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Antitumor activity of sulfated extracellular polysaccharides of *Ganoderma lucidum* from the submerged fermentation broth

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ABSTRACT

Water-soluble extracellular polysaccharides are known to possess weak or no *in vitro* antitumor activity. In this experiment, a mixture of extracellular *Ganoderma lucidum* polysaccharides (GLP) from the submerged fermentation broth was sulfated and studied on their antitumor activity. The sulfated GLP performed significant inhibition on the proliferation of assayed carcinoma cells in a dose-dependent manner, and present a degree of substitution-dependent suppressing to HepG2 cells. Meanwhile, the sulfated GLP presented remarkable but not dose-dependent inhibition on Heps hepatona in mice. With same degree of substitution, the sulfation protocol with aminosulfonic acid–pyridine yielded GLP sulfates with higher activity on HepG2 cells. In comparison, the native GLP showed no or little antitumor activity on the assayed cell lines but remarkable inhibition on suppressing the proliferation of rat Heps. The highest *in vivo* inhibition rate of 55.5% provided by sulfated GLP was observed on suppressing the proliferation of rat Heps.

1. Introduction

Ganoderma lucidum has been widely used in Asia medicine for thousand years, and it possesses various beneficial effects on immune function and implications for inhibition of tumor growth. Most researchers have focused on the *G. lucidum* polysaccharides (GLP) extracted from the fruiting bodies or spores and synthesized the sulfated polysaccharides with different sulfating agents (Bao, Zhen, Ruan, & Fang, 2002; Ye et al., 2008; Zhou et al., 2010). The variations and limitation on materials has made it hard to conclude the structure-relationship and urges an informative database of the experiment facts.

The submerged fermentation of *G. lucidum* is an efficient process to harvest gross of polysaccharides, supplying polysaccharides with constant or possibly desired components. The fermentation product provides mycelia polysaccharides and extracellular polysaccharides in the broth (Petre, Teodorescu, Tuluca, Bejan, & Andronescu, 2010; Tang, Zhang, Liu, Zhu, & Zhong, 2011) in which the water-soluble extracellular GLP is known to lack of *in vitro* antitumor activity comparing to the mycelia polysaccharides. Luckily, some inactive polysaccharides have been reported that their bioactivity can be stimulated by sulfation (Barahona, Chandia, Encinas, Matsuhiro, & Zuniga, 2011; Zou, Du, Li, Yang, & Zhang, 2010). However, there is much obscurity regarding the structure–function relationship. The structure factors include the sugar units in the polysaccharides, the degree of substitution (DS, average number of sulfated residues on each monosaccharide residue), the molecular weight and its distribution, as well as the sulfating reagents applied. Therefore, to stimulate the bioactivity of the extracellular polysaccharides, to enrich the database of structure–function relationship of polysaccharides and sulfated polysaccharides, we prepared the extracellular polysaccharides from the submerged fermentation broth of *G. lucidum* SB1997, synthesized the sulfated extracellular polysaccharides with different DS or same DS using different sulfating reagents; thereafter, investigated the antitumor activity of the extracellular polysaccharides and their sulfates, *in vitro* and *in vivo*, respectively.

2. Materials and methods

2.1. Materials and reagents

DEAE-Sepharose Fast-Flow was obtained from Pharmacia Co., Sweden; Trypsin, Amresco; 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Concanavalin A (ConA) were purchased from Sigma, USA. Roswell Park Memorial Institute (RPMI)-1640 medium, phosphate-buffered saline (PBS) and fetal bovine serum were purchased from Gibco, USA. Aminosulfonic

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acid, pyridine and N,N-dimethylformamide (DMF) were from Sinopharm Chemical Reagent Co., Ltd. All other chemical reagents were of analytical grade unless further stated.

2.2. Cell lines

The human hepatocytes L02, human hepatoma cell line (HepG2, BEL-7402), human gastric adenocarcinoma cell SGC7901, human oral cancer cell KB, human breast adenocarcinoma cell line MCF-7, rat hepatoma cell line Heps, and rat breast carcinoma cell line SHZ were provided by Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Science. The cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum, 1% glutamine (200 mmo1/L), penicillin (100 IU/mL) and streptomycin (100 mg/L) in a humidified 5% CO_2 atmosphere at 37 °C before use.

