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Preparation, characterization and bioactivities of derivatives of an exopolysaccharide from *Lachnum*

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ABSTRACT

An exopolysaccharide, obtained previously LEP-2b from *Lachnum* YM405, was phosphated and sulfated successfully. The derivatives named PLEP-2b and SLEP-2b, respectively, and their respective degree of substitution were 0.174 and 0.431. Phosphate groups -PO_3H_2 substituted at C-6 of 1,4-β-D-mannopyranose, C-5 of 2,6-β-D-1-OMe-mannofuranoside, C-3 of 1,6-β-D-galactopyranose, C-2 of 1-β-D-glucopyranose, and C-6 of 1,2-α-D-rhampyranose, while sulfate groups —SO_3H were mainly at C-6 of 1,4-β-D-Man*p*, C-6 of 1-β-D-Glc*p* and C-6 of 1,2-α-D-Rha*p*. Compared with LEP-2b, the scavenging effects of the derivatives, on hydroxyl radical and superoxide anion were significantly increased after the modifications, except for reducing power. Meanwhile, phosphorylated and sulfated modifications remarkably strengthened the inhibiting effect of LEP-2b on the proliferation of CT-26 murine colon carcinoma, Lewis lung carcinoma and human hepatocellular carcinoma HepG2 cells. The derivatives significantly enhanced the antioxidant and antitumor activities *in vitro*. Compared with sulfation, phosphorylation improved the inhibitory effect more contraposingly on some specific tumor cells.

1. Introduction

Fungal polysaccharides have caused much attention because of their various activities including antioxidant, antitumor, antivirus and immunoregulation, etc. What's more, they showed no cytotoxicity towards normal cells, even more, some of them have been clinically used (Wang et al., 2013a; Wang, Zhang, Yao, Zhao, & Qi, 2013a). The activities of the polysaccharides depend on their monosaccharide composition, glucosidic bonds, branch types, degree of polymerization, flexibility and configuration of the chains, and so forth (Chen, Xu, Zhang, & Zeng, 2009). So, the activities of the polysaccharides could be strengthened through remoulding the structure by chemical methods. Numbers of studies showed that chemical modifications could increase bioactivities of the polysaccharides (Abd El-Baky, ElBaroty, & ElBaz, 2009; Chen et al., 2011a,b; Gamal-Eldeen et al., 2006; Guo et al., 2013; Liu et al., 2012; Wang, Zhang, Zhang, Zhang, & Li, 2009), even introduce new ones (Martinichen-Herrero, Carbonero, Sassaki, Gorin, & Iacomini, 2005).

In recent years, we have found several polysaccharides owning antitumor and anti-aging activities from various strains of *Lachnum* sp., a genus of saprophytic fungi in the family *Hyaloscyphaceae* (Ye et al., 2012). The structure of the exopolysaccharide LEP-2b ($M_W \approx 2.8 \times 10^4$ Da) from *Lachnum* YM405 has also been studied. Its backbone was composed of 1,4-β-D-Man*p* (mannopyranose), 1,2-α-D-Rha*p* (rhampyranose), 1,6-β-D-Glc*p* (glucopyranose) and 1,6-α-D-Gal*p* (galactopyranose), and its three branches composed of 2,6-α-D-1-OMe-Man*f* (mannofuranoside), 2,6-β-D-1-OMe-Man*f*, 1,6-α-D-Gal*p*, 1,6-β-D-Gal*p* and 1-β-D-Glc*p* were, respectively, substitued at O-3 of Man*p* and O-2 and O-3 of the same Glc*p* on the backbone (He et al., 2014).

In this study, phosphorylated and sulfated modifications were, respectively, performed on LEP-2b, structural features of the derivatives were clarified, and their antioxidant and antitumor activities were also estimated at the same time. This work was expected to improve the activities of the polysaccharide by different chemical modifications and lay some theoretical foundations

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for the study of structure-function relationship as well as the development of polysaccharide functional foods and medicines.

2. Materials and methods

2.1. Materials

Lachnum YM405 was collected from Yun-nan Province, China. The strain were separated and preserved in Microbial Resource and Application Laboratory of the Hefei University of Technology. The exopolysaccharide LEP-2b was obtained preciously (He et al., 2014). CT26 colon carcinoma cells (CT26), Lewis lung carcinoma cells (LLC) and human hepatocellular carcinoma HepG2 cells (HepG2) were kindly provided by Institute of Immunology (Wei Haiming's lab), University of Science and Technology of China.

2.2. Reagents and instruments

SO_3-pyridine complex (≥92%, TCI Development Co., Ltd., Japan), RPMI 1640 medium (Thermo Fisher Scientific Inc, USA), high (25 mM) glucose DMEM medium (Mediatech Inc, China), fetal calf serum (HeFei BoMei Biotechnology Co., Ltd, China), MTT (methyl thiazolyl tetrazolium. Ruibio, Germany), dialysis bag (molecular cut off 13 kDa), other reagents were all analytically pure.

Fourier infrared spectrometer Nicolet 67 (Thermo Nicolet Co., USA), ultraviolet–visible spectrophotometer (Shimadzu Co., Japan), nuclear magnetic resonance spectrometer Bruker Avance AV-500 and Avance AV-400 (Bruker Co., Germany), iMark microplate absorbance reader model 680 (Bio-Rad Laboratories Inc., U.S.).

2.3. Preparation of LEP-2b derivatives

2.3.1. Polysaccharide phosphate

Sodium tripolyphosphate (8.57 g) and sodium trimetaphosphate (1.43 g) were mixed and used as a phosphorylating reagent and then dissolved in 100 mL distilled water together with 5.0 g sodium sulfate. Afterwards, 1.0 g LEP-2b was dissolved and pH of the solution was adjusted to 9.0 with $NaHCO_3$. Thermostatic water-bath at 80 °C was used for the phosphorylation reaction. Five hours later, Five-fold volume of 95% ethyl alcohol was used and standing for 24 h to precipitate the polysaccharide. After the precipitate was redissolved in water, it was, respectively, dialyzed against tap water and distilled water for 2 d and 1 d. Phosphorylated polysaccharide, namely the polysaccharide phosphate PLEP-2b, was obtained after filtration and lyophilization (Sun, Pan, Zeng, & Cao, 2011; Ye, Yuan, He, Du, & Ma, 2013).

