90 cm × 5 cm). The column was first eluted with distilled water followed by 0.3 M and then 0.5 M NaCl. The fractions obtained were combined according to the total carbohydrate content quantified by the phenol–sulfuric acid method. The main peak was further fractionated on a Sephadex G-200 column (100 cm × 5 cm) eluted with 0.1 M NaCl to yield three completely separated fractions. The main fraction was collected, dialyzed and lyophilized to get a white purified hedysari polysaccharide (HPS-1, 671 mg, 2.5% of the crude polysaccharide).

2.4. Homogeneity and molecular weight

The homogeneity and molecular weight of HPS-1 were determined on a Waters HPLC system (717 plus autosampler and 600 delta HPLC pump) equipped with a TSKgel 4000 PWXL column (7.8 mm× 300 mm) and a Waters 2414 Refractive Index Detector (RID). A sample solution (20 μl of 0.5%) was injected into each run, with 0.05 mol/L NaCl as the mobile phase at 0.8 ml/min. The HPLC system was precalibrated with T-series Dextran standards (T-10, T-40, T-70 and T-500).

2.5. Monosaccharide analysis

The monosaccharide was analyzed by gas chromatography (GC; GC-9A Shimadzu, Japan) (Li, Rong, & Wu, 2003). The HPS-1 was hydrolyzed by trifluoroacetic acid to monosaccharide. Monosaccharide was derivatized to acetylated aldononitriles. Xylose, glucose, rhamnose, mannose and galactose were also derivatized as standard. Acetyl inositol was used as the internal standard. Gas chromatography was used with a capillary column (OV-225, China) and detected with a flame ionization detector (FID). N2 was used as the carrier gas (40 ml/min). The injector temperature was kept at 250 °C (split injection 70:1), and the detector temperature was maintained at 235 °C. The GC station software was Zhida N2000 (Zhida, China).

2.6. Methylation analysis

The methylation analysis of HPS-1 (10.0 mg) was performed by the Hakomori method (Hakomori, 1964). The methylated polysaccharide was treated with 90% formic acid (3 ml) for 10 h at 100 °C in a sealed tube. After removal of the formic acid, the residues were heated with 2 M trifluoroacetic acid (2 ml) under the same conditions and the hydrolysate was concentrated to dryness. The methylated sugars were reduced with $NaBH_4$, acetylated with acetic anhydride and analyzed as the alditol acetates by GLC. The identification of the methylated sugars was analyzed by GLC–MS.

2.7. Periodate oxidation and smith degradation

The polysaccharide HPS-1 (10.0 mg) was dissolved in 0.015 M sodium metaperiodate (30 ml) and kept in the dark at 4 °C, the absorption at 223 nm being monitored every day. The reaction was completed after 120 h and ethylene glycol (0.2 ml) was added to the solution with stirring for 30 min to decompose the excess of the reagent. Consumption of $NaIO_4$ was measured by a spectrophotometric method (Chaplin & Kennedy, 1994; Dixon & Lipkin, 1954) and HCOOH production 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, 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 2 h. The hydrolysate was analyzed by GLC.

2.8. Partial hydrolysis

HPS-1 was partially hydrolyzed with a solution adjusted to pH 2.0 (20 ml) with aq. trifluoroacetic acid, at 100 °C, for 18 h. After neutralization with NaOH, a polymeric product (HPS-1-p) was obtained by precipitation with excess EtOH from a small volume of water, and then retained on dialysis with a M_r 2 kDa cut-off membrane. The periodate oxidation and Smith degradation of the precipitate fractions were followed using the same procedure as mentioned above.

2.9. Nuclear magnetic resonance spectroscopy

The freeze-dried polysaccharide was kept over P_2O_5 in vacuum for several days and dissolved in 99.96% D_2O. ^1H and ^{13}C NMR spectra were recorded with a Bruker AM 400 MHz spectrometer (operating frequencies 400.17 MHz for ^1H NMR and 100.62 MHz for ^{13}C NMR) at 30 °C. Chemical shifts were reported relative to DSS as the internal standard.