2.3. Preparation of G. lucidum polysaccharides (GLP)

G. lucidum SB1997 was obtained from the Laboratory of Biomass Resources, Jiangnan University (Wuxi, China), and incubated in a 250 mL flask containing 80 mL medium at 30 °C for 11 days at 150 rpm. The culture medium contained (g/L): glucose, 40; peptone, 4; K_2HPO_4, 1; KH_2PO_4, 1; $MgSO_4{\cdot}7H_2O$, 0.75; Vitamin B_1, 0.01. The initial pH of the medium was adjusted to 6.5. The supernatant of the fermentation broth was then precipitated with 95% ethanol at 4 °C in 24 h to harvest the crude polysaccharides. The precipitate was then dissolved in 30 mL distilled water and deproteinated using the Savage assay. Subsequently, the solution was dialyzed against distilled water for 3 days (M_w cut off = 3500 Da). Finally, the resulting polysaccharide solution was lyophilized to obtain the crude extracellular polysaccharides.

2.4. Preparation of polysaccharide sulfates

The extracellular polysaccharides were sulfated using aminosulfonic acid–pyridine or SO_3–pyridine as the sulfating reagent in this experiment. Aminosulfonic acid–pyridine was found to be the favorite because the smooth reaction and good yield. So unless stated, the GLP sulfate used in the experiment were synthesized with aminosulfonic acid–pyridine. Briefly, GLP (200 mg) was suspended and well mixed for 30 min in anhydrous DMF (50 mL) at room temperature in a three-neck flask. The sulfating reagent (10 mmol aminosulfonic acid well mixed with 370 mmol pyridine) was added drop wisely in 10 min and the mixture was stirred at 80 °C for another 3 h. The reaction mixture was precipitated and washed with 90% ethanol, and then dialyzed against distilled water for 24 h (M_w cut off = 3500 Da). Sulfated GLP was obtained as light brown powder after lyophilizing. Products with varied degree of substitution can be obtained by adjusting the dosage of the sulfating reagent.

The degree of substitution was determined based on the sulfate content of the molecule with $BaCl_2$–gelatin assay. Briefly, a calibration curve was prepared with sodium sulfate as the standard. 1 mg sulfated GLP was hydrolyzed with 1.0 M HCl (1 mL) for 5 h at 100 °C. Then 0.2 mL hydrolyzed sulfated GLP was transferred into a glass tube and reacted with 3.8 mL TCA (3%, w/v), and then 1.0 mL of protector solution (6.0 g NaCl, 0.5 mL HCl, 2.5 mL of 0.1% (w/v) gelatin and 0.03 g $BaCl_2$ in 47 mL distilled water) were added. The contents were stirred for 1 min and left for 15 min. The resulting $BaSO_4$ was measured turbidimetrically at 360 nm. The DS was calculated from the sulfur content using the formula as following:

$$DS = \frac{(162 \times SO_4^{2-}\%)}{(100 - 96/98 \times SO_4^{2-}\%)}$$

The structure of the sulfated GLP was analyzed with glycosyl residual composition analysis, IR, UV and NMR. The polysaccharides consist of rhamnose, arabinose, mannose, glucose and galactose with a molar ratio of 9:3:2:4:48. The main chain of the polysaccharide would contain α-D-Glc(1 → 6), α-D-Glc, α-D-Man, while rhamnose and arabinose residues would be in the side chain (unpublished data).