2.3.2. Polysaccharide sulfate

Pyridine (90 mL) was injected into a 250 mL three-necked bottle fitted with reflux condenser and thermometer. While the pyridine was being stirred and heated with a hot-plate magnetic stirrer, 7.5 ~ 9.0 g SO_3-pyridine complex was gradually added. When the temperature reached 90 °C, 1.5 g LEP-2b powder was added and stirred evenly at 90 °C for 1 h. The reaction liquid was cooled down and its pH was adjusted to about 7 using 3 mol L^{-1} NaOH, after which, five-fold volume of 95% ethanol was poured in it. Next day, the precipitate separated by centrifugation was redissolved and dialyzed against tap water and distilled water in turn for respective 2 d and 1 d. Sulfated polysaccharide, namely the polysaccharide sulfate SLEP-2b, was obtained after filtration and lyophilization (Tian, Li, Song, Zheng, & Li, 1995).

2.4. DS (degree of substitution) of substituent groups in the derivatives of LEP-2b

2.4.1. Determination of phosphate group content in PLEP-2b

Standard curve of phosphate anion (Sun et al., 2011): trimethylol aminomethane (Tris, 3.6 g) and $MgCl_2{\cdot}6H_2O$ (120 mg) were successively dissolved in 300 mL double distilled water, and a Tris buffer was obtained after the pH was adjusted to 7.0 with 1 mol L^{-1} HCl. Same volume of 20% V_C solution, 3 mol L^{-1} H_2SO_4 solution and 3% ammonium molybdate solution were measured and evenly mixed with one another to obtain a phosphorus quantitative reagent. 1.0000 g KH_2PO_4 was accurately weighed and dissolved in 100.00 mL distilled water. Diluted 100-fold, and a 0.1 mg mL^{-1} standard liquid of phosphate group was formed. 0, 0.50, 1.00, 1.50, 2.00, 2.50, 3.00, 3.50, 4.00, 4.50 and 5.00 mL of the standard solution were precisely transferred in to 11 colorimetric tubes in which distilled water was then supplemented and brought final volume to 5.00 mL. For each tube, after 3 mL Tris buffer was added and shaken well, 3 mL phosphorus quantitative reagent was soon added, which was followed by heat treatment in water bath at 45 °C for 30 min. Thereafter, the absorbance at 660 nm (A_{660}) was measured. A standard curve of A_{660} against the concentration of phosphate anion was drawn.

To determine the content of phosphate group, molybdenum blue colorimetry was adopted (Sun et al., 2011). The sample (0.1 g) was dissolved by the addition of 1 mL concentrated sulfuric acid and 1 mL concentrated nitric acid, and the solution was then heated until it was smoking. After cooling down the solution, 1 mL 30% H_2O_2 aqua was added for one more heat treatment. The above steps were repeated until no smoke emerged in beaker, and at the moment the solution was either transparent and colorless or faint yellow. One milliliter 6 mol L^{-1} hydrochloric acid was added to the cooled sample, and the acid was then totally decomposed by heating. The solution was transferred and brought final volume to 50 mL in a volumetric flask. According to the method mentioned in the last paragraph, 5 mL of the above solution was withdrawn to measure A_{660}. On the basis of regression equation of the standard curve, content of the phosphate anion was figured out.

2.4.2. Determination of the content of the sulfate group in SLEP-2b

Standard curve of sulfate anion: The K_2SO_4 dried to constant weight at 105 °C was precisely weighed (108.75 mg) and dissolved in 1 mol L^{-1} hydrochloric acid. Afterwards, it was brought to 100 mL with distilled water, hereby the standard stock solution (0.6 mg mL^{-1}) was obtained. A series of exact volumes (0, 0.04, 0.08, 0.12, 0.16 and 0.20 mL) of the solution were withdrawn and diluted with 1 mol L^{-1} HCl to an accurate 0.20 mL for each. 3.8 mL 3% trichloroacetic acid (TDA) and a barium chloride-gelatin solution (1 mL) that was composed of 0.5% gelatin and 1% $BaCl_2$ were added. After shaking up and standing at room temperature for 15 min, the absorbance at 360 nm was measured and recorded as A_1. Instead of barium chloride-gelatin solution, 1.0 mL 0.5% gelatin solution was added and the same procedure was repeated, herein the absorbance A_2 at 360 nm was measured. A standard curve of the difference value between A_1 and A_2 ($A_1 - A_2$) against the mass of sulfate anion was drawn.

Determination of the content of sulfate group: SLEP-2b (3 mg) was precisely weighed and dissolved in 3 mL 1 mol L^{-1} HCl aqua, then stayed in boiling water bath (100 °C) for 1 h. The sample solution (0.2 mL) was sucked out and its absorbance difference ($A_1 - A_2$) was calculated according to the above method used for standard curve of sulfate anion.

2.5. Calculation of degree of substitution (DS)

Content of substituent can be represented as $C = M_{Sub} \cdot DS/[162 - DS + M_{Sub}]$, so

$$DS = \frac{162 \times C + M_{Sub} \times C}{M_{Sub} + C} \quad (1)$$

162: relative molecular mass of one monosaccharide residue; M_{Sub}: molar mass of substituent; C: content of substituent; $M_{Sub\,1} = M(-PO_3H_2) = 81$; $M_{Sub\,2} = M(-SO_3H) = 81$.

2.6. Ultraviolet and infrared spectra of the derivatives of LEP-2b

LEP-2b, PLEP-2b and SLEP-2b were, respectively, dissolved in double distilled water to form a 4 μg mL^{-1} solution for each. Ultraviolet–visible spectrophotometer UV-2450 with a scanning wavelength range from 190 to 400 nm was used for full-wave band ultraviolet spectrum analysis of the three samples. Infrared (IR) absorption of LEP-2b, PLEP-2b and SLEP-2b were, respectively, measured with KBr pellet (mass ratio 1:100) by Fourier transform infrared spectrometer Nicolet 67 in a range from 4000 to 400 cm^{-1}.

