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Chemical characterization, antioxidant and antitumor activity of sulfated polysaccharide from *Sargassum horneri*

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ABSTRACT

Three water-soluble polysaccharide fractions (SHP30, SHP60, and SHP80) extracted from the *Sargassum horneri* were obtained by water extraction and radial flow chromatography. The high-performance gel-permeation chromatography analysis showed that the average molecular weight (Mw) of three polysaccharides were approximately 1.58×10^3, 1.92×10^3 and 11.2 KDa, respectively. Their *in vitro* antioxidant activities, antitumor activities were investigated and compared. Among these three polysaccharides, SHP30 with the highest sulfate content and intermediate molecular weight exhibited excellent antioxidant and antitumor activities in the superoxide radical assay, hydroxyl radical assay, reducing power assay, and MTT assay. Then, flow cytometry assay and quantitative real-time reverse transcription-PCR analysis suggested that the accumulation of cells in G_0/G_1 and *S* phase effecting apoptosis-associated gene expressions such as Bcl-2 and Bax might account for the growth inhibition of DLD cells by SHP30. Based on these results, we have inferred that sulfate content and molecular weight were the factors influencing antioxidant and antitumor activities.

1. Introduction

Seaweeds have caused an emerging interest in the biomedical area, mainly due to their contents of bioactive substances which show great potential and advantages as biological medicine production resource (Ye, Wang, Zhou, Liu, & Zeng; Chakraborty & Paulraj, 2010; Wijesinghe, Athukorala, & Jeon). In particular, polysaccharides from marine algae are known to exhibit various biological and physiological activities including antioxidant, anticoagulant, antiviral, antitumor, and antiinflammatory activities (Wang et al., 2012; Becker, Guimarães, Mourão, & Verli, 2007). Indeed, several species of brown algae have been found to contain polysaccharides and glycoproteins with bioactivities (Andradea et al., 2010; Ermakovaa et al., 2013; Ngo & Kim, 2013).

But only a limited numbers of algae species, however, are available for practical use. Among the remaining unexplored species is *Sargassum horneri*, a brown alga growing in the coastal sea, has been used as a food only in limited areas in Japan, but the processed product obtained by boiling has recently appeared in the Japanese market. Little information is available about its chemical composition and nutritional evaluation (Murakami et al., 2011). To enable the practical use of *Sargassum horneri* as a medicinal marine resource, it is important to obtain information about its chemical composition and bioactivities.

Antioxidant and antitumor activities of sulfated polysaccharide isolated from marine algae have been studied by many scientists (Vijayabaskar, Vaseela, & Thirumaran, 2012; Suresh et al., 2013). However, little information was available about the mechanisms and relationships between their biological activities. Therefore, the further evaluation of the polysaccharides bioactivities of algae seems to be imperative for the utilization of these marine resources.

In this study, three different fractions of polysaccharides were obtained by water extraction and radial flow chromatography (RFC) from one brown alga *Sargassum horneri*. The chemical and monosaccharide compositions of polysaccharides were determined. The *in vitro* antioxidant and antitumor activities of all the three polysaccharides were evaluated by superoxide radical assay, hydroxyl radical assay, 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) assay, 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, ferric reducing antioxidant power (FRAP) assay and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays using MKN45 gastric cancer cells and DLD intestinal cancer cells. Furthermore, the apoptotic effect of SHP30 on DLD cells was analyzed by flow cytometry assay and quantitative real-time reverse transcription-PCR analysis since many polysaccharides with antitumor activity act by inducing apoptosis.

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2. Materials and methods

2.1. Materials and chemicals

The *Sargassum horneri* was collected on the coast of Nanji Archipelago (Zhejiang province, China). The authentication of species was done by Prof. Xingqian Ye (Zhejiang University, Hangzhou, China). The collected samples were washed, air-dried and cultured in plastic bags at room temperature.

2,2-Azinobis-3-ethylbenzthiazoline-6-sulfonate (ABTS), sodium borohydride ($NaBH_4$), nitro blue tetrazolium (NBT), phenazine methosulfate (PMS), ferrous sulphate ($FeSO_4$), potassium ferricyanide ($K_3Fe(CN)_6$), hydrogen peroxide (HO), trichloroacetic acid (TCA), ferric chloride ($FeCl_3$), nicotinamide adenine dinucleotide-reduced (NADH), 1,1-diphenyl-2-picrylhydrazyl (DPPH), thiazolyl blue (MTT) and dimethyl sulfoxide (DMSO), were purchased from Sigma Chemical Co. Ion-exchange resins were obtained from Purolite International Ltd. The RFC column used in the experiment was purchased from Sepragen Corporation, USA. Real-time PCR Master Mix (SYBR Green) was purchased from Toyobo Co., Ltd. The FACSCalibur flow cytometry were purchased from Becton-Dickinson Co. All other chemical reagents used in this experiment were of analytical grade and purchased locally.

2.2. Analytical methods

The content of total sugar was determined by the method of phenol-sulfuric acid (Dubois, Gilles, Hamilton, Rebers, & Smith). The content of sulfate was determined by barium chloride–gelatin method (Kawai et al., 1969). The IR spectra of the polysaccharides were determined using a Fourier transform infrared spectrometer (FTIR) (PerkineElmer 16PC spectrometer, Boston, USA).

2.3. Extraction of polysaccharides

The air shade dried algae were smashed and screened through mesh size of 100, and were then treated with water extraction methods. Briefly, 30 g of algae powder was mixed with the distilled water at a mass/volume ratio of 1:65. The extraction time was 2 h, and the extraction temperature was 95 °C. After cooling and standing for 1 h, the slurry was filtered and centrifuged at 10,000 rpm for 10 min. Then the supernate was decanted and concentrated to an approximate volume of 180 mL by rotary evaporator. Afterwards, the crude polysaccharides were successively sub-fractionated by graded precipitation at final ethanol concentrations of 30%, 60% and 80%, and obtained by lyophylization.

2.4. Separation of polysaccharides by radial flow chromatography

Three kinds of polysaccharides were obtained after separation by radial flow chromatography, named SHP30, SHP60 and SHP80, respectively. The details were as follows: The above crude polysaccharides extracted from *Sargassum horneri* were configured to 10 mg/mL feed solution with distilled water, then the feed solution was centrifuged at 10,000 rpm, 4 °C for 15 min. The supernatant was pretreated through a membrane with the pore size of 0.45 μm. Then the polysaccharides separated by filtration were purified by ion-exchange chromatography in radial flow column. The crude samples were loaded onto the radial flow column at a flow rate of 5 mL/min. Then the column was washed with distilled water at a flow rate of 40 mL/min. Fractions (8 mL/each tube) were collected at a flow rate of 4 mL/min and monitored using the phenol-sulfuric acid method at 490 nm. After a process purification of the crude polysaccharides, resin regeneration was carried out. The ion exchange resins in the radial flow column were eluted with 2 mol/L NaCl solution at a flow rate of 40 mL/min until the absorbance at 280 nm of the eluent fell to the baseline, then washed with distilled water. At last, the polysaccharide was obtained after condensing under vacuum and drying by lyophylization.