2.5. Growth inhibition assay (MTT test)

The *in vitro* inhibition effects of polysaccharide sulfates on the cells were evaluated using MTT assay. The cells in logarithmic growth phase were digested with 0.25% trypsin and adjusted to 5×10^4/mL using RPMI1640 complete medium respectively. Before drug exposure, 150 μL of the cell sample was pipetted into each well of 96-well plates and cultured for 24 h at 37 °C in 5% CO_2. Then, cells were cultured with varied concentration of polysaccharide sulfates for 72 h at 37 °C in 5% CO_2. After drug exposure, the culture medium was removed and 100 μL of MTT reagent (diluted in culture medium, 0.5 mg/mL) was added. After 4 h incubation, the MTT/medium was removed and DMSO (150 μL) was added to dissolve the formazan crystals. Absorbance of the solution was recorded at 570 nm. Results were evaluated by comparing the absorbance of the wells containing polysaccharide sulfates treated cells with the absorbance of wells containing 0.1% DMSO alone (solvent control). Conventionally, cell viability was determined for each assay including blank wells that did not contain cells. All experiments were performed in triplicate. The inhibition rate was calculated according to the formula given below:

$$\text{Cell growth inhibition rate}\,(\%) = \frac{A_{570}\ \text{of control} - A_{570}\ \text{of sample}}{A_{570}\ \text{of control}} \times 100$$

2.6. In vivo antitumor test

The tumor was removed from the tumor-bearing mice and sampled with 0.9% NaCl into 10^8/mL. Qualified ICR mice weighting 20 ± 1 g were divided randomly into groups, with 5 male and 5 female in each group. The mice were injected subcutaneously with 0.2 mL cell suspension, respectively. The same volume of 0.9% aq NaCl was injected intraperitoneally into the negative control mice. The mice were administered intraperitoneally once a day for 8 d and weighed the next day. The block tumor, spleen and thymus were anatomically dissected and weighed respectively for calculating the inhibition rate, spleen index and thymus index by the following formulae. The mice were executed on the next day after the last injection; and the tumors, spleens and thymuses were excised. The tumor weights were compared with those in the control mice. The antitumor activity was valued as inhibition rate, spleen index and thymus index, calculated as following:

$$\text{Inhibtion rate}\,(\%) = \frac{A - B}{A} \times 100$$

where *A* is the average weight of the tumor from the negative control mice, while *B* is the average weight of the tumor from the sulfate treated mice.

$$\text{Spleen index} = \frac{\text{The average weight of the thymus from the mice (mg)}}{\text{The average weight of the mice (g)}}$$

$$\text{Thymus index} = \frac{\text{The average weight of the thymus from the mice (mg)}}{\text{The average weight of the mice (g)}}$$

2.7. Statistical analysis

The data were expressed as mean ± standard deviation (S.D.) and examined for their statistical significance of difference with

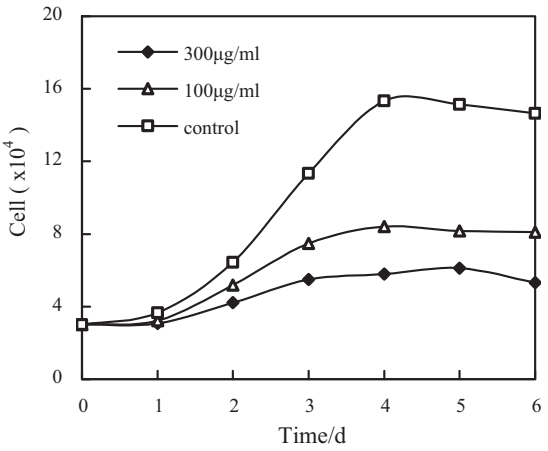

Fig. 1. Effects of GLP sulfate (DS = 1.46) on HepG2 cells growth.

Student's *t*-test. *P*-values of less than 0.05 were considered as statistically significant.

3. Results and discussion

3.1. Inhibition course of the polysaccharide sulfates on HepG2 cells line

Sulfated GLP is a mixture with distributed DS and molecular weights. First of all, the sulfated GLP (DS = 1.46) and the natural GLP were tested on HepG2 cells growth (Fig. 1); and then, fractions obtained by brine elution were assayed to chose the suitable fractions for subsequent experiments (Fig. 2). Fig. 1 showed that the GLP sulfate erased the logarithmic growth phase of the HepG2 cells comparing to the control, and the inhibition was dose-dependent. Thereafter, in the subsequent MTT test, 72 h was set as the measure time according to the inhibition course.