2.7. Nuclear magnetic resonance (NMR) spectra of the derivatives of LEP-2b

The samples (40 mg) LEP-2b, PLEP-2b and SLEP-2b were, respectively, dissolved in 2.0 mL D_2O for ^{13}C NMR experiment which was carried out at 125.76 MHz and 27 °C by nuclear magnetic resonance spectrometer Bruker Avance AV 500. In addition, NMR spectrometer Bruker Avance AV 400 was used to record the ^{31}P NMR spectrum of PLEP-2b at 161.97 MHz and 27 °C.

2.8. In vitro antioxidant activity of the derivatives of LEP-2b

2.8.1. Superoxide anion radical scavenging assay

Tris–HCl buffer (4.5 mL) at pH 8.2 was withdrawn and preheated by 25 °C water bath for 20 min. One milliliter polysaccharide (LEP-2b, PLEP-2b and SLEP-2b) solution at different concentrations of 0, 0.2, 0.4, 0.6, 0.8 and 1.0 mg mL^{-1} and 0.4 mL 25 mmol L^{-1} pyrogallic acid were sequentially added and evenly mixed. The reaction was performed at 25 °C for 5 min and stopped by 1 mL 8 mol L^{-1} HCl solution. In blank control group, acetone took the place of polysaccharide and also underwent the same rest procedure. Before the absorbance at 425 nm was measured, the absorbance of Tris–HC1 buffer was calibrated to zero.

Superoxide anion radical scavenging rate (%) = $[(A_0 - A_1)/A_0] \times 100\%$, in which A_0 and A_1, respectively, represented the absorbance of blank control and the sample. A relationship diagram was plotted with polysaccharide concentration as its abscissa and $\bullet O_2^-$ scavenging rate as its ordinate.

2.8.2. Hydroxyl radical scavenging assay

In 1 mL polysaccharide (LEP-2b, PLEP-2b and SLEP-2b) solution at different concentrations of 0, 0.2, 0.4, 0.6, 0.8 and 1.0 mg mL^{-1}, l mL 9 mmol L^{-1} $FeSO_4$ and 1 mL 9 mmol L^{-1} salicylic acid ethanol solution were added. The reaction was initiated by 1 mL 8.8 mmol L^{-1} and running at 37 °C for 0.5 h. The absorption was measured at 510 nm with distilled water as reference. To calculate the scavenging rate, the absorbance value of 8.8 mmol L^{-1} H_2O_2 solution was taken as the background absorption of the polysaccharides in consideration of the absorbance of polysaccharide itself.

Hydroxyl radical scavenging rate (%) = $\{[A_0 - (A_X - A_{X0})]/A_0\} \times 100\%$, where A_X was the absorbance of the solution with the polysaccharide (LEP-2b, PLEP-2b and SLEP-2b) in it, and A_0 was the absorbance of blank control, and A_{X0} was the background absorbance of the polysaccharide solution without H_2O_2. A graph was plotted with polysaccharide concentration as its abscissa and •OH scavenging rate as its ordinate.

2.8.3. Reducing power assay

One milliliter polysaccharide (LEP-2b, PLEP-2b and SLEP-2b) solution at different concentrations of 0, 0.2, 0.4, 0.6, 0.8 and 1.0 mg mL^{-1}, 0.5 mL 1% $K_3[Fe(CN)_6]$ and 0.2 mL 0.2 mol L^{-1} PBS (pH = 6.6) were mixed in test tubes and kept in 50 °C water bath for 20 min. After it was cooled down to room temperature, 1 mL 10% trichloroacetic acid was added, which was followed by centrifugation at 3000 r min^{-1} for 10 min. Supernatant (1.5 mL) was sucked up, after which, 3 mL distilled water and 0.2 mL 1% $FeCl_3$ aqua were successively added in and well shaken. Five minutes later, absorption at 700 nm (A_{700}) was measured using distilled water as a blank control for the zero set. A greater absorbance value means a stronger reducing power of the determinand. *Vc* at the same concentration with the polysaccharide was used as a positive control. Reducing power curve was drawn with polysaccharide concentration as its abscissa and A_{700} as its ordinate.

2.9. In vitro antitumor activity of the derivatives of LEP-2b (MTT assay)

Tumor cells (CT26, LLC and HepG2 cells) in the logarithmic phase of growth were digested by 0.25% pancreatin. After the concentration was adjusted to 1×10^4 individual mL^{-1}, the cell was transferred to 96-well plates containing 100 μL of cell suspension per well. After 24 h of the cultivation, supernatant was withdrawn, and 200 μL medium with polysaccharides of different concentrations of 0.5, 1, 5, 10, 50, 100 and 150 μg mL^{-1} were added in. In the standard control wells, 200 μL medium without polysaccharides was injected, but only 200 μL DMSO in blank control wells.

Cells in 96-well plates were grown in a humidified 5% CO_2 incubator at 37 °C. Forty eight hours later, the medium was removed, instead, Serum-free medium (180 μL) and MTT (20 μL, 5 mg mL^{-1}) were added, which was followed by incubation at 37 °C for 4 h. Microplate reader was used to measure the absorbance (A) at 630 nm. Inhibition ratio was calculated from the following formula. All determinations were carried out in triplicate.

$$\text{Inhibition ratio} = \left\{ \frac{\left[(A_{control} - A_{blank}) - (A_{drug} - A_{blank})\right]}{(A_{control} - A_{blank})} \right\} \times 100\%$$

2.10. Statistical analysis

All data were processed with statistical software DPS v6.55 and represented as mean ± standard deviation. *T*-test was used to analyze the variance between groups. $p < 0.05$ indicated a significant difference, and $p < 0.01$ indicated a very significant one.

3. Results and discussion

3.1. DS of the substituent in the derivatives of LEP-2b

Regression equations of the standard curve of phosphate anion (Fig. 1A) and standard curve of sulfate anion (Fig. 1B) were $Y = 0.0506X + 0.0315$ and $Y = 0.875X + 0.1346$, hereby, the contents of PO_4^{3-} from PLEP-2b and SO_4^{2-} from SLEP-2b were, respectively, calculated to be 6.79% and 17.07%. Therefore, the contents of phosphate group $-PO_3H_2$ and sulfate group $-SO_3H$ were accordingly 5.79% and 14.40%. So, in accordance with Eq. (1), the DS of $-PO_3H_2$ in PLEP-2b (DS_P) were 0.174, and the DS of $-SO_3H$ in SLEP-2b (DS_S) were 0.431.