2.5. Molecular weight and monosaccharide composition analysis

The relative molecular weight of SHP was estimated on an Agilent 1100 HPLC system equipped with a refractive index detector (RID) and a TSK-GEL G3000SWxl column (7.5 × 300 mm, Tosoh Corp., Tokyo, Japan). The column was eluted with 0.01 mol/L sodium phosphate buffer (pH 7.0) containing 0.2 mol/L Na_2SO_4 at a flow rate of 0.7 mL/min. Pullulan P-800, P-400, P-200, P-100, P-20, P-10 and P-5 were used as the standards for molecular weight measurement.

The monosaccharide compositions of three sulfated polysaccharides were obtained by reductive hydrolysis. First, 20 mg of sample was hydrolyzed with 2 M trifluoroacetic acid (TFA) at 110 °C for 3 h. The excess acid was removed by vacuum evaporation with methylalcohol (MeOH) after the hydrolysis. The hydrolyzed products were reduced by $NaBH_4$ and acetylated with acetic anhydride, and the standard monosaccharide was derived with same method. Then the alditol acetates were analyzed by gas chromatography (GC) using an Agilent 7890N instrument equipped with an HP-5 capillary column (30 m × 0.32 mm × 0.25 μm) and a flameionization detector (FID). The applied temperature program was as follows: Oven temperature was initially set at 120 °C, increasing to 240 °C at a rate of 10 °C/min and then held at 240 °C for 6.5 min. The heater temperatures of both injector and detector were kept at 250 °C Nitrogen was used as the carrier gas. Quantitation was calculated from the peak area using response factors.

2.6. Antioxidant activities in vitro

2.6.1. Superoxide radical assay

The superoxide radical scavenging abilities of the sample were assessed by the method of Robak and Gryglewski (1988), with a minor modification. Samples were firstly dissolved in distilled water at different concentrations. Then, 0.1 mL of sample solution, 1 mL of 16 mM Tris–HCl (pH 8.0) containing 557 μM NADH, 1 mL of 16 mM Tris–HCl (pH 8.0) containing 45 μM PMS, and 1 mL of 16 mM Tris–HCl (pH 8.0) containing 108 μM NBT were mixed. The reaction mixture was incubated at room temperature for 5 min and the absorbance was read at 560 nm by a spectrophotometer against blank samples. Decreased absorbance of the reaction mixture indicated increased superoxide anion-scavenging activity. The capability of scavenging the superoxide anion radicals was calculated using the following equation:where A_0 is the absorbance of mixture solution without sample; A_i is the absorbance of the test sample mixed with reaction solution.

2.6.2. Hydroxyl radical scavenging assay

The hydroxyl radical assay of samples was measured according to the method described by Ghiselli, Nardini, Baldi, and Scaccini (1988), with a minor modification. Briefly, sample was dissolved in distilled water at 0.5–6 mg/mL. 0.1 mL of sample solution was mixed with 0.6 mL of reaction buffer [0.2 M phosphate buffer (pH 7.4), 2.67 mM deoxyribose, and 0.13 mM EDTA], 0.2 mL of 0.4 mM ferrous ammonium sulfate, 0.05 mL of 2.0 mM ascorbic acid, and 0.05 mL of 20 mM H_2O_2 were then added to the reaction solution. The reaction solution was incubated at 37 °C for 15 min, and then 1 mL of 1% thiobarbituric acid and 1 mL of 2.0% trichloroacetic acid were added. Afterwards, the mixture reacted at 100 °C for 15 min and cooled down with ice. The presence of hydroxyl radical was detected by monitoring absorbance at 532 nm. The control group contained all the reaction reagents without the samples was prepared and measured as A_0. A_i was the result of samples, and A_j

contained all the samples except that the phosphate buffer (20 mM pH 7.4) was replaced with milliliter phosphate buffer (20 mM pH 7.4, containing 0.1 mmol ferric chloride, 0.1 mmol EDTA, 2.8 mmol deoxyribose), 0.1 mL Vit C (1 mmol) and 0.5 mL hydrogen peroxide (20 mM). The hydroxyl radical scavenging ratio was calculated by the following equation:

2.6.3. ABTS radical scavenging activity assay

The ABTS assay was also employed to measure the antioxidant activity of all the three kinds of polysaccharides (Re et al., 1999). Briefly, ABTS radical cation solution was prepared by 16 h of reaction of ABTS (7 mmol) with potassium persulfate (2.45 mmol) at room temperature in dark. The solution was diluted with ethanol to obtain an absorbance of 0.700 ± 0.005 at 734 nm. The decolorisation assay started by mixing the diluted ABTS solution (2 mL) with different polysaccharides (20 μL, 0.5–6 mg/mL). The mixture was allowed to react at 25 °C for 6 min and the absorbance at 734 nm was recorded as A_i. A control sample containing the same amount of ethanol and ABTS radical was measured as A_0. The absorbance of polysaccharides solution and ethanol with the same amount of ABTS was recorded as A_j. Fresh stocks of ABTS solution were prepared every five days due to self-degradation of the radical. The activity to scavenge ABTS radical was calculated as a percentage according to the following equation:

2.6.4. DPPH radical scavenging activity assay

The free-radical scavenging capacity of the sulfated polysaccharides was analyzed using the DPPH test (Souza et al., 2012). Briefly, 0.2 mL of MeOH and 0.3 mL of various sample concentrations (0.5–6 mg/mL) dissolved in MeOH were mixed in a 10 mL test tube. DPPH (2.5 mL of 75 μM in MeOH) was then added to achieve a final volume of 3.0 mL. The solution was kept at room temperature for 30 min, and the absorbance at 517 nm was measured. The DPPH scavenging effect was calculated as follows:

$$\mathrm{DPPHscavengingrate}(\%) = [1 - (A_i - A_j)/A_0] \times 100\%$$

Here, the mixture of 3 mL of DPPH-ethanol solution plus 0.1 mL sample solution was used as A_i, the mixture of 3 mL ethanol plus 0.1 mL sample solution was used as a blank A_j and the mixture of 3 mL of DPPH-ethanol solution plus 0.1 mL ethanol was used as a negative control A_0.

All the results of the four radical scavenging activity assays were summarised in Fig. 2.

