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Catalytic synthesis and antitumor activities of sulfated polysaccharide from *Gynostemma pentaphyllum* Makino

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ABSTRACT

A native polysaccharides (GPP2) was isolated from *Gynostemma pentaphyllum* Makino. Four sulfated derivatives of GPP2 (GPP2-s1 to s4) were prepared by chlorosulfonic acid method with ionic liquids (ILs) as solvent and 4-dimethylaminopyridine (DMAP) as catalyst. The structures of the sulfated derivatives were analyzed by FT-IR spectra and ^{13}C NMR spectra, which indicated that the sulfated groups were introduced mainly at the C-6 position as well as at the C-2 position. The products showed different degree of substitution (DS) ranging from 0.79 to 1.34, and different weight-average molecular mass (Mw) ranging from 8.64 to 11.2 Da. The sulfated derivatives inhibited the growth of HepG2 cells and Hela cells *in vitro* significantly. Furthermore, they had no obvious influence on 293 cells, which indicated that they had low toxicity. Flow cytometric studies revealed that treatment of the sulfated derivatives with Hela cells could mediate the cell-cycle arrest in the *S* phase.

1. Introduction

Polysaccharides from plants are not only safe, biocompatible, but also biodegradable, which own many kinds of biological activities, such as enhancing immunity, anti-cancer, anti-inflammatory, anti-oxidation (Gern, Wisbeck, Rampinelli, Ninow, & Furlan, 2008; Guo et al., 2010; Sun et al., 2008; Sun, Liang, Zhang, Tong, & Liu, 2009; Tong et al., 2009; Yang, Zhao, Yang, & Ruan, 2008). Biological activities of polysaccharides depend on sugar unit, glycosidic bond of the main chain, the types and polymerization degree of the branch, flexibility and configuration of the chains in their molecule (Lu, Wang, Hu, Huang, & Wang, 2008). Therefore, molecular modification and structure improvement of polysaccharide were considered as a way to enhance the biological activities of polysaccharide (Xing et al., 2005).

To extend the use of polysaccharides, various chemical modifications, such as sulfation, acetylation and alkylation, have been widely studied because of industrial and scientific interests. Especially, sulfated polysaccharides comprise a complex group of macromolecules with a wide range of important biological properties. The sulfated polysaccharides could not only enhance the water solubility but also change the molecular structure, which would have a great influence on their biological activities (Chaidedgumjorn et al., 2002; Qiu, Tang, Tong, Ding, & Zuo, 2007; Shi, Nie, Chen, Liu, & Tao, 2007). Many studies confirmed that the biological activities of the Chinese herbal polysaccharides could be improved by the sulfated method. For example, Zhao-Mei Wang synthesized the sulfated bagasse cellulose (BCS). BCS attained significant anticoagulation activity, causing a dose-dependent prolongation of coagulation time and inhibition of FIIa and FXa activities in human plasma (Wang, Li, & Chen, 2009; Wang, Li, Xiao, & Wu, 2009). Yu-hong Liu extracted the native polysaccharide from *Phellinus ribis* and synthesized a sulfated derivative. PRP-SI–IV showed significant inhibition effects on HepG2 cells in comparison with the native non-sulfated polysaccharide (PRP) (Liu et al., 2009). Yang extracted a polysaccharide from the mycelium of a marine filamentous fungus *Phoma herbarum* YS 4108 (YCP) and synthesized its two sulfated derivatives YCP-S1 and YCP-S2. The result showed that sulfated YCP was more potent than YCP at protecting erythrocytes against oxidative damage hemolysis (Yang, Gao, Han, & Tan, 2005).

Gynostemma pentaphyllum Makino (*Cucurbitaceae*) is a perennial liana and grows widely in Southern China, Japan, India and Korea. It is a well known edible and medicinal plant (Hu, Chen, & Xie, 1996). Recently, polysaccharide obtained from *G. pentaphyllum* Makino (GPP) has attracted great attention owing to its antitumor activities (Zhou, Liang, & Hu, 2001), anti-gastric ulcer effect (Rujjanawate, Kanjanapothi, & Amornlerdpison, 2004), immunomodulatory effect (Qian, Wang, & Tang, 1998), anti-oxidant properties (Cai, Zhang, & Wang, 2005), and treating hyperlipidemia (Birgitte, Per, & Zhao, 1995). In the present study, we synthesized a series of sulfated derivatives of GPP (GPPS), and hoped that sulfated modification could improve the biological activities of GPP. The activity of these sulfated polysaccharides