2.10. Cell lines

Human hepatocellular carcinoma HEP-G2 cells and human gastric cancer MGC-803 cells were obtained from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Science. HEP-G2 and MGC-803 cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 mg/L) (Hsu, Kuo, & Lin, 2004; Tomatsu, Ohnishi-Kameyama, & Shibamoto, 2003) in a humidified 5% CO_2 atmosphere at 37 °C.

2.11. Cell proliferation assay

The proliferation of MGC-803 and HEP-G2 cells was determined using the colorimetric MTT assay as described previously (Mosmann, 1983). Briefly, cells were seeded at a density of 3×10^3 cells/well in a 100 μl volume of the medium in 96-well plates and allowed to attach for 24 h. The dosages of HPS-1 on the selected cell lines were in the range of 50–400 μg/ml while the negative controls were treated with the medium only. MTT (5 g/L) 20 μl was added 48 h later. After incubation at 37 °C for 4 h, the supernatant was aspirated, and 150 μl DMSO was added to each well. Absorbance was measured at 570 nm by a 96-well microplate reader (Mode 680, Bio-Rad, Tokyo, Japan). The percent viability of the treated cells was calculated as follows: (A_{570} of treated cells/A_{570} of untreated cells) × 100%.

2.12. Statistical analysis

Data were expressed as means ± SD. Data in all the bioassays were statistically evaluated by Student's t test and $P < 0.05$ was considered significant.

3. Results and discussion

3.1. Isolation and structural analysis

The yield of the crude water-soluble polysaccharide from the roots of HP was 0.67% of the fresh material. The crude polysaccharide was separated and sequentially purified through DEAE-Sephadex A-25 and Sephadex G-200, each giving a single elution peak, as detected by the phenol–sulfuric acid assay. The main fraction (HPS-1) was collected for subsequent analyses.

HPS-1 appeared as a white powder, $[\alpha]_D^{16}$ +192° (*c* 1.0, H_2O). It had a negative response to the Bradford test and no absorption at 280 or 260 nm in the UV spectrum, indicating the absence of protein and nucleic acid. Elemental analysis found it to be free of nitrogen, indicating it was a neutral polysaccharide. In Fig. 1, the GPC profile showed a single and symmetrically sharp peak, indicating that HPS-1 was a homogeneous polysaccharide, with a weight-average molecular weight of 9.4×10^4 Da. The total sugar content of HPS-1 was determined to be 96.60%. As determined by *m*-hydroxydiphenyl colorimetric method and GC, the polysaccharide did not contain uronic acid. HPS-1 was composed of only glucose monomers, as detected by GLC of the alditol acetate derivatives of the components of the HPS-1 hydrolysate. The relatively high positive value of optical rotation (+192°) suggested the dominating presence of α-form glycosidic linkages in HPS-1 (Zhao, Kan, Li, & Chen, 2005).

In Fig. 2, the FTIR spectrum of HPS-1 showed a strong band at 3420.19 cm^{-1} attributed to the hydroxyl stretching vibration of the polysaccharide. The band at 2930.83 cm^{-1} was due to C—H stretching vibration. The broad band at

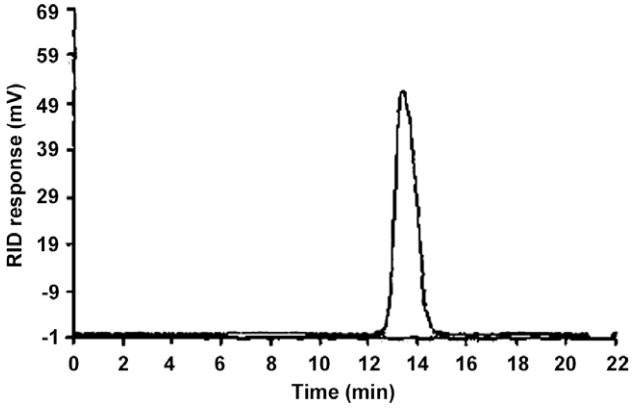

Fig. 1. Profile of HPS-1 in HPGPC. The GPC profile showed a single and symmetrically sharp peak, indicating that HPS-1 was a homogeneous polysaccharide, with a weight-average molecular weight of 9.4×10^4 Da.