2.6.5. FRAP assay

The reductive potential of samples was determined according to the method of Tounsi et al. (2011) with some modification. 2.5 mL samples (0.5–6 mg/mL), 2.5 mL pH 6.6 phosphate buffer (0.2 mol/L) and 2.5 mL 1% (w/v) $K_3Fe(CN)_6$ were incubated at 50 °C for 20 min. The reaction was terminated by adding 2.5 mL trichloroacetic acid (10%, w/v), and centrifuged at 2000 rpm for 10 min. The upper layer (5.0 mL) was mixed with 5 mL water and 1 mL 0.1% (w/v) $FeCl_3$. The mixture was shaken, and its absorbance was measured at 700 nm against a blank. The increase of reaction mixture absorbance indicates an increase of reduction capability.

2.7. Antitumor activities in vitro

The inhibition effects of SHP30, SHP60 and SHP80 on the growths of MKN45 gastric cancer cells and DLD intestinal cancer cells were evaluated *in vitro* by MTT assay (Xin et al., 2012). Cells in their exponential growth phase were seeded into flat-bottomed 96-well plates at a density of 1×10^5 cells per well and incubated for 24 h at 37 °C in CO_2 incubator, then were washed with 0.10 mL of PBS. Afterwards, 100 μL of different concentrations of polysaccharides (0.5, 1.0, 2.0, 4.0 and 6.0 mg/mL), prepared in culture medium, was added to each well. After 48 h of exposure, the polysaccharide-containing medium was removed, washed with 0.10 mL of PBS and replaced by fresh medium. The cells in each well were then incubated in culture medium with 0.02 mL of 5 mg/mL solution of MTT for 4 h. After the media were removed, 0.15 mL of DMSO was added to each well. The absorbance was read at 570 nm using enzyme-linked immuno sorbent assay (ELISA) plate reader. The inhibition ratio of the treated cells was calculated based on the following formula:

$$\mathrm{Inhibitionratio}(\%) = (1 - A_{570}\mathrm{oftreatedcells}/A_{570}\mathrm{ofcontrolcells}) \times 100\%$$

2.8. DLD cell cycle analysis

Based on the results in MTT assays, SHP30 exhibited better inhibitory effect on DLD cell. Therefore, the working mechanisms of SHP30 on DLD cell were further studied.

The effect of SHP30 on DLD cell cycle phase distribution was assessed using flow cytometry by the methods of Ma et al. (2013) with some modification. Briefly, the cells were seeded in 25 cm^2 flasks (7×10^5 cells/flask) and allowed to adhere for 48 h, followed by treatment with different concentrations (0, 1 and 2 mg/mL) of SHP30 for 48 h. After treatment, the cells were harvested by trypsinization, washed with PBS twice, centrifuged at 2000 rpm for 5 min and fixed overnight in 70% ethanol solution at 4 °C. The fixed cells were washed twice with PBS, resuspended in 1 mL of PBS containing RNaseA (100 U) and propidium iodide (PI, 50 μg/mL), and incubated at room temperature for 60 min in the dark. The stained cells were then transferred to flow tubes, and the DNA contents in the cells were analyzed by FACSCalibur flow cytometry.

2.9. Quantitative real-time reverse transcription-PCR analysis

The mRNA expression levels for Bax and Bcl-2 were determined by quantitative real-time reverse transcription-PCR (Ma et al., 2013; Hou et al., 2008). Total cellular RNA was isolated from Trizol reagent according to the manufacturer's instructions. RNA concentration and purity were estimated from the optical density at 260 and 280 nm, respectively. cDNA was synthesized using random primer and Prime Script reverse transcriptase. For quantitative analysis of mRNA of Bcl-2 and Bax, the primers used in the reaction and β-actin gene served as endogenous reference were as follow: β-actin, 5'-GCAGAAGGAGATCACTGCCCT-3' and 5'-GCTGATCCACATCTGCTGGAA-3'; Bax, 5'-GACCCGGTGCC-TCAGGATGC-3' and 5'-GTCTGTGTCCACGGCGGCAA-3', Bcl-2, 5'-ATCCAGGATAACGGAGGC-3' and 5'-CAGCCAGGAGAAATCAAAC-3'. Quantitative PCR for target gene was carried out using SYBR green qPCR kit on a fluorescent temperature cycler. cDNA templates (2 μL) were amplified in a final volume of 20 μL containing the SYBR Green PCR Master mix and primer. Melt-curve analysis was used to confirm amplicon specificity. The length of the amplified product was confirmed using 2% agarose gel electrophoresis. Relative quantification was performed using the $2^{-\Delta\Delta ct}$ method (Livak & Schmittgen, 2001). Relative expression ratios were expressed as fold changes of mRNA abundance in the treatment group compared with the model control group.

2.10. Statistical analysis

All experiments were performed in triplicate and the results were the average of three independent experiments. Statistical analysis was done by using the Statistical Analysis Systems

Table 1
The chemical composition and monosaccharide composition of SHP.

Samples		SHP30	SHP60	SHP80
Chemical composition	Total sugars (%)	19.23	47.05	69.16
	Sulfate (%)	19.41	13.15	11.4
Monosaccharide (Molar ratio)	Glucose	24.95	48.04	100
	Rhamnose	60.63	32.19	0
	Mannose	8.09	6.93	0
	Galactose	6.33	10.01	0
	Gylose	0	2.83	0
Average molecular weight (KDa)		1.58×10^3	1.92×10^3	11.2