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depended on structural parameters, such as the degree of substitution (DS) (Vogl, Paper, & Franz, 2000), the weight-average molecular mass (Mw) (Yanga, Dua, Yan, Li, & Hub, 2003), the position of sulfation (Alban & Franz, 1994), and glycosidic branching (Zhang, Zhang, Wang, & Cheungb, 2003). In most previous studies, the sulfation of polysaccharide was carried out in either aqueous solution or organic reagents. However, polysaccharide sulfation in aqueous solution was going on very slowly and needed longer time to achieve dynamic equilibrium, leading to the depolymerization of polysaccharide. On the contrary, the solubility of polysaccharide was very poor in organic solvents, leading to lower DS. It is acknowledged widely that ionic liquids (ILs) have miscibility with water and organic solvents, which could offer good solubility for polysaccharide. If polysaccharide was dissolved in ILs, the reaction would be carried out in a homogenous system, which would be beneficial to sulfated modification. Thus, the dissolution of polysaccharide by ILs is a much better choice for chemical modification. Furthermore, polysaccharide in the strong acid circumstance would be depolymerized severely due to longer reaction time and lose the original biological activities. To solve this problem, catalyst was added into the reaction for increasing the reaction rate as soon as possible and decreasing the depolymerization of polysaccharide.

To the best of our knowledge, there was still no reported study on sulfated modification of polysaccharide in ILs with catalyst. In our study, GPP was modified by chlorosulfonic acid–pyridine method with an ionic liquid 1-butyl-3-methylimi-dazolium chloride ([C_4mim]Cl) as solvent and 4-dimethylaminopyridine (DMAP) as catalyst in the reaction system. Polymer characteristics, including DS, Mw and chain conformation, and *in vitro* antitumor activities of the sulfated derivatives and the native polysaccharide, were compared.

2. Materials and methods

2.1. Materials and reagents

The crude GPP from *G. pentaphyllum* Makino (collected from the mountain area in Weinan City, Shaanxi Province, China) was obtained from Shaanxi Lixin Biotechnology Co. (China).

Papain was from Beijing Huamei Biotechnology Co., Ltd. (China). 4-Dimethylaminopyridine (DMAP) was purchased from Guoyao Chemical Reagent Co. (China). 1-Butyl-3-methylimidazolium chloride [C_4mim]Cl was purchased from Shanghai Cheng Jie Chemical Co. (China). Chlorosulfonic acid (CSA), pyridine and N,N-dimethylformamide (DMF) were analytical grade and were obtained from Gansu Yinguang Chemical Industry Co. (China). SephadexG-100 was from Pharmacia Co. (Sweden). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was from Sigma Co. (France). 5-Fluorouracil (5-Fu) was from Shanghai Haiqu Chemical Co. Ltd., China.

2.2. Purification of GPP

The crude GPP was purified as follows: the protein was removed by the Sevage method, combined with papain. After centrifugation, the supernatant was dialyzed by using distilled water for 48 h. Through SephadexG-100, two purified polysaccharides (GPP1, GPP2) were obtained.

The contents of GPP were measured by Vitriol–Phenol (Xu et al., 2005) taking anhydrous glucose as standard control. GPP1 had too low contents and was given up. As a result, GPP2 was the only used for our experiments, and Mw of GPP2 was 9.3 kDa. Shu-liang Song reported that GPP2 was composed of rhamnose and xylose, and their mol ratio was rhamnose:xylose = 1:12.25. The glucosidicbond configuration of GPP2 was mainly α-configuration (Song, Ji, Liang, & Wang, 2008).

2.3. Sulfated modification of GPP2

2.3.1. Preparation of sulfating reagent

CSA was dripped in anhydrous pyridine, under agitating and cooling condition in ice water bath (Wang, Li, & Chen, 2009; Wang, Li, Xiao, et al., 2009). All processes were completed in 20 min.

2.3.2. Dissolution of GPP2 in the ionic liquid

[C_4mim]Cl was pre-melted in a flat-bottomed flask agitated with a mechanical stirrer, and heated to 80 °C in an oil bath. Dry GPP2 was dispersed into the pre-melted [C_4mim]Cl (100 mg GPP2/1500 mg [C_4mim]Cl) under vacuum to ensure complete dissolution of GPP2 with constant stirring for 1 h at 80 °C.