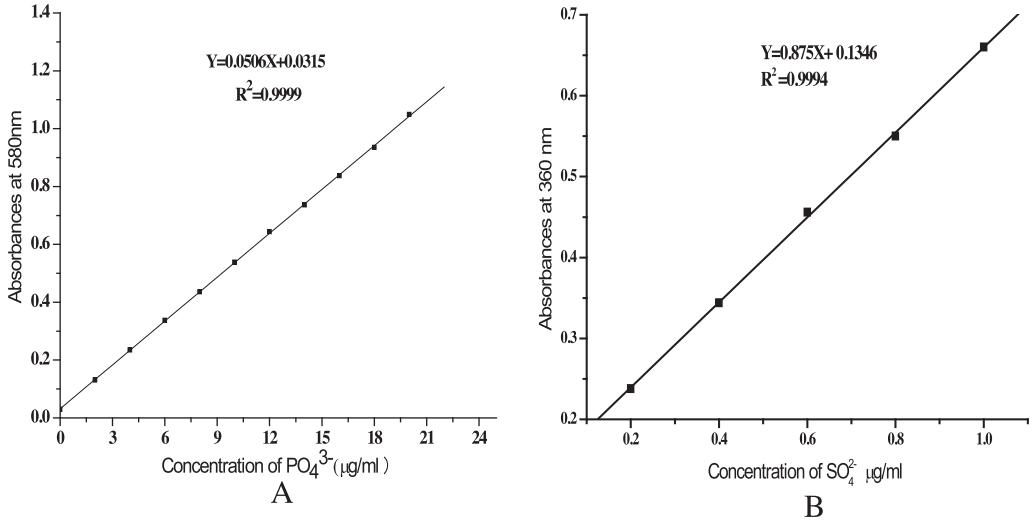

Fig. 1. Standard curves of phosphate anion (A) and sulfate anion (B).

3.2. Structural features of the derivatives of LEP-2b

Compared with LEP-2b, ultraviolet maximal absorption of PLEP-2b showed an obvious redshift from 192 to 196 nm due to the introduction of phosphate group, while the change of maximal absorption of SLEP-2b could be hardly observed, but a broad peak was newly arisen around 258 nm (Fig. 2A), which was caused by $n \rightarrow \pi^*$ transition of sulfate in SLEP-2b (Yang, Du, Wen, Li, & Hu, 2003), indicating that sulfate group was connected with LEP-2b.

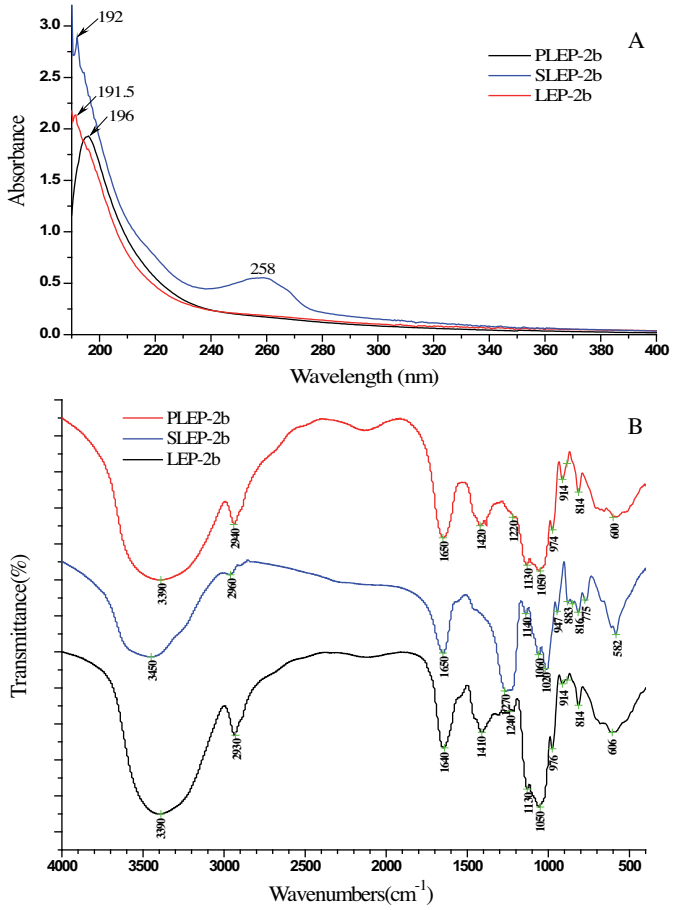

Fig. 2. Ultraviolet (A) and IR (B) spectra of LEP-2b, PLEP-2b and SLEP-2b.

Absorption peaks at 3400 cm^{-1}, 2940 cm^{-1} and 1410 cm^{-1} were, respectively, caused by stretching vibration of O—H, stretching vibration and bending vibration of C—H. After phosphorylation, a weak absorption due to P=O stretching vibration showed at 1220 cm^{-1} (Wang et al., 2013a,b) as well as a P—O—C stretching vibration peak near 1000 cm^{-1} (Fodor & Newman, 1979) (Fig. 2B), which was overlapped by characteristic absorption (1050 cm^{-1}) of pyranoid sugar residues (He et al., 2014). But, the profile, number, intensity and width of other absorption peaks were basically the same with those of LEP-2b, indicating that the fundamental structure did not change after phosphorylated modification, which probably due to the mild reaction condition and the low DS (DS_P = 0.174) of phosphate group in PLEP-2b.