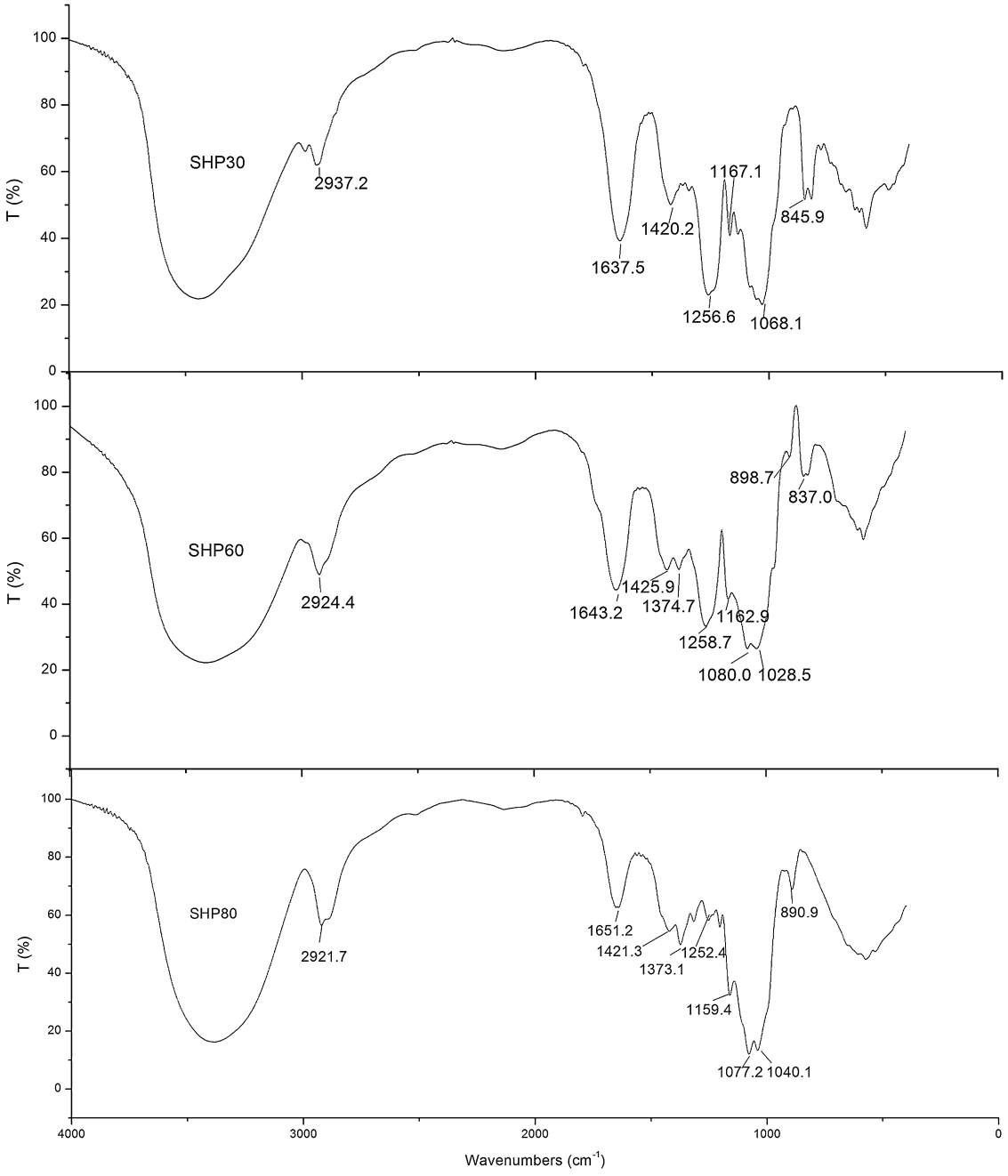

Fig. 1. FTIR spectra of SHP.

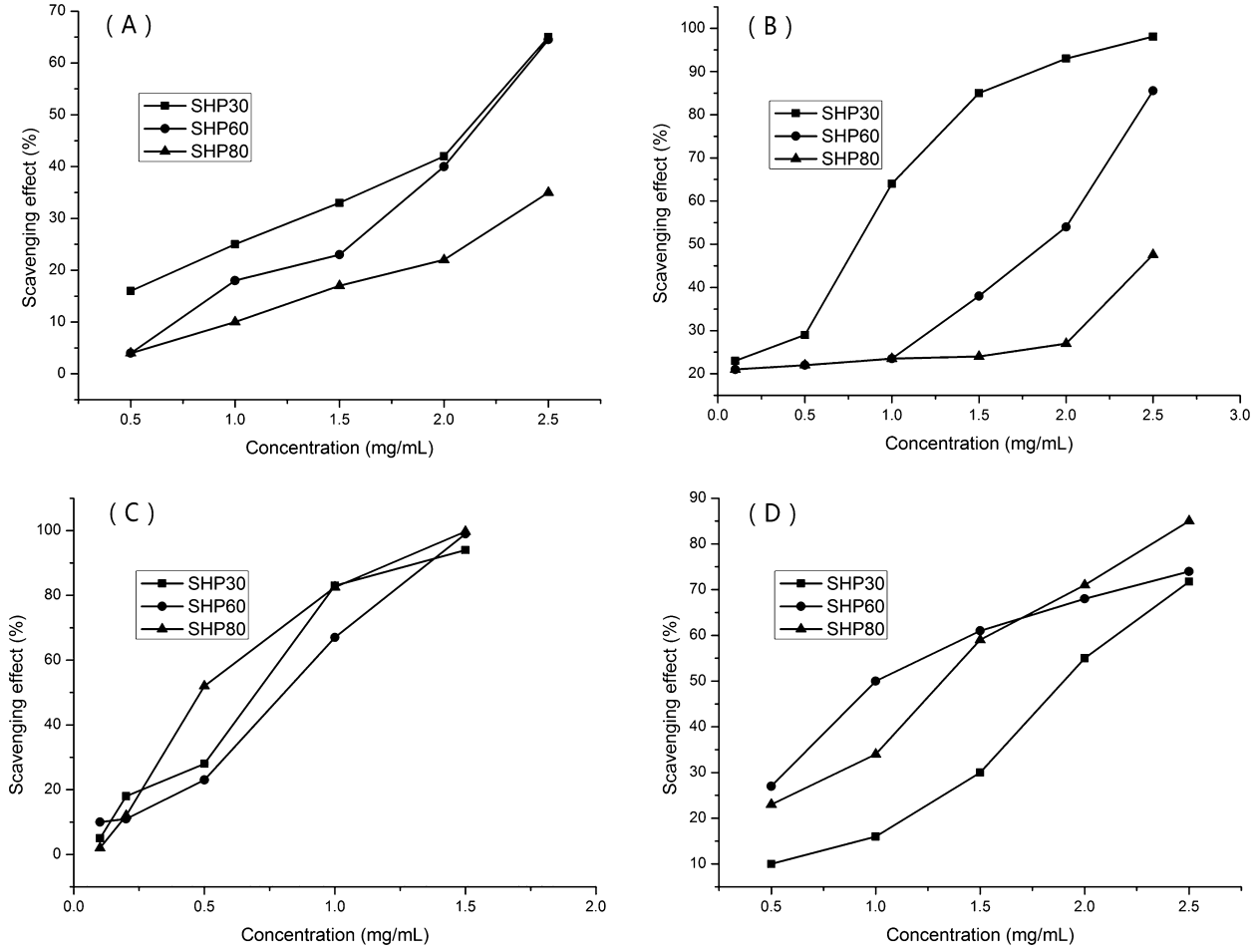

Fig. 2. Scavenging effects of SHP on superoxide radicals (A), hydroxyl radicals (B), ABTS radicals (C), and DPPH radicals (D).

(OriginPro 8.5) software package. The results obtained were analyzed using one-way analysis of variance (ANOVA) for mean differences among the samples.

3. Results and discussion

3.1. Chemical analysis

The chemical compositions of all the samples were given in Table 1. In general, the three polysaccharides possessed high sulfate content, relatively low total sugar. The content of sulfate decreased in the order SHP30>SHP60>SHP80. And the polysaccharide recovery yield of SHP30, SHP60 and SHP80 after water extraction, alcohol precipitation and separation by radial flow chromatography were 3.73%, 4.15% and 5.67%, respectively. According to the monosaccharide composition analysis, they were all heteropolysaccharides, and there were glucose in every polysaccharide. Among these three polysaccharides, rhamnose was the main sugar unit in SHP30, while glucose was the main sugar unit in SHP60 and SHP80. Besides the main sugar unit, some other monosaccharide unit also took up a high percentage (e.g. glucose in SHP30, rhamnose in SHP60).