2.3.3. Sulfation reaction

100 mg GPP2 was dissolved in ILs, and the sulfating reagent was added. Then 10 mg DMAP was added to ensure appropriate modification on the polysaccharides. The mixture was stirred for 1 h at 30 °C. Later, the mixture was cooled to room temperature and the pH value was adjusted to 7–8 with 2.5 mol/L NaOH solution. The mixtures were dissolved in water, and were dialyzed (molecular weight cutoff 3.0 kDa) by using distilled water for 24 h to remove DMAP, pyridine, salt and potential degradation products. Four sulfated GPP2-s (GPP2-s1 to GPP2-s4) with different DS were obtained after lyophilizing, then kept in dryness box.

GPP2 was dissolved in DMF, and GPP2-s5 was obtained by chlorosulfonic acid–pyridine method without catalyst and ILs in the reaction.

2.4. Characterization of GPP2-s

Mw of the sulfated derivatives were determined by high-performance gel-filtration chromatography (HPGFC) on a Waters 2695 instrument equipped with three WATO columns, and a Waters 2414 Refractive Index Detector (RID). 1 mg/ml sample solution (GPP2-s solution) was injected in each run with using 0.05 mol/L Na_2SO_4 as the mobile phase. The analysis was performed at 25 °C and a flow rate of 0.7 ml/min. The HPGFC system was calibrated with T-series Dextran standards (T-5, T-25, T-50, T-150 and T-270).

The elemental compositions of C, H and S in the sulfated derivatives was determined by an elemental analyzer (Vario EL, Elementar Co., Germany), and DS which represented the average number of sulfate residues on each monosaccharide residue, was calculate:

$$DS = \frac{162 \times S\%}{32 - 102 \times S\%}$$

FT-IR spectra (KBr pellets) of GPP2 and GPP2-s were recorded with a FLS920 FT-IR spectrophotometer (Edinburgh, England) in the range 4000–500 cm^{-1} using the KBr-disk method. ^{13}C NMR spectra of 40 mg/ml solutions in D_2O were recorded on a Bruker Avance 600 MHz spectrometer (Germany) at 40 °C with 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as internal standard.

2.5. Cell lines and culture

The human hepatoma cell line (HepG2), uterine cervix carcinoma cell line (Hela) and human embryonic kidney cell line (293) were provided by the Biology Preservation Center in Shanghai Institute of Materia Medica and maintained with RPMI 1640 medium containing 10% fetal bovine serum and 100 ng/ml, each of penicillin and streptomycin at 37 °C in a humidified atmosphere with 5% CO_2.

2.6. Growth inhibition assay

The inhibition effects of the sulfated derivatives on the HepG2 cells, Hela cells and 293 cells were studied by using MTT method *in vitro*. They (5×10^3 cells/well) were incubated for 12 h, respectively, before addition of the sulfated derivatives. Then the sulfated derivatives with varied concentration (100, 200, 500, 1000 and 2000 μg/ml) or 5-Fu (25 μg/ml) were added. After incubation for 48 h at 37 °C, the culture medium was removed and 100 μl of MTT reagent (diluted in culture medium, 0.5 mg/ml) was added. Following incubation for 4 h, the MTT/medium was removed carefully and DMSO (100 μl) was added to each well for dissolving the formazan crystals. Absorbance of the colored solution at 570 nm was measured on a microplate photometer (PerkinElmer Victor3). All experiments were performed in triplicate. Results were evaluated by comparing the absorbance of the wells containing compound treated cells with the absorbance of wells containing 0.1% DMSO alone (solvent control). 5-Fu was used as positive control. To validate the induction of apoptosis by identifying morphological features, phase-contrast microscopy was used in cultures of HepG2 cells or Hela cells treated with the sulfated derivatives.

The inhibition rate (IR) was calculated according to the formula below:

$$\text{Growth inhibition rate}\,(\%) = \frac{1 - A_{\text{drug-blank}}}{A_{\text{control-blank}}} \times 100$$

2.7. Cell-cycle analysis

The effects of the sulfated derivatives on Hela cell-cycle were assessed by flow cytometry. The cells (5×10^3 cells/well) were incubated on a 6-well plate with GPP2-s3 in the concentration range from 100 to 2000 μg/ml. After the incubation of 48 h, Hela cells were washed with phosphate-buffered saline (PBS) twice, fixed in 70% cold ethanol, and stored at −4 °C overnight. Previous to the analysis, the fixed cells were washed with PBS twice and stained with 200 μl of 1.12 mg/ml propidium iodide (PI) (Bender MedSystems Inc., Burlingame, CA). The stained cells were then transferred to flow tubes by passing through nylon mesh with a pore size of 40 μm, and were analyzed on a flow cytometer (EPICS XL, U.S. COULTER).