While after sulfated modification, the characteristic peaks of carbohydrate rings changed to some extent, such as C—H stretching vibration peak of methylene near 2930 cm^{-1} (namely the characteristic absorption peak of C-6 on sugar residues) and C—H angular vibration peak of methyl and methylene at 1410 cm^{-1} were both obviously weakened (Fig. 2B). The steric hindrance effect of hydroxyl at C-6 was weak and did not play a role in the formation of hydrogen bond that could stabilize a higher structure, so the hydroxyl was more active (Wang & Fang, 1998), therefore substitution at C-6 easily occurred, as a result, had a relatively significant influence on methylene (Han, Xu, & Tao, 2005). Besides, in the spectrum of SLEP-2b, a newly arisen broad absorption peak at 1270 cm^{-1} was caused by stretching vibration of S=O in —O—SO_3H, and the absorption at 816 cm^{-1} was a result of C—O—S stretching vibration, overlapping the absorption peak (814 cm^{-1}) caused by C—H bending vibration of α-configurated sugar residues (He et al., 2014). Based on the above results, the conclusion could be drawn that sulfated modification was successfully done, furthermore, sulfate groups probably substituted at C-6 of the sugar residues.

Comparing the ^{13}C NMR spectra of PLEP-2b and LEP-2b (Fig. 3A and B), the following signal peaks almost entirely disappeared: δ 64.01 (C-6 of 1,4-β-D-Man*p*), 70.06 (C-5 of 2,6-β-D-1-OMe-Man*f*), 78.35 (C-3 of 1,6-β-D-Gal*p*), 73.58 (C-2 of 1-β-D-Glc*p*) and 21.58 ppm (C-6 of 1,2-α-D-Rha*p*) (Table 1). But the signals at δ 71.50 and 78.26 ppm were strengthened, what's more, a new peak showed up at δ 77.82 ppm (Fig. 3B). So, it was known that phosphate groups were successfully connected to C-6 of 1,4-β-D-Man*p*, C-5 of 2,6-β-D-1-OMe-Man*f*, C-3 of 1,6-β-D-Gal*p*, C-2 of 1-β-D-Glc*p* and C-6 of 1,2-α-D-Rha*p*.

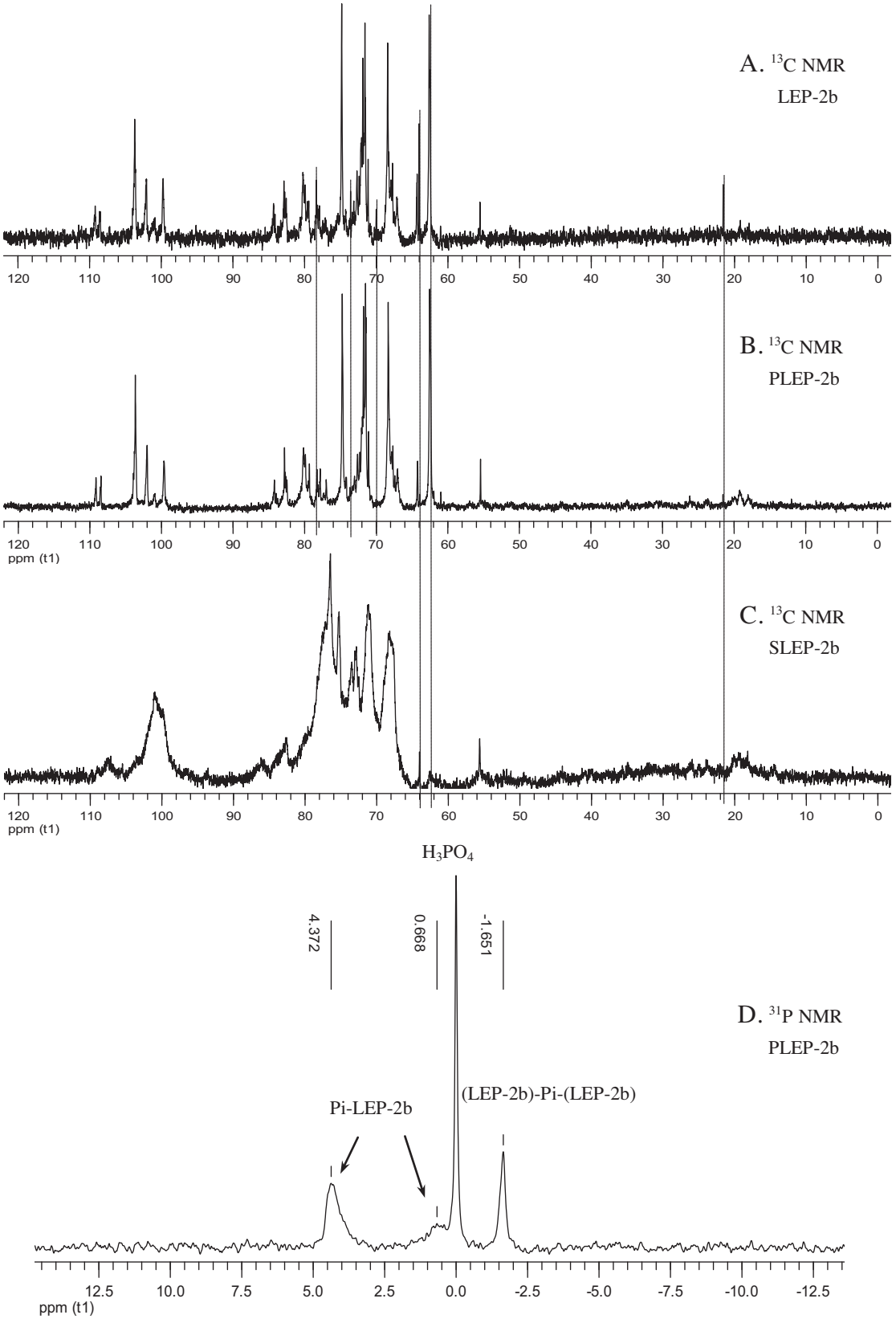

Fig. 3. ^{13}C NMR spectra of LEP-2b (A), PLEP-2b (B) and SLEP-2b (C), and ^{31}P NMR spectrum of PLEP-2b (D) at 27 °C (Pi: phosphate group).

Compared with LEP-2b, various carbon signals in ^{13}C NMR spectrum of SLEP-2b seriously overlapped with one another (Fig. 3C) because of the impact of sulfate group that the change of chemical environment around every group weakened the ^{13}C NMR resonance signals (Chen et al., 2006). Although it could not be analyzed in detail, it could be clearly identified that the peaks at δ 64.01 (C-6 of 1,4-β-D-Man*p*), 62.50 (C-6 of 1-β-D-Glc*p*), 62.61 (C-6 of 1-β-D-Glc*p*) and 21.58 ppm (C-6 of 1,2-α-D-Rha*p*) were almost totally

Table 1
^{13}C NMR chemical shift (unit: ppm) data of LEP-2b (He et al., 2014).