As aforementioned, GLP sulfate consists of fractions with different molecular weights and so on. Brine of higher concentration elutes molecules with lower molecular weight and higher DS. Hence, the eluting fractions were tested on their *in vitro* effect on HepG2 (Fig. 2A) and L-02 (Fig. 2B) cell line, respectively. However, there was no obvious difference found between the fractions. Therefore, the GLP sulfate was employed in the subsequent experiments without any further separation.

3.2. Effect of the degree of substitution and sulfating reagents on the antitumor activity

Some natural polysaccharide sulfates have exhibited potential *in vivo* antitumor activity, such as Heparin, a highly sulfated hetero polysaccharide mixture extracted from mammalian tissues (Lins et al., 2009; Mourier, Guichard, Herman, & Viskov, 2011). An unverified hypothesis is that the reason for natural polysaccharide sulfates performing gentle bioactivity is their lower DS. As mentioned before, many efforts have been made on testing the antitumor activity of sulfated polysaccharides. But still not many results can conclude clear relationships between DS and the inhibition activity. Moreover, no literature has compared the performances of polysaccharide sulfates with same DS, which were obtained with different sulfating reagents. Hence, GLP sulfate with different DS obtained with aminosulfonic acid–pyridine or SO_3–pyridine were tested for their inhibition activity on HepG2 cells.

3.2.1. Sulfating agent: aminosulfonic acid–pyridine (A), SO_3–pyridine (B)

As in Fig. 3, regardless the sulfation protocol used, all assayed sulfated GLP presented a dose and DS dependence of inhibition on HepG2 cell line with an unordinary inhibition. To date, this is one of the few reports which found the clear dose and DS dependence. Liu et al. sulfated polysaccharides isolated from *Phellinus ribis* with the DS range from 0.62 to 2.02 (Liu et al., 2009); they have found that the native polysaccharides hardly inhibited the growth of HepG2 cells, and the sulfate with a DS of 0.62 even enhanced the cell growth; while the polysaccharide sulfates with DS higher than 1.52 had stronger but DS-independent inhibition effects. Similarly, Bao et al. synthesized sulfated *Hypsizigus marmoreus* polysaccharides with DS ranging from 0.11 to 1.06 (Bao, Choi, & You, 2010). The inhibitory activity toward cancer cell growth (AGS cell) was found enhanced up to 34% with the polysaccharide sulfates, as compared to native polysaccharides. Bao did not conclude the coincidence between the DS and the antitumor effect either. In their experiment, the antitumor activity was enhanced with the increase of DS up to 0.30. Wang, Zhang, Yu, and Cheung (2009) reported that sulfated *G. lucidum* fruit body polysaccharides; and found the polysaccharides sulfate (DS = 0.94) inhibited the *in vitro* proliferation of Sarcoma 180 (S-180) tumor cells in a dose-dependent manner.

In short, compromising with the literatures, at least we can conclude all polysaccharides sulfate reported so far have the antitumor activity if their DS > 1. And it still needs more researches on the DS-function assay.