The molecular weight of the polysaccharide was determined by high performance liquid chromatography. The equation of the standard curve was Log Mp = 10.25 + 0.654 t (where Mp represented the peak molecular weight, while t represented retention time). The molecular weight of four polysaccharides fractions was estimated to be SHP30 1.58×10^3 KDa, SHP60 1.92×10^3 KDa, and SHP80 11.2 KDa, respectively.

The FT-IR spectra of the three polysaccharides were shown in Fig. 1. The broad bands centered at 2920–2940 cm^{-1} were assigned to C—H stretching vibrations (Chen et al., 2013). The bands at 1650 cm^{-1} were due to the bending vibrations of HOH, and the bands at 1419 cm^{-1} originated from the bending vibrations of O—H bond (Chen et al., 2013). The peaks at 1380–1355 cm^{-1} in SHP60 and SHP80 were because of ester sulfate (—S=O) (Souza et al., 2012). The peaks around 1250 cm^{-1} were unsymmetrical carbonyl stretching (Zhao, Zhang, Guo, & Wang, 2013), and the peaks between 1200 cm^{-1} and 1000 cm^{-1} were attributed to the sugar ring and glycosidic bond C—O stretching vibrations. In SHP60 and SHP80, the main absorptions of C—O stretching (1160 cm^{-1}, 1080 cm^{-1}, and 1030 cm^{-1}) showed that the characteristics of sugar structures were pyranose configuration, and the spectrum at approximately 890 cm^{-1} indicates agar specific characteristics and is mainly associated with C—H bending at the anomeric carbon of 3-linked-β-D-galactose residues (Wongpraserta, Rudtanatip, & Praiboon, 2014). In SHP30 and SHP60, the peaks around 840 cm^{-1} were assigned to Galactose 4-sulfate (Souza et al., 2012), this was consistent with monosaccharide composition analysis. These results indicated that SHP possesses typical absorption peak of polysaccharides.

3.2. Antioxidant activities in vitro

3.2.1. Superoxide radical assay

The superoxide radical is a highly toxic species that can be generated by numerous biological and photochemical reactions.

Superoxide radicals may decompose to form singlet oxygen and hydroxyl radicals (Chen, Zhang, Jiang, Mu, & Miao). In this work, scavenging activities of all the three samples on superoxide anion were presented in Fig. 2(A). Obviously, all the samples had significant scavenging effects with dose-effect relationship. The scavenging effect of three polysaccharides on superoxide radical increased in the order of SHP80< SHP60< SHP30 at all concentrations, and the effects were 65.0%, 64.5% and 35.0% at the concentration of 2.5 mg/mL, respectively.

3.2.2. Hydroxyl radical assay

Among the reactive oxygen species, the hydroxyl radical is the most reactive and induces severe damage to the adjacent bimolecular. So it is essential to eliminate hydroxyl radical for antioxidant defense in many fields. Hydroxyl radicals are mainly responsible for the oxidative injury of bimolecular generated by reaction of Fe(II) complex with H_2O_2 in the presence of acid. Salicylic acid has the ability to absorb •OH to bring coloring material. Added hydroxyl radical scavengers compete with salicylic acid, which makes the content of coloring material down. The method was used to evaluate the hydroxyl radicals scavenging ability of natural compounds (Yang, Liu, Wu, Chen, & Wang).

In this study, the scavenging effects of all samples were shown in Fig. 2(B). As the figure showed, like the superoxide radical assay, the effects of scavenging hydroxyl radicals were in a concentration-dependent manner, and the scavenging effect of SHP30 is always far higher than SHP60 and SHP80. But unlike the superoxide radical assay, with the increasing of polysaccharide concentration, the scavenging activity of SHP30 increases sharply, and smooth-out when the scavenging rate reaches 93%. The scavenging activities of SHP60 and SHP 80 were nearly identical when polysaccharides concentration is below 1.0 mg/mL. SHP60 showed a prompt and continuous increase, and SHP 80 was cruising for slow growth at a concentration of 1.0–2.5 mg/mL. Above all, the SHP30 and SHP60 showed excellent radical scavenging performances; SHP80 had a relatively low effect with a scavenging rate of 47.57% while SHP30's hydroxyl scavenging rate was 98.07%, and SHP60's was 85.56% at the concentration of 2.5 mg /mL. All the data in superoxide radical assay and hydroxyl radical assay indicated that the antioxidant activities of all the samples were related to their ability to scavenge superoxide radical and hydroxyl radical.

3.2.3. ABTS radical assay

ABTS radical assay is a decolorization assay applicable to both lipophilic and hydrophilic antioxidants, including flavonoids, hydroxycinnamates, carotenoids, and plasma antioxidants. The preformed radical monocation of $ABTS^+$ is generated by oxidation of ABTS with potassium persulfate and is reduced in the presence of such hydrogen-donating antioxidants. The influences of both the concentration of antioxidant and duration of reaction on the inhibition of the radical cation absorption are taken into account when determining the antioxidant activity (Re et al., 1999). In this paper, the ABTS radical scavenging activities of the three samples in different concentrations were tested and the results were summarised in Fig. 2(C). Concentration-dependent radical scavenging activity was also observed. And in general, the three polysaccharides samples exhibited satisfactory ABTS radical scavenging activities at all tested concentrations. Unlike performances in superoxide radical assay, hydroxyl radical assay DPPH radical assay and FRP assay, the scavenging effects of SHP30, SHP60 and SHP80 on ABTS radical could be as high as above 94% at the relatively low concentration of 1.5 mg/mL. And the scavenging effects of three polysaccharides on superoxide radical increased in the order of SHP30< SHP60< SHP80 at all concentrations. Different radical scavenging activities

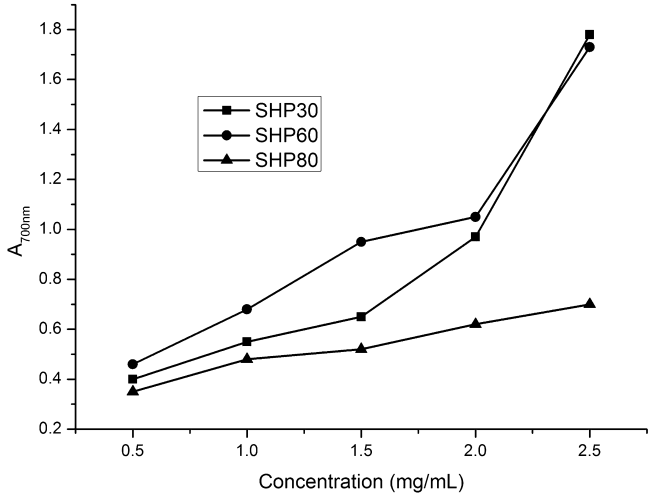

Fig. 3. Total reductive potential of SHP.

of SHP30 in different evaluation systems might suggest that the other factor had a certain effect on antioxidant activity.