Residue	C-1	C-2	C-3	C-4	C-5	C-6
→4)-β-Man*p*-(1→	102.05	80.23	72.68	80.32	71.66	64.01
→3,6)-α-Man*p*-(1→	100.87	68.71	68.71	67.06	71.71	67.45
→2)-α-Rha*p*-(1→	100.99	78.19	71.13	73.66	71.13	21.58
→6)-β-Glc*p*-(1→	103.83	73.58	84.30	70.00	74.81	68.40
→2,3,6)-β-Glc*p*-(1→	103.59	73.12	84.33	68.56	74.99	67.93
→6)-β-Gal*p*-(1→	102.08	82.55	78.35	71.57	71.74	68.71
→2)-α-1-OMe-Man*f*-(6→	108.50	84.12	78.26	82.88	72.38	64.33
→2)-β-1-OMe-Man*f*-(6→	109.22	84.30	77.92	82.97	70.06	64.28
→6)-α-Gal*p*-(1→	99.73	71.50	72.04	71.86	72.14	70.00
→1)-β-Glc*p*	103.70	73.58	78.35	71.13	78.02	62.50 & 62.61

disappeared (Table 1, Fig. 3C). In consequence, sulfate group —SO_3H was identified to be at C-6 of 1,4-β-D-Man*p*,C-6 of 1-β-D-Glc*p* and C-6 of 1,2-α-D-Rha*p*. In Fig. 3D, the peak at δ 0 ppm was the signal of external standard H_3PO_4 (Abeygunawardana, Bush, & Cisar, 1991). In combination with the results of ^{13}C NMR analysis, ^{31}P in phosphate group —PO_3H_2 at C-3 of 1,6-β-D-Gal*p* residue in LEP-2b phosphomonoester gave rise to the resonance at δ 4.372 ppm, and ^{31}P in -PO_3H_2 at C-6 of 1,4-β-D-Man*p* and 1,2-α-D-Rha*p* residues gave rise to the resonance at δ 0.668 ppm (Muhrbeck & Tellier, 1991). Both of the two resonance peaks were relatively broad, which probably because the resonances of ^{31}P in —PO_3H_2 at other sites, like C-5 of 2,6-β-D-1-OMe-Man*f* or C-2 of 1-β-D-Glc*p*, also contributed to the peaks at δ 4.372 and 0.668 ppm. Resonance at δ −1.651 ppm was assigned to ^{31}P in LEP-2b phosphodiester (Garrido et al., 2012).

To sum up, two types of polysaccharide phosphate (PLEP-2b) phosphomonoester and phosphodiester of LEP-2b were obtained as well as a polysaccharide sulfate (SLEP-2b). Although, the position of phosphodiester bonds in the phosphodiester was unknown, it was certain that phosphate groups —PO_3H_2 substituted at C-6 of 1,4-β-D-Man*p*, C-5 of 2,6-β-D-1-OMe-Man*f*, C-3 of 1,6-β-D-Gal*p*, C-2 of 1-β-D-Glc*p*, and C-6 of 1,2-α-D-Rha*p* in the phosphomonoester, while sulfate groups —SO_3H were mainly at C-6 of 1,4-β-D-Man*p*, 1-β-D-Glc*p* and 1,2-α-D-Rha*p*. These substitutions by phosphate or sulfate groups not only significantly altered the original primary structure of the polysaccharide including main and branch chains, but also affected its high order structures, including chain flexibility, spatial configuration and so on, based on which, the activities, such as antioxidation or antitumor, were probably notably changed (Huang, Kong, Wang, & Hu, 2007; Nie & Xie, 2011).

3.3. *In vitro antioxidant activity of the derivatives of LEP-2b*

Superoxide anion radical ($\bullet O_2^-$) scavenging rate of LEP-2b, PLEP-2b and SLEP-2b increased with their increasing concentration, and all attained their respective maximum value 45.2%, 63.9% and 66.7% at 1 g L^{-1} (Fig. 4A). According to the comparison of half maximal effective concentration (EC_{50}) of the three polysaccharides, it could be concluded that scavenging effects of PLEP-2b and SLEP-2b on $\bullet O_2^-$ were significantly stronger than that of LEP-2b, moreover, the scavenging rate of PLEP-2b was slightly greater than that of SLEP-2b (Table 2). Scavenging effect of LEP-2b, PLEP-2b and SLEP-2b on •OH enhanced with their growing concentration, and PLEP-2b exhibited the highest ability to scavenge •OH. The effect of LEP-2b, PLEP-2b and SLEP-2b, respectively, reached 43.2%, 69.5% and 58.6% at 1 g L^{-1} (Fig. 4B). Further considering EC_{50} of the three polysaccharides, it was concluded that the scavenging activities of PLEP-2b and SLEP-2b were stronger than that of LEP-2b. Compared with LEP-2b, reducing powers of SLEP-2b and PLEP-2b were greater and were all concentration dependent (Fig. 4C). Reducing powers of PLEP-2b and SLEP-2b, respectively, reached 0.316 and 0.376 at 1 g L^{-1}, which were indistinctively greater than that of LEP-2b (0.255).

In LEP-2b, glucosidic bonds among mannose, galactose and glucose residues were easily cut off by free radical oxidation, therefore it had a scavenging effect on •OH and $\bullet O_2^-$ to some extent (Zhao et al., 2011).