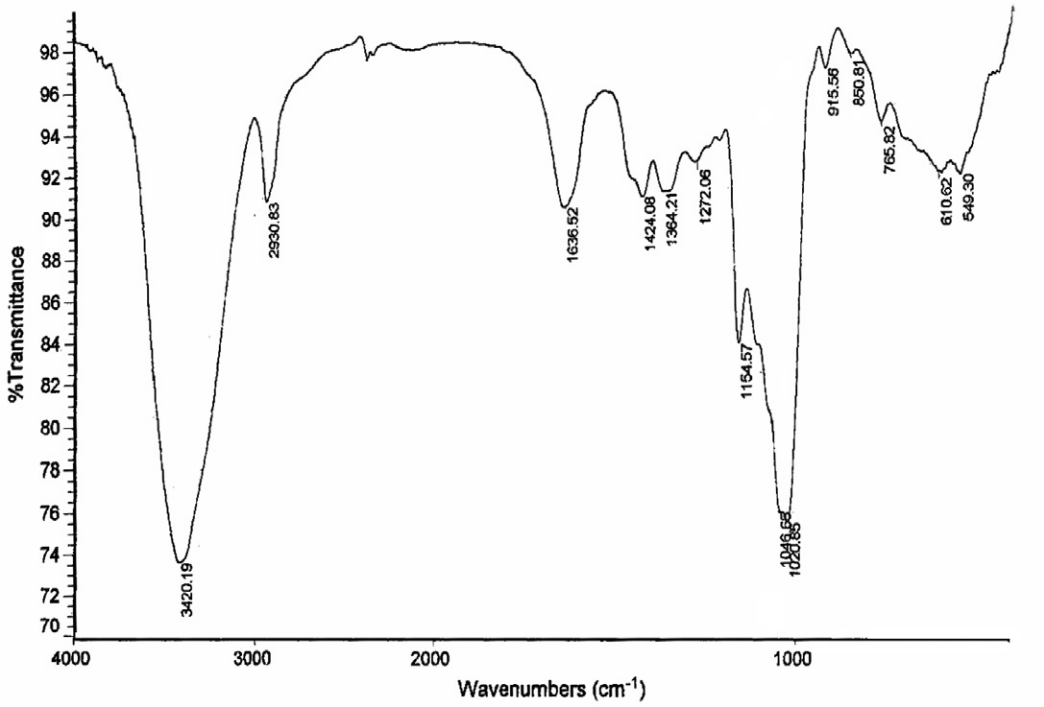

Fig. 2. FTIR spectrum of HPS-1. The FTIR spectrum of HPS-1 showed a strong band at 3420.19 cm^{-1} attributed to the hydroxyl stretching vibration of the polysaccharide. The band at 2930.83 cm^{-1} was due to C—H stretching vibration. The broad band at 1636.52 cm^{-1} was due to the bound water. The band at 850.81 cm^{-1} was ascribed to α-type glycosidic linkages in the polysaccharide. The bands at 850.81 and 915.56 cm^{-1} were characteristic of (1 → 4)-α-glucan. The absorptions at 1020.85, 1046.68 and 1154.57 cm^{-1} also indicated α-pyranose form of the glucosyl residue.