3.2.4. DPPH radical assay

The DPPH free radical is a stable free radical that is widely used as a tool for estimating the free-radical scavenging activities of antioxidants. The role of an antioxidant is to remove free radicals. One mechanism through which this is achieved involves donating hydrogen to a free radical, and hence, its reduction to an unreactive species. Addition of hydrogen removes the odd electron feature which is responsible for radical reactivity (Wang, Gao, Zhou, Cai, & Yao, 2008). Antioxidant activities of the compounds were tested and summarised in Fig. 2(D). The results demonstrated that the sulfated polysaccharides had noticeable effects on inhibiting the formation of these radicals with dose-effect relationship. It is interesting to find that the scavenging effect of SHP30 was always lower than the SHP60 and SHP80, unlike other antioxidant experiments *in vitro* where the high antioxidant activity of SHP30 have been determined. And the scavenging rates of SHP80, SHP60 and SHP30 on DPPH radical were 85.01%, 73.96% and 71.74% at the concentration of 2.5 mg/mL, respectively.

3.2.5. FRAP assay

It has been reported that an electron-donating reducing agent contributes to antioxidant activity by its capacity to donate an electron to free radicals, which results in neutralization of the radical, and the reduced species subsequently acquires a proton from solution. Another reaction pathway in electron donation is the reduction of an oxidized antioxidant molecule to regenerate the "active" reduced antioxidant (Zhang et al., 2013; Wang et al., 2008).

The reducing power of all samples was shown in Fig. 3. Higher absorbance value means stronger reducing power. As the figure showed, the reducing power of SHP correlated well with increasing concentrations. In addition, the reducing power of SHP30 and SHP60 was more pronounced than that of SHP80, absorption of 1.78, 1.73 and 0.71 for SHP30, SHP60 and SHP80 at the concentration of 2.5 mg/mL, respectively. The data on the reducing power of different contents suggested that they were likely to contribute the observed antioxidant effect.

It has been reported that sulfate content had a significant effect on superoxide radical, hydroxyl radical scavenging effects and reducing power (Qi et al., 2005; Zou et al., 2008). The enhanced radical scavenging activity of the sulfated polysaccharides over

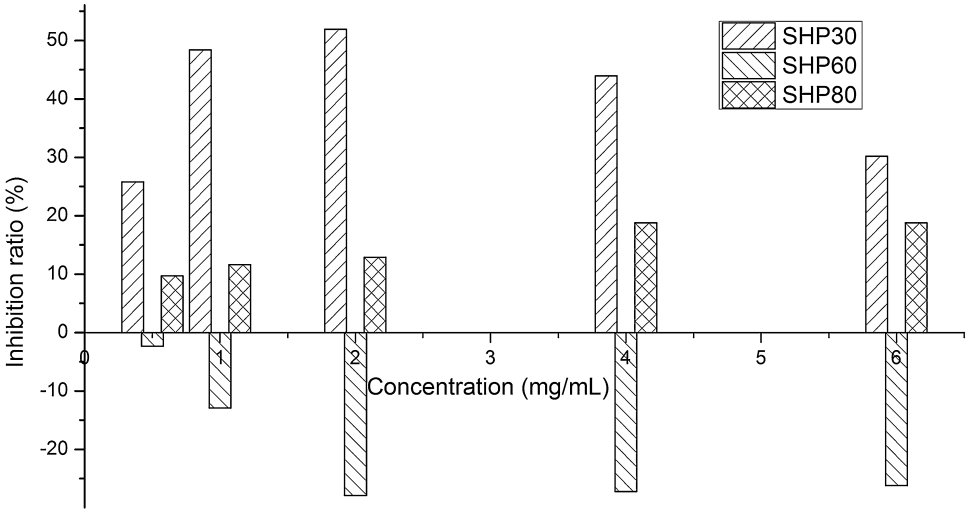

Fig. 4. Inhibition effects of SHP on MKN45 cells.

the neutral form may be due to that the sulfate group may act as an electrophile which promotes the intramolecular hydrogen abstraction. That was consistent with our study. Fig. 2(A) and (B) and Fig. 3 demonstrated that the scavenging effects were proportional to sulfate content, SHP30 with the highest sulfate content showed the highest antioxidant activity. The data in the superoxide radical assay, hydroxyl radical assay and reducing power assay combined with structural properties suggested that $-OSO_3H$ groups of polysaccharides might act as electron donors, and the hydroxyl of the $-OSO_3H$ group was active, which can react with free radical and thereby terminate radical chain reactions (Chang, Hsu, & Chen).

In addition, the antioxidant activities were not a function of a single factor but a combination of several factors, such as molecular weight (Zhang et al., 2013; Sun, Wang, Shi, Ma). It was reported that molecular weight had a significant effect on the antioxidant activity of ulvan, and ulvan with low molecular weight had stronger antioxidant activity (Qi et al., 2005). In the ABTS radical assay and DPPH radical assay, SHP80 with low molecular weight showed the best performances while SHP60 had the highest molecular weight and lowest scavenging rates on ABTS radical. This might be related to low molecular weight and good solubility. Further, SHP30 with the highest sulfate content and intermediate molecular weight showed the highest antioxidant activity in superoxide radical assay, hydroxyl radical assay and reducing power assay. According to these points, we can infer that the sulfate content is not the only factor around antioxidant activities of polysaccharides and the polysaccharide can show good radical scavenging effects in antioxidant tests only when sulfate content is high and molecular weight is relatively low.

3.3. Antitumor activities in vitro

3.3.1. Growth inhibition of polysaccharides on MKN45 gastric cancer cells

The inhibitory effects of natural products are generally evaluated by testing their ability to inhibit the proliferation of the cancer cells directly, or their ability of inducing immune cells to secrete cytokines that can act on the cancer cells. After incubated with SHP for 24 h at the concentrations from 0.5 to 6.0 mg/mL, the inhibition effects of MKN45 cells were observed and compared with the control group. The inhibition ratios of the three samples in different concentrations against the human gastric cancer cell MKN45 were summarised in Fig. 4. The SHP30 and SHP80 exhibited some inhibition effect at the concentrations from 0.5 to 6.0 mg/mL. There was no dose-dependency relationship between the inhibition ratios and concentrations, that was different from antioxidant assays. It was deserved to note that SHP30 with the highest sulfate content and intermediate molecular weight exhibited relatively higher inhibition ratios(51.92%, 2 mg/mL) than SHP80 at all concentrations, which still had growth inhibition percentages of 18.8% on MKN45 cells even at a high concentration of 6.0 mg/mL. On the contrary, the SHP60 promotes the MKN45 cells to grow at the concentrations from 0.5 to 6 mg/mL. These results suggested SHP30 had an acute cytotoxic effect on the MKN45 cells while SHP60 and SHP80 presented relatively low antitumor activities against MKN45 cells.