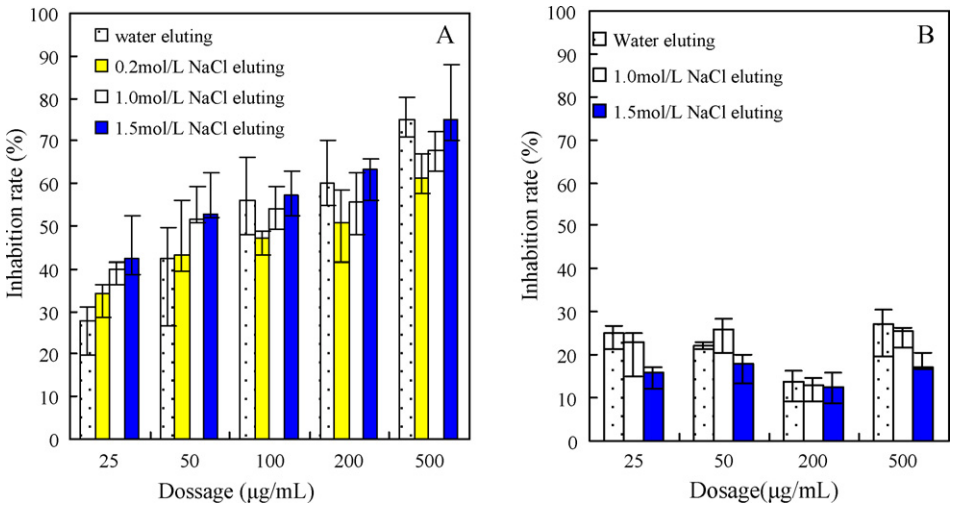

Fig. 2. Inhibition of sulfated GLP (DS = 1.46) fractions on HepG2 (A) and L-02 (B) cell lines.

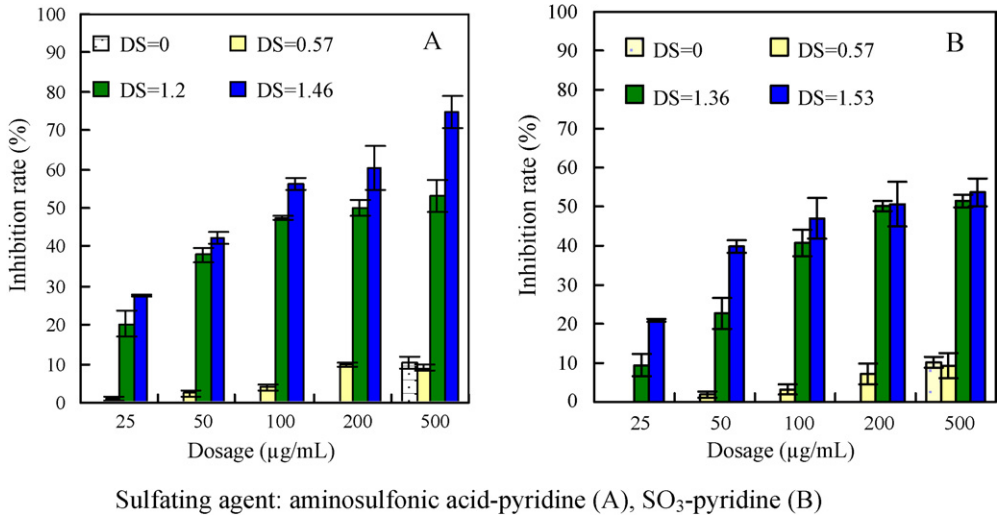

Fig. 3. Effect of DS of GLP Sulfate on HepG2 cell line.

Moreover, two groups GLP sulfate of similar DS prepared by using aminosulfonic acid–pyridine or SO_3–pyridine was tested as in Fig. 4. The different sulfating reagent yielded different molecules even that their DS are the same. In the case of DS about 1.4, the sulfation product using aminosulfonic acid–pyridine has bigger molecular weight ($M_w = 1.9 \times 10^4$; molecular weight distribution index $d = 4.34$) than that obtained with SO_3–pyridine ($M_w = 1.6 \times 10^4$, $d = 3.34$). Unlike Wang's result (Wang et al., 2009) in which the molecular weight of the sulfated polysaccharides was lower ($M_w = 10.1 \times 10^4$) than the native polysaccharides ($M_w = 13.3 \times 10^4$), the M_w of native extracellular GLP in our experiment is lower than that of sulfates (1.1×10^4) with a wider distribution ($d = 16.56$). So for HepG2 cells, the bigger molecules with same DS actually have more $-OSO_3$ groups in the molecule and present stronger activity. As for SHZ cells, there is no obvious difference between the sulfates' performance.

So we may conclude that the higher DS and dose of GLP sulfate provided stronger inhibition on HepG2 cells in a range, but different sulfating agents induced different activities because the product difference. In general, aminosulfonic acid–pyridine yielded more active GLP sulfate.