1636.52 cm^{-1} was due to the bound water (Park, 1971). The band at 850.81 cm^{-1} was ascribed to α-type glycosidic linkages in the polysaccharide (Barker, Bourne, Stacey, & Whiffen, 1954). The bands at 850.81 and 915.56 cm^{-1} were characteristic of (1 → 4)-α-glucan. The IR spectrum, together with the high positive value of the specific rotation indicated the presence of α-glycosidic linkages in the HPS-1 (Bao, Duan, Fang, & Fang, 2001; Tsumuraya & Misaki, 1979). The absorptions at 1020.85, 1046.68 and 1154.57 cm^{-1} also indicated α-pyranose form of the glucosyl residue.

Periodate oxidation of HPS-1 resulted in the values of 1.04 mol periodate consumed and 0.10 mol formic acid produced per sugar residue. After further Smith degradation of the periodate-oxidized HPS-1, the glycerol and erythritol were found with molar ratio 1.1:8.5 by GLC after conversion to the corresponding alditol acetates. It was thus deduced that 1 →, (1 → 6)- and (1 → 4,6)-amounted to 11.5%, with (1 → 4)-linked glycosyl bonds amounting to 88.5%, respectively. HPS-1 was partially hydrolyzed with 0.3 M TFA. After periodate oxidation and Smith degradation, only erythritol was found, indicating HPS-1 was a polysaccharide with a (1 → 4)-linked backbone.

The fully methylated product of HPS-1 was hydrolyzed with acid, converted into alditol acetates and analyzed by GLC and GLC–MS (Table 1). HSP-1 furnished three types of glucose derivatives in a relative molar ratio of 1.0:8.9:0.9 according to the peak areas. The overall results suggested that the polysaccharide HPS-1 was a glucan with a (1 → 4)-linked backbone and (1 → 6)-linked branches. This was also in accordance with the mode of linkage of glucose present in the polysaccharide by periodate oxidation and Smith degradation.

The ^{1}H NMR spectrum of HPS-1 showed two anomeric protons at δ 5.33 and 4.89, which were assigned as (1 → 4)-α-D-Glcp and (1 → 6)-α-D-Glcp, respectively. This confirmed that the sugar residues were linked α-glycosidically, which is consistent with presence of an IR band

Table 1
GC and GC–MS data of methylated HPS-1

Component	T_R[a]	Molar ratio	MS (*m*/*z*)	Linkage
1,5-di-acetyl-2,3,4,6-tetra-*O*-Me-Glc	1.0	1.0	43,45,71,87,101,117,129,145,161,205	Glcp-(1 →
1,4,5-tri-acetyl-2,3,6-tri-*O*-Me-Glc	2.6	8.9	43,45,87,99,101,113,117,233	→ 4)-Glcp-(1 →
1,4,5,6-tetra-acetyl-2,3-di-*O*-Me-Glc	4.3	0.9	43,101,117,127,261	→ 4,6)-Glcp-(1 →

[a] Retention time of alditol acetate relative to 1,5-di-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.

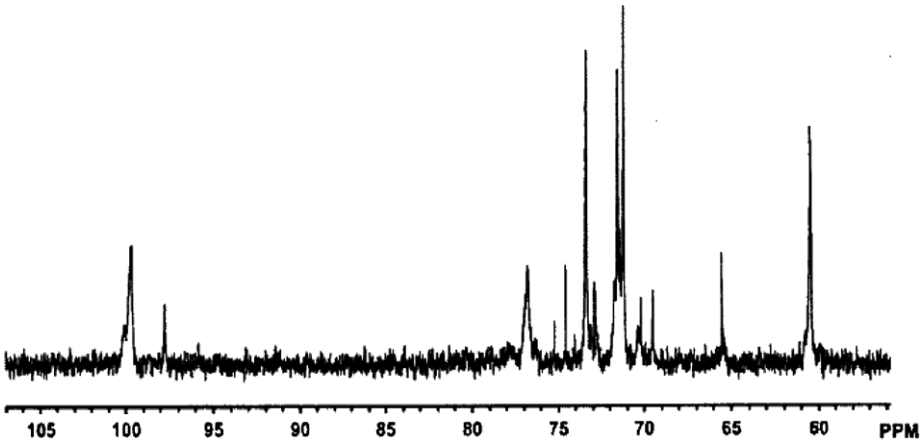

Fig. 3. ^{13}C NMR spectrum of HPS-1. The α-configuration of the D-glucosyl groups was clearly confirmed by the presence of two anomeric peaks in the regions δ 99.9 and 97.8 ppm from ^{13}C NMR experiments.