3. Results and discussion

3.1. Characterization of the sulfated derivatives

Mw and DS of the sulfated derivatives were investigated (Table 1). Results showed that Mw of the sulfated derivatives increased from 8.64 to 11.2 kDa, and DS increased from 0.79 to 1.34. It implied that hydroxyl groups were substituted successfully by sulfated groups in the polysaccharide.

Compared with GPP2, the molecular mass decrease of polysaccharide after sulfated modification with ILs as solvent had not been observed. On the contrary, the molecular mass of GPP2-s1 and GPP2-s2 had been a little increase. It showed that the superiority of ILs as solvent for sulfated modification of polysaccharide could avoid the depolymerization in the homogeneous system. Furthermore, the reaction could achieve dynamic equilibrium as soon as possibly due to addition of DMAP. However, the depolymerization of polysaccharide happened severely in organic solvent DMF.

Table 1
Characterization of GPP2-s.

GPP2-s[a]	CSA:PD[b]	Time (h)	Mw (kDa)	Elemental analysis (%)			DS
				C	H	S	
GPP2-s1	1:5	1.0	11.2	16.25	3.14	10.47	0.79
GPP2-s2	1:4	1.0	10.9	17.95	3.52	12.04	0.98
GPP2-s3	1:3	1.0	8.96	17.21	3.37	13.49	1.20
GPP2-s4	1:2	1.0	8.64	16.37	3.15	14.38	1.34
GPP2-s5	1:5	2.5	6.02	15.63	3.29	13.07	1.13

[a] Sulfation of polysaccharide from *Gynostemma pentaphyllum* Makino.
[b] The ratio of chlorosulfonic acid to pyridine in sulfating reagent.

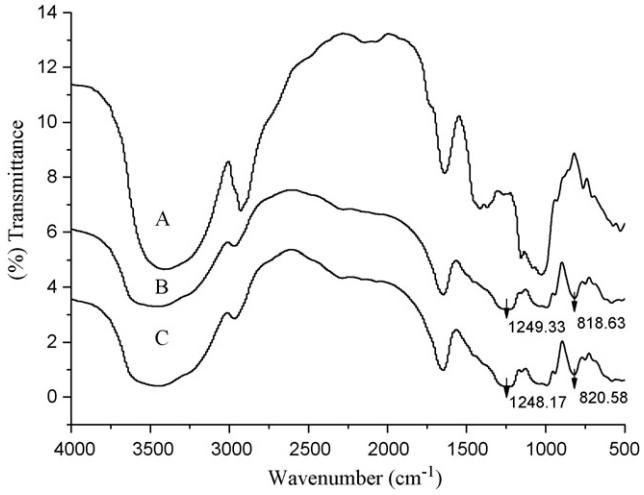

Fig. 1. FT-IR spectra of GPP2 and its sulfated derivatives. (A) GPP2, (B) GPP2-s1, (C) GPP2-s5.

The FT-IR spectra of the native GPP2 and the sulfated derivatives were shown in Fig. 1. Compared with GPP2, two new strong absorption peaks appeared at 1246 cm^{-1} and 816 cm^{-1} for the sulfated derivatives, assigned to the S=O asymmetric stretching and C–O–S symmetric vibrations, respectively. These absorptions indicated that the sulfated modification in the samples had actually taken place.

The sulfated position of polysaccharide was usually determined by ^{13}C NMR spectra. The ^{13}C NMR spectra of the native GPP2 and its derivative (GPP2-s1) were shown in Fig. 2. Compared with the signals of GPP2, it was found that the signals of GPP2-s1 became more complicated in ^{13}C NMR spectra. If the carbon directly attached to an electron-withdrawing sulfate group, it would shift to a lower field position. On the contrary, the carbon indirectly attached to sulfate group would shift to higher field position (Gamazade et al., 1997). The new peak of GPP2-s1 at 68.5 represented the signals of C-6s; the peak at 75.3 represented the signal of C-2s. The peak at 62.9 was weakened, which indicated that C-6 had been substituted by the sulfate group, whereas C-2 had been substituted partially. We believed that the C-6 position was more active than the C-2 position due to the steric hindrance. Furthermore, a new peak at 94.7 was assigned to C-1′, which showed that C-2 had been