Compared with LEP-2b, the ability of PLEP-2b to scavenge $\bullet O_2^-$ was significantly enhanced, which was similar to the result reported by Guo et al. (2013). However, there were also studies showed that after phosphorylation, the scavenging effect of polysaccharides on $\bullet O_2^-$ did not significantly strengthened (Liu et al., 2013; Wang et al., 2013a,b), or even opposite, weakened by phosphorylation (Zhang et al., 2009). This phenomenon illustrated that mere introduction of phosphate group may not increase the ability to scavenge $\bullet O_2^-$, other factors like DS and substituent position also strongly affected the scavenging ability. Hydroxyl radical generated from superoxidation by hydrogen peroxide with metal ions, usually Fe^{2+} or Cu^{2+}, and phosphate group with strong nucleophilic properties could chelate metal ions, so the polysaccharide exhibited a stronger scavenging activity after phosphorylation (Wang et al., 2009). Compared with LEP-2b, reducing power of PLEP-2b showed a slight increase, which was similar with the results of the literatures (Liu, Luo, Ye, & Zeng, 2012; Wang et al., 2009), however, not as significant as the results of Chen et al. (2011). But, there was also another study indicated that reducing power nearly lost after phosphorylated modification (Zhang et al., 2009). The reason why phosphorylation brought about various results, even contradictory ones, was possibly owing to the fact that electron donating ability of the polysaccharide was enhanced by the phosphate group at some specific sites of the residues, but weakened at others (Liu et al., 2012).

Compared with LEP-2b, SLEP-2b exhibited a stronger ability to eliminate free radicals (•OH and $\bullet O_2^-$), which resembled other studies (Tsiapali et al., 2001; Wang et al., 2010; Zou et al., 2008). It was reported that polysaccharide with different DS of sulfate group could improve the reducing power to varying degrees (Wang et al., 2010; Zou et al., 2008). So, we deduced that, because of the relatively low DS, sulfated modification did not significantly improve the reducing power of LEP-2b. However, the optimum degree of DS of SLEP-2b which is able to bring the strongest antioxidant activity still needs more study.

3.4. *In vitro antitumor activity of the derivatives of LEP-2b*

Table 2 half maximal effective concentration (EC_{50}) of LEP-2b, PLEP-2b and SLEP-2b scavenging free radicals, and their half maximal inhibitory concentration (IC_{50}) against the tumor cells. The result of MTT assay showed that, compared with LEP-2b, the cytotoxicity of PLEP-2b and SLEP-2b towards CT26, LLC and HepG2 cells were significantly heightened and behaved in a dose-dependent manner (Fig. 4D–F; Table 2). Inhibition rates of LLC cells growth attained as high as 79.5% and 66.38% when treated with PLEP-2b and SLEP-2b at 150 μg mL^{-1} (Fig. 4E). The calculated half maximal inhibitory concentration (IC_{50}) of PLEP-2b against CT26, LLC and

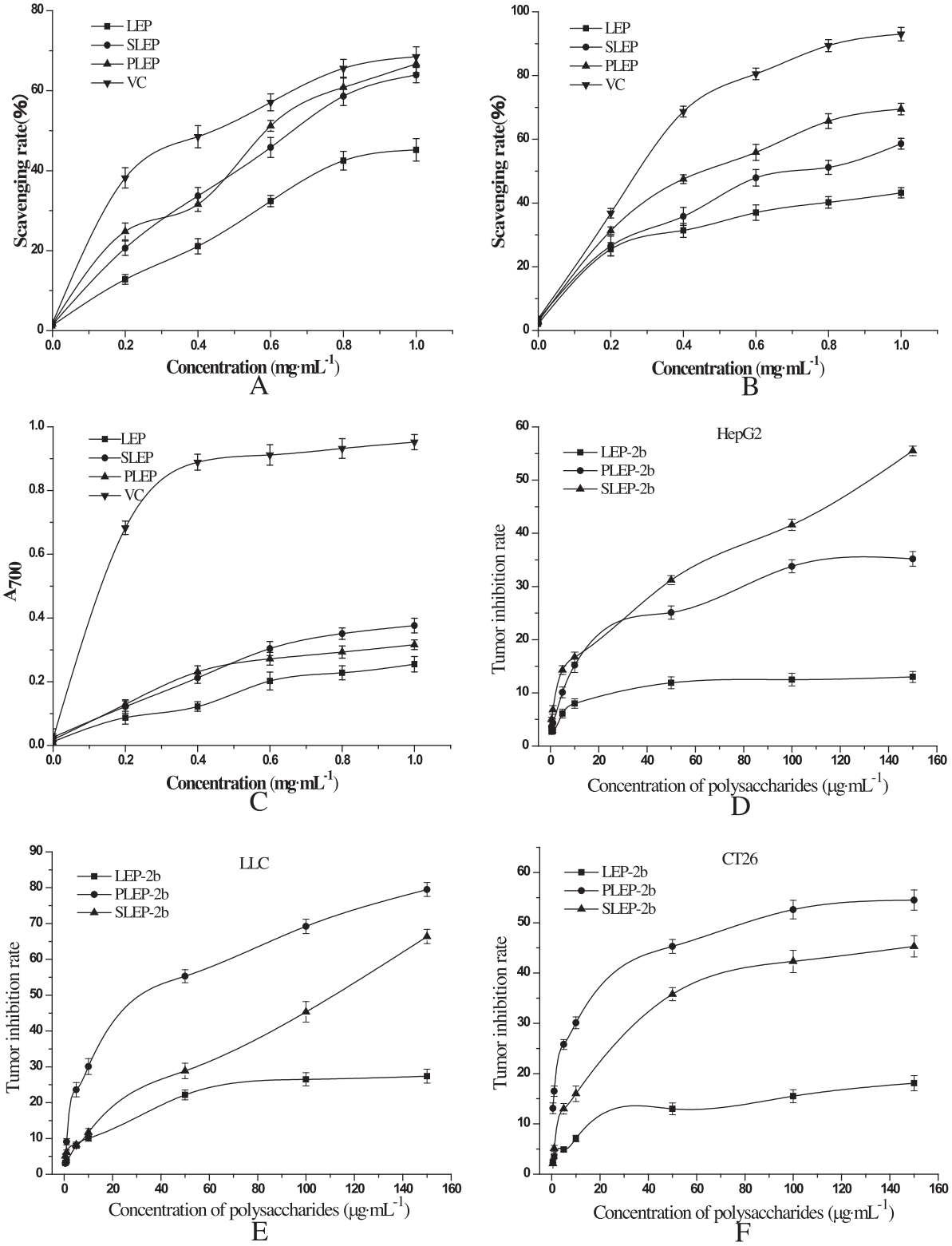

Fig. 4. Scavenging effect of LEP-2b, PLEP-2b and SLEP-2b on $\bullet O_2^-$ (A), •OH (B) and their reducing power (C); inhibitory effect on the proliferation of CT26 colon carcinoma cells (CT26, D), Lewis lung carcinoma cells (LLC, E) and Human hepatocellular carcinoma HepG2 cells (HepG2, F).

