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Characterization and antitumor activity of a polysaccharide from *Strongylocentrotus nudus* eggs

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Abstract

A water-soluble polysaccharide named as SEP was isolated from the eggs of Dalian purple sea urchin *Strongylocentrotus nudus* by hot water extraction, anion-exchange and gel-permeation chromatography and tested for its antitumor activity. Its structural characteristics were investigated by FTIR, HPLC, NMR spectroscopy, GLC–MS, methylation analysis, Periodate oxidation and Smith degradation. Based on the data obtained, SEP was found to be an α-(1 → 4)-D-glucan, with a single α-D-glucose at the C-6 position every nine residue, on average, along the main chain. The glucan has a weight-average molecular weight of about 1.95×10^6 Da. Pharmacological studies revealed SEP could inhibit the growth of Sarcoma 180 tumor remarkably *in vivo*. SEP also stimulated the spleen lymphocyte proliferation in S180-bearing mice, indicating its antitumor effect may be related to immunomodulation.

Keywords: Sea urchin; *Strongylocentrotus nudus*; Eggs; D-Glucan; Structure; Antitumor

1. Introduction

Many polysaccharides and polysaccharide–protein complexes have been isolated from mushrooms, fungi, yeasts, algae, lichens and plants, and their biological activities have attracted more attention recently in the biochemical and medical areas due to their immunomodulatory and anti-cancer effects (Ooi & Liu, 2000). The great majority of chemical compounds, which have been identified as cytotoxic to cancer cells, are also toxic to normal cells. In view of the need for new anti-cancer compounds with low toxic potential, numerous polysaccharides from different biological origins have been investigated for antitumor and immunomodulating activities (Kim et al., 1996; Han et al., 2001). It is generally accepted that polysaccharides enhance various immune responses *in vivo* and *in vitro*. In many oriental countries, several immunoceuticals composed of polysaccharides have been accepted such as lentinan, schizophyllan and krestin (Liu, Ooi, & Fung, 1999; Borchers, Stern, Hackman, Keen, & Gershwin, 1999).

Sea urchins, belonging to echinoderm, are in the same group of animals as starfish, brittle stars and sea cucumbers (Zhang, Liao, & Wu, 1964). Sea urchin roes or eggs are a kind of favorite seafood for their good taste and high nutrition in China. According to traditional Chinese medicine, sea urchins can prevent from cardio-vascular diseases and enhance immunity. The Dalian purple sea urchin, *Strongylocentrotus nudus*, is mainly distributed in Huang-Bo Sea and is native to North China. The present paper was concerned with the isolation, chemical characterization and evaluation of the antitumor activity of a glucan from *S. nudus* eggs.

2. Experimental

2.1. Material

Sea urchins (*S. nudus*) were collected from Huanghai Sea, China and transported to the laboratory packed in

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ice. The shell, spines and intestine were immediately removed and the eggs were kept frozen at −20 °C. The voucher specimen of this marine animal is deposited in the School of Life Science and Technology, China Pharmaceutical University, Nanjing, China.

2.2. General methods

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

2.3. Extraction and fractionation of polysaccharide

A total of 2 kg of sea urchin eggs were first treated with acetone (1:1, 2000ml × 3) to remove fat and pigments. The pellets were extracted with distilled water (3000 ml) at 90 °C every 6 h for three times. The supernatants were collected by centrifugation and concentrated in vacuo. The concentrated solution was deproteinated by a combination of papain enzymolysis and Sevag method. The crude polysaccharide fraction (11.22 g) was obtained through precipitation with 4 vol of ethanol and desiccation in vacuo. The precipitate was redissolved in distilled water and applied to DE52 (OH^-) anion exchange chromatography column (3.5 × 30 cm), eluting at a flow rate of 60 ml/h successively with distilled water and a gradient of 0 → 2 mol/L NaCl. The yielded fractions were combined according to the total carbohydrate content quantified by the phenol–sulfuric acid method. The main peak was further fractionated on a Sephacryl S-400 column (1.6 × 80 cm) eluted with 0.05 mol/L NaCl at a flow rate of 27 ml/h to yield two completely separated fractions. The main fraction was collected, dialyzed and lyophilized to get a white purified sea urchin eggs polysaccharide (SEP, 707 mg, 6.3% of the crude polysaccharide).