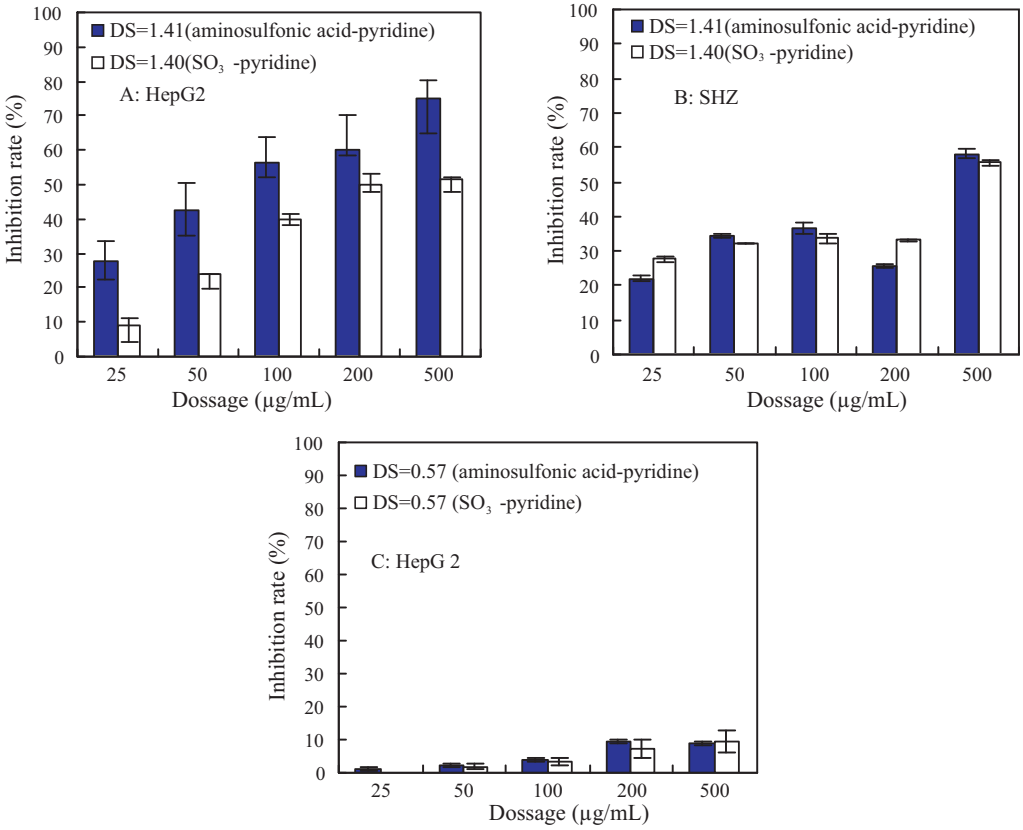

Fig. 4. Inhibition on HepG2 (A and C) and SHZ (B) cell lines by GLP sulfate of same DS.

Table 1
In vitro inhibition of extracellular GLP sulfate on the carcinoma cells.

Samples	Dosage (μg/mL)	Inhibition rate (%)					
Control		KB ND[a]	SGC-7901 ND	HePG2 ND	BEL-7402 ND	MCF-7 ND	SHZ ND
GLP	25	ND	ND	ND	9.33	19.94	ND
	50	ND	4.36	ND	11.19	21.14	1.81
	500	ND	6.57	ND	18.33	16.60	10.4
Sulfated GLP (DS = 1.46)	25	8.89	10.13	27.81	17.64	20.60	42.90
	50	19.66	13.77	42.26	24.23	30.09	52.30
	500	34.46	46.60	74.79	55.46	40.20	85.88

[a] Not detectable.

Table 2
Antitumor effect of GLP on the transplanted Heps.[a].