HepG2 cells, respectively, were 85.78 μg mL^{-1}, 29.97 μg mL^{-1} and 437.48 μg mL^{-1}. Compared with LEP-2b, IC_{50} of PLEP-2b against the three types of tumor cells were all remarkably decreased, but varied from one another, what's more, PLEP-2b showed the strongest cytotoxicity to LLC cells (Table 2). As well as PLEP-2b, IC_{50} of SLEP-2b significantly decreased, but it exhibited relatively strong inhibiting effect on all of the tumor cells without any significant difference among one another. Polysaccharide phosphates were reported to have favorable inhibitory effect on BGC-823 human gastric cancer cells and mouse sarcoma 180 (S180) cells (Huang and Zhang, 2011; Liu et al., 2012), which was considered to be close to our result. Through our research, a deeper speculation was made that

Table 2
Half maximal effective concentration (EC_{50}) of LEP-2b, PLEP-2b and SLEP-2b scavenging free radicals, and their half maximal inhibitory concentration (IC_{50}) against tumor cells.

Free radicals	Polysaccharide	EC_{50} (mg·mL^{-1})	Tumor cells	Polysaccharide	IC_{50} (μg mL^{-1})
•O_2^-	LEP-2b	1.15	CT26	LEP-2b	8,816.27
	PLEP-2b	0.59		PLEP-2b	85.78
	SLEP-2b	0.64		SLEP-2b	154.52
•OH	LEP-2b	1.76	LLC	LEP-2b	1,002.29
	PLEP-2b	0.44		PLEP-2b	29.97
	SLEP-2b	0.71		SLEP-2b	139.83
–	–	–	HepG2	LEP-2b	36,104.07
	–	–		PLEP-2b	437.48
	–	–		SLEP-2b	139.82

phosphorylated polysaccharide was likely to only show strong cytotoxicity to some specific tumor cells, rather than all.

Inhibition of the sulfated polysaccharide we used here against HepG2 cells were extremely close to the results of other related reports (Abd El-Baky et al., 2009; Gamal-Eldeen, Amer, & Helmy, 2006; Tao, Zhang, & Cheung, 2006; Ye, Wang, Zhou, Liu, & Zeng, 2008). Contrasting the characteristics of these polysaccharides, we found it that the polysaccharides either very resembled one another in the aspects of molecular weight (M_w) and DS (Gamal-Eldeen et al., 2006; Tao et al., 2006; Ye et al., 2008) or had a extraordinarily similar monosaccharide composition (Abd El-Baky et al., 2009; Gamal-Eldeen et al., 2006). Therefore, Mw, DS and monosaccharide composition of the sulfated polysaccharides had significant influences on their antitumor activities *in vitro*. The inhibitory effect of the sulfated polysaccharide SLEP-2b on CT26 cells was similar with that of the sulfated polysaccharide reported by Athukorala et al. (2009), but differed quite a bit in their Mw and monosaccharide composition, which possibly caused by the structural differences between chemically modified sulfated polysaccharides and natural ones, or we could put it in this way that the chemical modification altered some special structures of natural polysaccharides. For instance, after sulfated modification with chemical method, the ring conformation of the polysaccharide may distorted which was conducive to form a covalent bond. Meanwhile, the curled conformation of the polysaccharide, which was caused by the mutually repel effect of same charged group, would become stretch or rigid (Deng, Yang, Wang, Xu, 2000). In other words, the structure of the sulfated polysaccharide would be more orderly than before. These may the causes of differences in biological activity before and after the modification (Deng et al., 2000).

Phosphorylated modification could significantly improve the inhibitory effect of polysaccharides on the proliferation of tumor cells *in vitro*, especially LLC cells, and sulfated modification also facilitated the *in vitro* antitumor activity of the polysaccharide. However, some great distinctions from other reports, even opposition, still existed (Chen et al., 2011a,b, 2010a,b; Liu et al., 2009). These results suggested that the inhibition of phosphorylated or sulfated polysaccharide was a combined result contributed by its Mw, monosaccharide composition, DS, solubility and chain conformation, rather than just one factor of them.

Taken together, phosphorylation and sulfation significantly enhanced both antioxidant and antitumor activities of the polysaccharide, which was in accordance with the results that free radicals played very important roles in tumor genesis and tumor promotion. For instance, free radicals can act as initiators and/or promoters, cause DNA damage, and alter the cellular antioxidnt defense system. Antioxidants, the free radicals scavengers, however, are shown to be anticarcinogens (Sun, 1990). Moreover, oxygen free radicals (ORF) related lesions can stimulate the development of cancer, as well as a large body of evidence suggests important roles of OFR in the expansion of tumor clones and the acquisition of malignant properties (Dreher & Junod, 1996). So, the enhancement of antitumor activity probably partly resulted from the promoted scavenging effect of the polysaccharide on •O_2^- and •OH.

4. Conclusions

After the exopolysaccharide LEP-2b from *Lachnum* YM 405 was chemically modified, phosphate groups —PO_3H_2 substituted at C-6 of 1,4-β-D-Man*p*, C-5 of 2,6-β-D-1-OMe-Man*f*, C-3 of 1,6-β-D-Gal*p*, C-2 of 1-β-D-Glc*p* and C-6 of 1,2-α-D-Rha*p*, and sulfate groups —SO_3H mainly substituted at C-6 of 1,4-β-D-Man*p*, C-6 of 1-β-D-Glc*p*, C-6 of 1,2-α-D-Rha*p*. Both phosphorylated and sulfated modifications significantly enhanced the antioxidant and antitumor activities *in vitro*, and compared with sulfated modification, phosphorylated modification improved the inhibitory effect on some specific tumor cells more contraposingly. The bioactivities of the polysaccharide derivatives were not only related to DS and substitution site, but also other factors like molecular weight, monosaccharide composition, solubility and chain conformation. The phosphorylated and sulfated polysaccharides were expected to develop into functional foods or polysaccharide drugs.

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