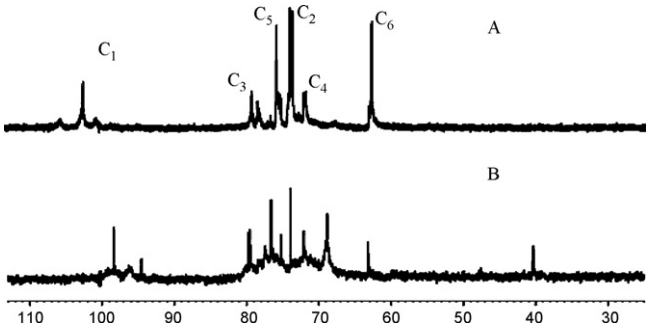

Fig. 2. ^{13}C NMR (600 MHz) spectra of GPP2 and its sulfated derivatives. (A) GPP2; (B) GPP2-s1.

Table 2(a)
Growth inhibition of GPP2 and its sulfated derivatives against HepG2 cells *in vitro*.

Group	Result	C[b] (μg/ml)					
		25	100	200	500	1000	2000
GPP2	IR[a] (%)		3.7 ± 0.9	10.4 ± 0.7	13.9 ± 1.5	19.7 ± 1.3	22.9 ± 2.1
GPP2-s1	IR (%)		4.5 ± 1.4	12.1 ± 0.7	18.5 ± 2.3	22.7 ± 1.7	29.2 ± 2.3
GPP2-s2	IR (%)		7.1 ± 1.4	11.5 ± 1.2	21.7 ± 1.6	24.9 ± 1.5	36.3 ± 2.4
GPP2-s3	IR (%)		11.4 ± 1.1	17.2 ± 2.3	21.5 ± 1.9	35.2 ± 2.1	42.7 ± 2.3
GPP2-s4	IR (%)		10.1 ± 0.6	18.4 ± 2.4	29.3 ± 1.4	40.5 ± 2.2	46.4 ± 2.8
GPP2-s5	IR (%)		5.3 ± 1.1	11.9 ± 1.2	16.1 ± 1.0	21.8 ± 1.5	33.9 ± 3.1
5-Fu	IR (%)	47.5 ± 1.6					

[a] IR, growth inhibition rate.
[b] *C*, concentration of GPP2 and its sulfated derivatives.

Table 2(b)
Growth inhibition of GPP2 and its sulfated derivatives against Hela cells *in vitro*.

Group	Result	C[b] (μg/ml)					
		25	100	200	500	1000	2000
GPP2	IR[a] (%)		−1.2 ± 0.4	6.1 ± 1.2	8.6 ± 1.1	11.3 ± 1.5	13.7 ± 1.1
GPP2-s1	IR (%)		5.2 ± 0.9	12.8 ± 2.3	18.4 ± 1.8	25.3 ± 1.1	28.8 ± 1.4
GPP2-s2	IR (%)		11.2 ± 1.7	17.9 ± 2.2	27.1 ± 1.4	34.8 ± 2.7	40.5 ± 1.6
GPP2-s3	IR (%)		16.4 ± 1.2	25.5 ± 1.3	40.3 ± 2.3	48.6 ± 1.6	61.5 ± 2.1
GPP2-s4	IR (%)		14.3 ± 1.7	19.6 ± 2.7	32.2 ± 1.9	39.9 ± 1.0	54.2 ± 1.6
GPP2-s5	IR (%)		7.3 ± 1.5	14.2 ± 2.1	18.9 ± 2.2	23.2 ± 1.6	34.2 ± 1.4
5-Fu	IR (%)	72.3 ± 1.3					

[a] IR, growth inhibition rate.
[b] *C*, concentration of GPP2 and its sulfated derivatives.

substituted, and could influence the adjacent C-1 to split into two peaks. New peaks at 70–80 meant that sulfation of other positions had occurred besides C-6 and C-2.

3.2. Growth inhibition of the sulfated derivatives on HepG2 cells

It was reported that the introduction of sulfated groups to polysaccharide should change its physicochemical characterization and chain conformation, and the sulfated polysaccharides were beneficial to the enhancement of their antitumor activity (Lin et al., 2004; Wang et al., 2004). In the present study, the growth inhibitory effects of GPP2 and the five sulfated derivatives against HepG2 cells *in vitro* were first examined (Table 2(a)). The native GPP2 was then determined to exhibit a relatively lower antitumor activity against HepG2, but all the sulfated derivatives showed higher antitumor activities than the native one. In the concentration range from 100 to 2000 μg/ml, GPP2-s3 and GPP2-s4 significantly inhibited the growth of HepG2 cells. Furthermore, GPP2-s4 significantly exhibited higher inhibition ratios than other fractions at all concentrations. Especially, at the concentration of 2000 μg/ml, the inhibition activity of GPP2-s4 was the highest with an inhibition ratio of 46.4 ± 2.8%. This suggested that antitumor activities could be improved by sulfation. However, GPP2-s5 exhibited a relatively lower inhibition activity from 100 to 2000 μg/ml.