2.4. Homogeneity and molecular weight

The homogeneity and molecular weight of SEP was determined on a Waters HPLC system (717 plus autosampler and 600 delta HPLC pump) equipped with a TSKgel 4000 PW_{XL} column (7.8 × 300 mm) and a Waters 2414 Refractive Index Detector (RID). A sample solution (20 μl of 0.5%) was injected in each run, with 0.05 mol/L NaCl as the mobile phase at 0.8 ml/min. The HPLC system was precalibrated with T-series Dextran standards (T-10, T-40, T-70, T-500 and T-2000).

2.5. Monosaccharide composition

The polysaccharide sample (10 mg) was hydrolyzed in 2 M trifluoroacetic acid (2 ml) at 120 °C for 2 h in an oven. The hydrolyzate was then converted into its respective alditol acetates as previously described (Blakeney, Harris, Henry, & Stone, 1983), and analyzed by gas–liquid chromatography (GLC) using a Hewlett–Packard model 6890 instrument equipped with a HP-5 capillary column (30 m × 0.25 mm) and a flame-ionization detector, and programmed from 150 to 220 °C at 2 °C /min and from 220 to 280 °C at 30 °C/min. Peaks were identified and estimated with *myo*-inositol as the internal standard. Quantitation was carried out from the peak area, using response factors.

2.6. Methylation analysis

The polysaccharide was methylated three times by the Needs and Selvendran (1993) The pre-methylated product was depolymerized with 90% HCOOH at 100 °C for 6 h and further hydrolyzed with 2 M TFA at 110 °C for 3 h. The partially methylated residues were reduced and acetylated. The resulting products were analyzed by GLC–MS. The GLC temperature program was isothermal at 150 °C, followed by a 3 °C/min gradient up to 220 °C and 30 °C/min up to 280 °C. Methylated alditol acetates were identified by their fragment ions in GLC–MS and by relative retention time on GLC, and the molar ratios were estimated from the peak areas and the response factors (Sweet, Shapiro, & Albersherm, 1975; Tsumuraya, Misaki, Takaya, & Torii, 1978).

2.7. Periodate oxidation and smith degradation

The polysaccharide SEP (10.0 mg) was dissolved in 0.015 M sodium metaperiodate (30 ml) and kept in the dark at 4 °C, respectively, with the absorption at 223 nm monitored every day. The reaction was completed after 120 h and ethylene glycol (0.2 ml) was added to the solution with stirring for 30 min to decompose the excess of the reagent. Consumption of $NaIO_4$ was measured by a spectrophotometric method (Dixon & Lipkin, 1954; Chaplin & Kennedy, 1994) and HCOOH production was determined by titration with 0.01 M NaOH. The reaction mixture was dialyzed against distilled water, and the nondialysate was reduced with $NaBH_4$ (25 mg, 12 h). The pH was adjusted to 5.0, the solution was dialyzed, and the nondialysate was lyophilized, and then hydrolyzed with 2 M TFA at 110 °C for 2 h. The hydrolysate was analyzed by GLC.

2.8. Partial hydrolysis

SEP was partially hydrolyzed with a solution adjusted to pH 2.0 (20 ml) with aq. trifluoroacetic acid, at 100 °C, for

18 h. After neutralization with NaOH, a polymeric product (SEP-p) was obtained by precipitation with excess EtOH from a small volume of water, and then retained on dialysis with a M_r 2 kDa cut-off membrane. The periodate oxidation and Smith degradation of the precipitate fractions was followed the same procedure as mentioned above.