3.3.2. Growth inhibition of polysaccharides on DLD intestinal cancer cells

The cytotoxicities of SHP30, SHP60 and SHP80 were determined in the human intestinal cancer DLD cells. After the treatment of the cells with increasing concentration of the three samples, cell proliferation was evaluated using MTT assay. As shown in Fig. 5, SHP30 and SHP60 exhibited significant inhibition effect, and the highest inhibition ratios were 85.3% and 76.37% at the concentration of 4.0 mg/mL, respectively. Furthermore, the highest inhibition ratios of SHP80 were approximately 44% against DLD cancer cells at the concentration of 6.0 mg/mL, and no obvious concentration-dependent relationship was observed. Consistent with the results on MKN45 cells, the SHP30 sample had the relatively higher inhibition ratio, and SHP80 showed low antitumor activity, but unlike the results on MKN45 cells, SHP 60 showed obvious inhibition effect on DLD intestinal cancer cells. After analyzed, the cause might be that the mechanism of SHP60 was not available for MKN45 cells, while it could promote cancer cell growth as a nutrient.

3.4. Effect of SHP30 on cell cycle distribution of DLD cells

To determine whether the inhibition of SHP30 on DLD cells proliferation involved cell cycle changes, we studied the cell cycle phase distribution by flow cytometry. As shown in Fig. 6(A)–(C), a special DNA peak (usually called apoptotic peak) appeared in the

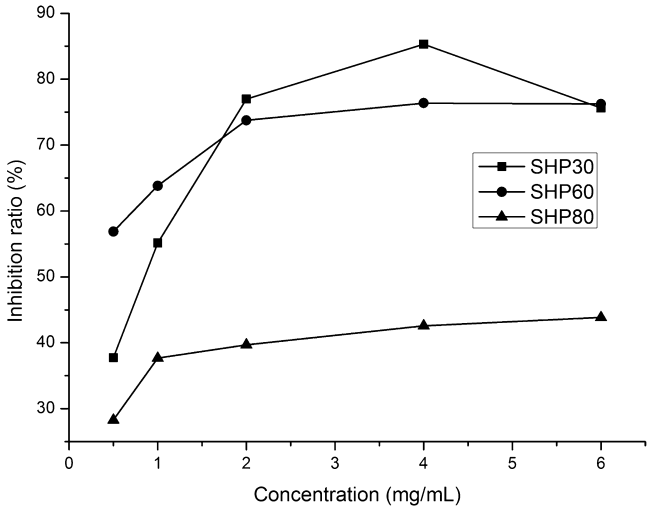

Fig. 5. Inhibition effects of SHP on DLD cells.

cells undergoing apoptosis. The cell cycle phase distribution of DLD cells after treatment for 48 h with 1 or 2 mg/mL of SHP 30 was shown in Fig. 6(D), and a significant increase of the cell population in the G_0/G_1 phase was observed from 48.35% to 83.85%, *S* phase was observed from 11.77% to 51.65%, G_2/M phase was observed from 0% to 4.38%. All the data were supported by Chen et al. (2011). The result indicated that the accumulation of cells in G_0/G_1 and *S* phase might account for the growth inhibition of DLD cells by SHP30.

3.5. Effects of SHP30 on Bax and Bcl-2 mRNA expressions in DLD cells

In order to explore the possible mechanisms of SHP30-induced apoptosis, the mRNA expression levels of apoptosis-related genes such as Bax and Bcl-2 in DLD cells were examined using real-time fluorescence quantitative PCR. As shown in Fig. 7, after treatment for 48 h with 2 mg/mL of SHP30, relative mRNA expression levels of Bax (Fig.7(A)) significantly increased by 1.67-fold, compared with that of the control. In contrast, relative mRNA expression levels of Bcl-2 gene (Fig.7(B)) markedly decreased by 0.41- and 1.94-fold for