3.3. Growth inhibition of the sulfated derivatives on Hela cells

In vitro inhibition ratio of Hela cells growth by GPP2 and the five sulfated derivative at different concentration (100, 200, 500, 1000 and 2000 μg/ml) were shown in Table 2(b). The results indicated that GPP2 had no obvious influence on Hela cells. The growth of Hela cells could be inhibited by the sulfated derivative at a concentration as low as 100 μg/ml, and a dose-dependent manner was observed up to 2000 μg/ml. GPP2-s3 exhibited strongest inhibition ratio against Hela cells proliferation at the concentration of 2000 μg/ml, reached 61.5 ± 2.1%, and there was an increase in the percentage of cells being shrunk observed with phase-contrast microscopy. The difference in the antitumor activity may be attributed to their different DS and molecular weights. It did not seem that polysaccharide with higher sulfate content exhibited stronger antitumor activity. In our paper, GPP2-s3 with DS of 1.20 showed the highest activity, suggesting a moderate DS of the sulfated derivatives was necessary for a high antitumor activity *in vitro*. However, GPP2-s5 had found a relatively lower inhibition activity from 100 to 2000 μg/ml. We believed that GPP2-s5 would be possibly depolymerized due to longer reaction time in the strong acid circumstance, and the structure of polysaccharide was damaged severely.

Table 2(c)
Growth inhibition of GPP2 and its sulfated derivatives against 293 cells *in vitro*.

Group	Result	C[b] (μg/ml)					
		25	100	200	500	1000	2000
GPP2	IR[a] (%)		−22.3 ± 1.5	−19.7 ± 2.6	−17.3 ± 2.1	−16.9 ± 1.2	−12.6 ± 1.3
GPP2-s1	IR (%)		−17.3 ± 0.6	−13.6 ± 1.3	−11.1 ± 0.7	−4.2 ± 1.2	3.6 ± 1.1
GPP2-s2	IR (%)		−18.3 ± 1.3	−10.6 ± 1.6	−3.1 ± 0.8	5.3 ± 1.7	11.8 ± 1.9
GPP2-s3	IR (%)		−6.4 ± 0.7	−0.05 ± 0.1	4.2 ± 1.3	14.6 ± 0.6	18.4 ± 1.1
GPP2-s4	IR (%)		−9.5 ± 1.2	−2.7 ± 1.1	0.4 ± 0.2	7.4 ± 1.3	16.9 ± 1.7
GPP2-s5	IR (%)		−10.3 ± 0.1	1.2 ± 0.4	3.5 ± 1.2	11.8 ± 1.3	13.7 ± 1.1
5-Fu	IR (%)	51.2 ± 1.9					

[a] IR, growth inhibition rate.
[b] *C*, concentration of GPP2 and its sulfated derivatives.

3.4. Growth inhibition of the sulfated derivatives on 293 cells

293 cells were normal cells in healthy volunteers, the growth inhibitory effects of GPP2 and the five sulfated derivatives against 293 *in vitro* were examined (Table 2(c)). The results indicated that GPP2 and the five sulfated derivatives had no obvious influence on 293 cells. Interestingly, GPP2-s1 could stimulate proliferation of 293 cells, maybe the lower degree of sulfation was beneficial to the growth of 293 cells. This implied that they had no direct cytotoxicity to non-cancerous cells.