Table 2
^{1}H and ^{13}C NMR chemical shifts of polysaccharide HPS-1 in D_2O

Residue	δ $^{13}C/^{1}H$ (ppm)[a]					
	1	2	3	4	5	6
(1 → 4)-α-D-Glcp	99.9	71.6	73.4	76.9	71.3	60.7
	5.33	3.54	3.87	3.56	3.74	3.70
(1 → 6)-α-D-Glcp	97.8	71.8	73.0	69.6	70.3	65.7
	4.89	3.48	3.63	3.43	3.84	3.64

[a] In ppm downfield relative to the signal for DSS.

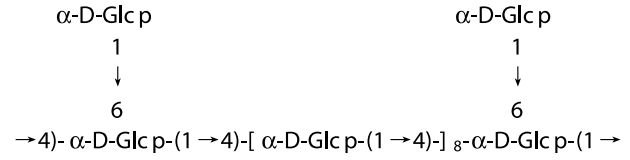

Fig. 4. Predicted structure of polysaccharide HPS-1. The results of the present investigation showed that the polysaccharide of HPS-1 was a D-glucan containing α-(1 → 4)-linked backbone, branched α-(1 → 6)-linkage.

850.81 cm^{-1}. The chemical shifts from 3.4 to 4.0 ppm were assigned to protons of carbons C-2 to C-6 of glycodidic ring (Chauveau, Talaga, Wieruszeski, Strecker, & Chavant, 1996). The α-configuration of the D-glucosyl groups was clearly confirmed by the presence of two anomeric peaks in the regions δ 99.9 and 97.8 ppm from ^{13}C NMR experiments (Fig. 3). The ^{1}H and ^{13}C NMR chemical shifts of HPS-1 are shown in Table 2.

On the basis of the above-mentioned results, it can be concluded that HPS-1 is composed of a repeating unit having the possible structure as shown in Fig. 4.

3.2. Anti-tumor activity of HPS-1

The anti-tumor activity of the polysaccharide was usually believed to be a consequence of the stimulation of the cell-mediated immune response (Ooi & Liu, 2000). For instance, immunostimulatory activities were found in the polysaccharides from *Panax ginseng*, *Ganoderma lucidum*, *Coriolus versicolor*, etc., which suggested that immunostimulatory effects might be the main mechanism for

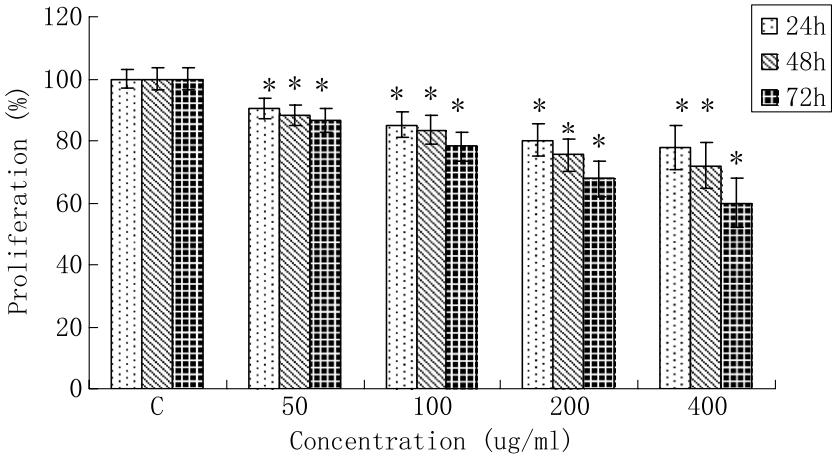

Fig. 5. Effect of HPS-1 on HEP-G2 cell growth. At the concentrations from 50 to 400 μg/ml, HPS-1 significantly inhibited the proliferation of HEP-G2 cells ($^{*}P < 0.05$) and the effects were in a concentration-dependent manner.