Sample	Dose (mg/kg d)	Mouse weight (g) (before/after drug exposure)	Average weight of the tumor (g)[c]	Inhibition rate (%)	Spleen index	Thymus index[d]
Control[b]	0.5 mL × 8	20.5/28.5	3.09 ± 0.53	ND	94.77 ± 52.20	28.08 ± 5.22
Sulfated GLP (DS = 1.46)	0.5 × 8	19.9/28.5	1.39 ± 0.42	55.02	108.89 ± 27.80	42.15 ± 11.12
	2.0 × 8	19.8/28.7	1.74 ± 0.44	43.69	111.42 ± 31.20	45.26 ± 15.60
GLP	0.5 × 8	19.8/27.5	2.05 ± 0.84	33.66	123.81 ± 29.90	40.05 ± 7.48
	2.0 × 8	20.1/28.4	2.12 ± 0.48	31.39	119.76 ± 32.40	42.27 ± 12.90

[a] X ± SD, $n = 10$, no mouse was dead after the test.
[b] 0.9%NaCl.
[c] $P < 0.001$, comparing to the negative control.
[d] $P < 0.01$ comparing to the negative control.

To further verify the antitumor effect, more carcinoma cell species were tested as following.

3.3. Antitumor activity of the GLP sulfate on carcinoma cells

The antitumor activity of GLP sulfate (DS = 1.46) over the human hepatocytes L02, human hepatoma cell line (HepG2, BEL-7402), human gastric adenocarcinoma cell SGC7901, human oral cancer cell KB, human breast adenocarcinoma cell line MCF-7, and rat breast carcinoma cell line SHZ were also tested in this experiment. As shown in Table 1, the GLP sulfate was effective dose-dependently on all the assayed carcinoma cells. In comparison, the native GLP presented no obvious antitumor effect, which agrees to most of the findings aforementioned above.

3.4. In vivo effect of the GLP sulfate on transplanted Heps

Polysaccharides and their sulfated derivatives have been counted their *in vivo* antitumor effects on their anti-inflammatory effect (Ananthi et al., 2010; Siqueira et al., 2011; Tao, Zhang, Han, Zeng, & Ding, 2009; Weber et al., 2010; Zhou et al., 2010). The antitumor effect of GLP on the transplanted Heps is presented in Table 2.

As shown in Table 2, both native and sulfated GLP presented remarkable inhibition on the transplanted Heps in mice, indicating the sulfation of GLP did not bring dramatic difference to the antitumor effect. In addition, the *in vivo* antitumor effect was not dose-dependent, which could be responded from Tao's result. Tao's experiment demonstrated that both the native polysaccharide and its sulfated derivatives using low dose (20 mg/kg) increased production of tumor necrosis factor alpha (TNF-alpha) in mouse plasma while the native one decreased the amount of TNF-alpha at high does (60 mg/kg) with or without lipopolysaccharide stimulation.

4. Conclusions

A mixture of extracellular GLP was harvested from the submerged fermentation broth, consisting of rhamnose, arabinose, mannose, glucose and galactose with a molar ratio of 9:3:2:4:48. Sulfation endowed the dose-dependent inactive extracellular GLP with *in vitro* antitumor activity, with no toxicity to normal human hepatic cell line (L02), but did not dramatically enhance their inhibition on Heps hepatona in mice. The *in vitro* antitumor activity of GLP sulfate was DS-dependent on HepG2 cells.

In general, aminosulfonic acid–pyridine yielded GLP sulfate with higher activity. The GLP sulfates presented significant dose-dependent inhibition on the proliferation of KB, SGC-7901, BEL-7402, HepG2, MCF-7 and SHZ cells, showing an inhibition rate of 34% was the minimum for all cell lines at 500 μg/mL. The inhibitory rate of the polysaccharide sulfates over HepG2, BEL-7402, and SHZ cells reached 74.79%, 55.46%, and 58.88%, respectively. Both native and sulfated GLP presented remarkable but not dose-dependent *in vivo* inhibition. The *in vivo* inhibition rate of 55.5% was observed on suppressing the proliferation of rat Heps, at the dosage of 0.5 mg/kg d for 8 days.

Acknowledgements

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