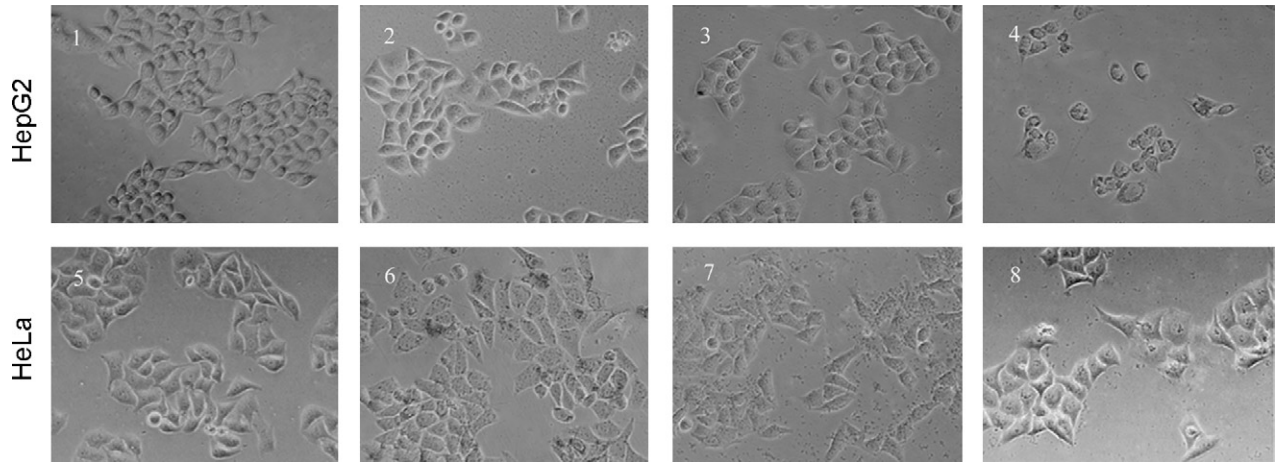

Fig. 3. Morphological characteristics of HepG2 cells and Hela cells treated with GPP2-s3. HepG2 cells and Hela cells were seeded into tissue culture flask and treated with increasing concentrations of GPP2-s3. 1–4 was HepG2 cells: (1) control, (2)–(4) concentration of GPP2-s3 was 100, 1000 and 2000 μg/ml, respectively. 5–8 was Hela cells: (5) control, (6)–(8) concentration of GPP2-s3 was 100, 1000 and 2000 μg/ml, respectively. All cells were photographed after 48 h drug treatment.