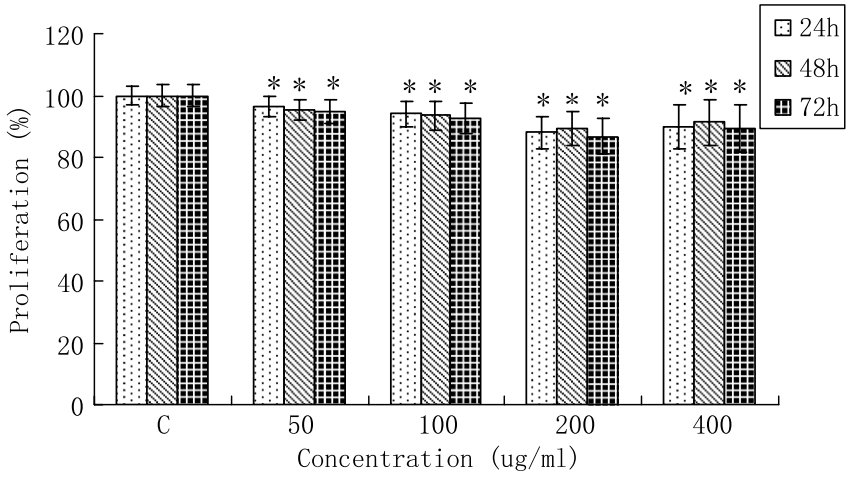

Fig. 6. Effect of HPS-1 on MGC-803 cell growth. HPS-1 had significant suppressing activity on MGC-803 cells ($^{*}P < 0.05$), there was no clear relationship between the concentrations and the effects.

the anti-tumor activities of polysaccharides (Cao & Lin, 2004; Ho, Konerding, Gaumann, Groth, & Liu, 2004; Shin et al., 2002). But some polysaccharides, such as polysaccharides from *Phellinus linteus* (Li et al., 2004) and *Cordyceps sinensis* (Chen, Shiao, Lee, & Wang, 1997), could directly inhibit the proliferation of cancer cell in vitro. In this study, we investigated the anti-tumor activities of HPS-1 against two kinds of human solid cancer cell lines, MGC-803 and HEP-G2 in vitro. Figs. 5 and 6 showed the effects of the polysaccharide on the growth of HEP-G2 and MGC-803 cells. At concentrations from 50 to 400 μg/ml, HPS-1 significantly inhibited the proliferation of HEP-G2 cells ($P < 0.05$) and the effects were in a concentration-dependent manner. At the highest concentration of 400 μg/ml, HPS-1 had the inhibition ratio of 40.0%. However, although HPS-1 also had significant suppressing activity on MGC-803 cells ($P < 0.05$), there was no clear relationship between the concentrations and the effects. The highest inhibition ratio on MGC-803 cells was 13.2% between four dosages, which was far lower than the ratio (34.3%) on HEP-G2 cells at the same concentration (200 μg/ml). From above, HPS-1 displayed significant anti-tumor activity, especially in human HEP-G2 cells.

4. Conclusion

The results of the present investigation showed that the polysaccharide of HPS-1 was a D-glucan containing α-(1 → 4)-linked backbone, branched α-(1 → 6)-linkage. Preliminary pharmacological tests suggested that HPS-1 exhibited significant anti-tumor activities in vitro. The structural and pharmacological results obtained might help enlarge the knowledge of the relationship between the structure and anti-tumor effects of polysaccharides.

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