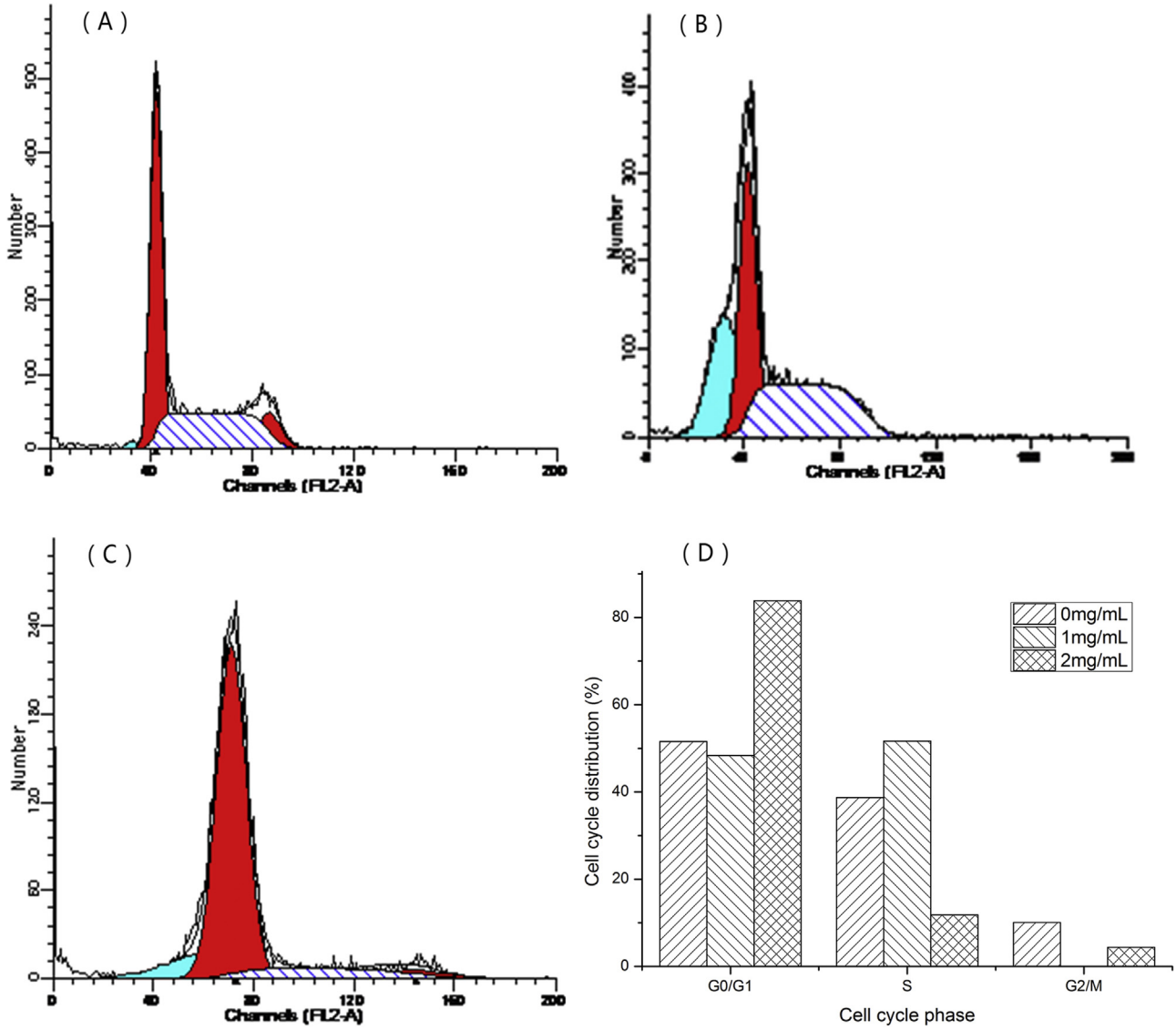

Fig. 6. Cell cycle proportions of SHP30-treated DLD cells. (A–C) The appearance of sub-G1 peak (apoptotic peak) was observed after the treatment with 0, 1 or 2 mg/mL SHP30 for 48 h, respectively; (D) Bar graph summarizes the data on cell cycle distribution.

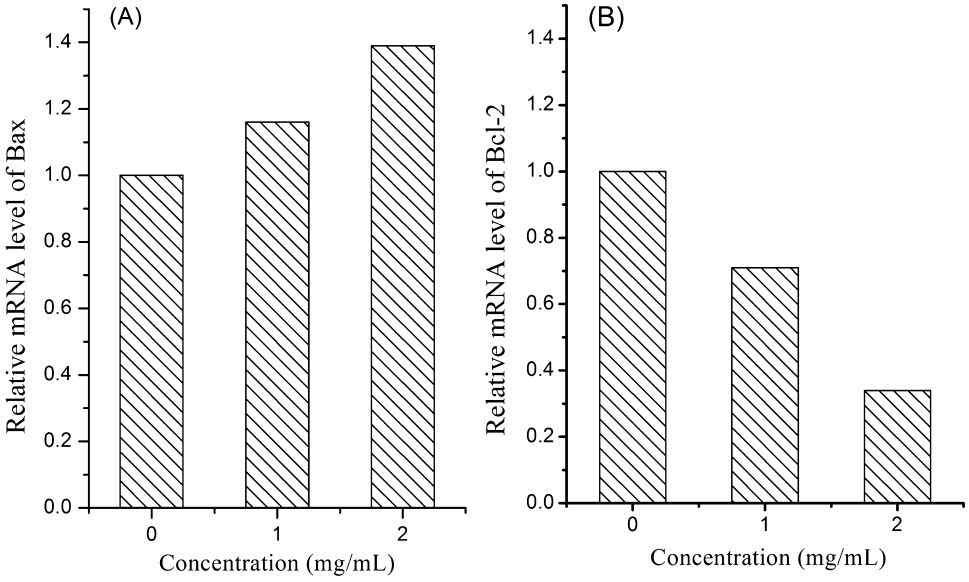

Fig. 7. Relative mRNA levels of Bax and Bcl-2 in SHP30-treated DLD cells.

the treatments with 1 and 2 mg/mL of SHP30, respectively. All the results suggested that SHP30 might induce apoptosis of DLD cells through up-regulating of Bax mRNA levels and down-regulating of Bcl-2 mRNA level, and then possibly affect levels of corresponding proteins (Gao et al., 2008; Li, Chong, & Wang).

It has been reported that the polysaccharides with higher sulfate contents exhibited stronger antitumor activities *in vitro* (Xu, Ye, Sun, Tu, & Zeng; Suresh et al., 2013). That is supported by our study. Consistent with these results in the superoxide radical assay, hydroxyl radical assay and reducing power assay, SHP30 which had highest sulfate contents showed markedly higher antitumor activities against the DLD cells and MKN45 cells. And Fig. 7 showed that the SHP60 with the highest molecular weight didn't inhibited but promoted the MKN45 cells to grow. Therefore, sulfate content maybe not the only the factor that determines antitumor activities of three polysaccharides. Combined with results of antioxidant tests *in vitro*, we inferred that the higher antitumor and antioxidant activities of the three polysaccharides may be related to comprehensive effects of their sulfate contents and molecular weight.

Furthermore, based on the good performance of SHP30 on MTT assays and the results in DLD cell cycle analysis and quantitative real-time reverse transcription-PCR analysis, we speculated that the main inhibition mechanism of SHP30 on DLD cells is arresting cell cycle at G_1 period, S period and lead to apoptosis which might be in relation to the regulation of apoptosis-associated gene expressions in SHP30-treated cells. This is supported by other related researches (Ma et al., 2013; Chu, Mao, Feng, Liu, Geng).

But the exact correlation between the chemical characteristics and bioactivities of polysaccharides needs further investigation.

4. Conclusions

In conclusion, the three sulfated polysaccharides exhibited obvious antioxidant, antitumor activities and dose-effect relations were observed. Specifically, SHP30 the highest sulfate content and the intermediate molecular weight had consistently excellent antioxidant and antitumor performances in the superoxide radical assay, hydroxyl radical assay, reducing power assay, and MTT assay while there has been nothing extraordinary in ABTS radical assay and DPPH radical assay. SHP30 might induce apoptosis of DLD cells through regulation of apoptosis-associated gene expressions such as Bcl-2 and Bax. SHP60 had been performing well in scavenging radicals and inhibition on DLD intestinal cancer cells, however, showed the negative inhibition ratio on MKN45 gastric cancer cells even at all concentrations. At the same time, SHP80 who had the lowest sulfate content and the lowest molecular weight showed high activity in scavenging radical assays especially in ABTS radical assay and DPPH radical assay, but in the superoxide radical assay, hydroxyl radical assay, reducing power assay, and MTT assay, its performance was not satisfactory.

These results indicate that the combined action of sulfate content and molecular weight may influence their antioxidant and antitumor activities. Our findings provided a novel understanding of the antiproliferative mechanisms of the polysaccharide fragment from *Sargassum horneri*. And that might be helpful for developing a novel antioxidant and antitumor agent. Further studies about the structure-bioactivities of the polysaccharides are in progress.

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