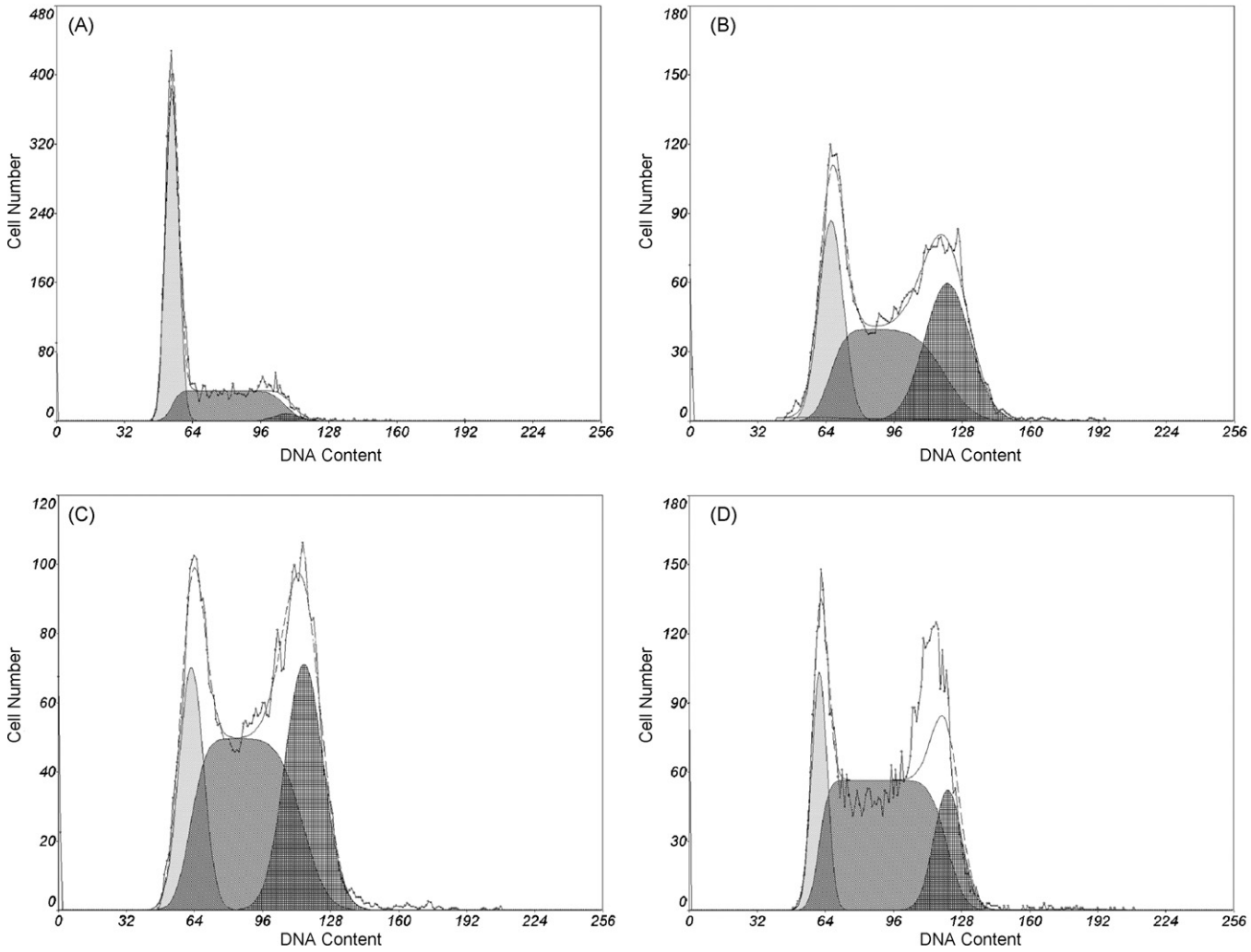

Fig. 4. Cell-cycle analysis of Hela cells treated with GPP2-s3. (A) Control; (B)–(D) concentration of GPP2-s3 was 100, 1000 and 2000 μg/ml, respectively.

Table 3
Cell-cycle perturbations induced by GPP2-s3.

Group	Concentration (μg/ml)	Cell-cycle phase (%)		
		G_0/G_1	S	G_2/M
GPP2-s3	100	25.05%	43.18%	31.77%
	1000	19.32%	50.20%	30.49%
	2000	18.05%	65.46%	16.50%
Control	0	59.54%	37.98%	2.48%

5-Fu, a well-known anti-cancer agent, was included for comparison. *In vitro* 5-Fu showed high antitumor activities against HepG2 cells and Hela cells growth at lower concentration in Tables 2(a) and 2(b). Meanwhile, it showed high toxic and killed the normal cells (293 cells) as well as tumor cells in Table 2(c).

3.5. Changes in HepG2 cells and Hela cells morphological features

Fig. 3 showed that HepG2 cells and Hela cells treated with GPP2-s3 were accompanied by morphological changes. Two days after treatment with GPP2-s3, the average inhibitory ratio against HepG2 cells and Hela cells increased with the increase of the concentration. Moreover, it was clear that the distinctive morphological features of cells included detachment and cell shrinkage. After cells were killed, cytoplasm came out. We believed that HepG2 cells and Hela cells death induced by GPP2-s3 was very obvious.

3.6. Cell-cycle arrest and apoptosis induction of Hela cells by GPP2-s3

Apoptosis is a physiological and crucial process that is regarded as the preferred way to eliminate cancer cells. Fig. 4 and Table 3 showed the effect of GPP2-s3 on the cell-cycle phases (G_0/G_1, S, and G_2/M) of Hela cells. GPP2-s3 induced accumulation of Hela cells in the S phase, indicating apoptotic phenomenon, as well as led to the S phase arrest. In the cell-cycle analysis, a significant increase of the cell population in the S and G_2/M phase was observed from 37.98% and 2.48% of the control group to 65.46% and 16.5% for GPP2-s3, respectively, with the concentration of 2000 μg/ml. These results suggested that the sulfated derivatives could inhibit the growth of Hela cells through cell-cycle arrest in the S phase and induced the apoptosis.

4. Conclusions

Four sulfated derivatives of GPP2 from *G. pentaphyllum* Makino were prepared by chlorosulfonic acid method with ILs as solvent and DMAP as catalyst. FT-IR spectra and ^{13}C NMR spectra indicated that the sulfated modification had actually occurred. The sulfated derivatives showed different DS ranging from 0.79 to 1.34, and different Mw ranging from 8.64 to 11.2 kDa.

Compared with GPP2-s5, the sulfated derivatives exhibited obvious inhibition effects on HepG2 cells and Hela cells *in vitro*. Flow cytometric studies revealed that treatment of the sulfated derivatives with Hela cells could mediate the cell-cycle arrest in the S phase. It was determined that the sulfated derivatives of the native GPP2 could improve its antitumor activity. They had no obvious influence on 293 cells, which indicated that the sulfated derivatives had low toxicity. Thus, it could be expected that the sulfated derivatives of GPP2 have an assistant role in the treatment of cancer in the further.

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