2.9. Nuclear magnetic resonance spectroscopy

The freeze-dried polysaccharide was kept over P_2O_5 in vacuum for several days and dissolved in 99.96% D_2O. ^1H and ^{13}C NMR spectra were recorded with a Bruker DRX Avance 500 MHz spectrometer (operating frequencies 500.13 MHz for ^1H NMR and 125.76 MHz for ^{13}C NMR) at 30 °C. Chemical shifts were reported relative to internal DSS at δ_H 0.00 ppm for ^1H spectra and δ_C 21.745 ppm for ^1C spectrum. Standard homo- and heteronuclear correlated 2D techniques were used for general assignments of SEP: COSY, NOESY and HSQC (Brisson et al., 2002). The NOESY mixing delay was 200 ms.

2.10. Assay of antitumor activity

2.10.1. Animals and treatment

Female Kunming mice (6–8 weeks old) were purchased from the Qinglongshang Experimental Animal Center (Nanjing, China. Quality certificated Number: SCXK 2002-0018). The mice were housed under normal laboratory conditions, i.e., room temperature, 12/12-h light–dark cycle with free access to standard rodent chow and water. Sixty mice were randomly divided into six groups, each group consisting of 10 animals. One group was normal control. And seven-day-old Sarcoma 180 (S180) ascites (0.2 ml, 2×10^6 cells) were transplanted subcutaneously into the right axilla of each mouse of the rest groups. The mice were treated as following: normal control group (normal saline); model control group (normal saline); three SEP groups (16, 8 and 4 mg/kg body weight); positive control group (Cyclophosphamide, 20 mg/kg body weight). All the groups were administered by intraperitoneal injection in 0.2 ml every day for seven days, starting 24 h after tumor implantation.

2.10.2. Tumor weight, thymus and spleen index

Twenty-four hours after the last drug administration, mice were sacrificed by cervical dislocation. Spleen, thymus, and tumor weights in the mice were measured. The antitumor activity *in vivo* was expressed as an Inhibitory rate calculated as $[(A-B)/A]\times100\%$, where A and B were the average tumor weight of the model control and experimental groups, respectively.

2.10.3. Splenocyte proliferation assay

Splenocytes taken from mice of all the groups were suspended in a solution of RPMI-1640 medium containing 10% newborn bovine serum (NBS), 100 μg/ml penicillin, 100 UI streptomycin, and 5 μg/ml concanavalin A (ConA). Cells suspension (2×10^6 cells/ml) was placed in a 96-well flat-bottomed microplate with 200 μl per well. The cells were cultured in 37 °C, 5% CO_2 for 72 h, and further incubated for 4 h with 20 μl per well of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT: 5 mg/ml; Sigma, USA). A total of 100 μl acidified isopropylalcohol was added to the culture and homogenized for at least 10 min to fully dissolve the colored material. The absorbance at 570 nm was measured in an ELISA reader (Bio-Rad Model 6800, USA) (Mossmann, 1983).

2.10.4. Statistical analysis

The data were expressed as means ± SD Significance of difference was evaluated with one-way ANOVA, followed by Dunnett's test to statistically identify differences between the control and treated groups.

3. Results and discussion

3.1. Isolation and structural analysis

The yield of the crude water-soluble polysaccharide from the eggs of sea urchin was 0.56% of the fresh material. The crude polysaccharide was separated and sequentially purified through DE52 and Sephacryl S-400, each giving a single elution peak, as detected by the phenol–sulfuric acid assay. The main fraction (SEP) was collected for subsequent analyses.

SEP appeared as a white powder, $[\alpha]_D^2 0+210^\circ$ (c 0.2, H_2O). It had a negative response to the Bradford test and no absorption at 280 or 260 nm in the UV spectrum, indicating the absence of protein and nucleic acid. Elemental analysis (Wt%): Found C, 39.12; H, 6.68; N, 0.00, indicating it was a neutral polysaccharide. The GPC profile (Fig. 1) showed a single and symmetrically sharp peak, indicating that SEP was a homogeneous polysaccharide, with a weight-average molecular weight of 1.95×10^6 Da. The total sugar content of SEP was determined to be 99.4%. As determined by *m*-hydroxydiphenyl colorimetric method and GC, the polysaccharide did not contain uronic acid. SEP was composed of only glucose monomers, as detected by GLC of the alditol acetate derivatives of the components of the SEP hydrolyzate. The relatively high

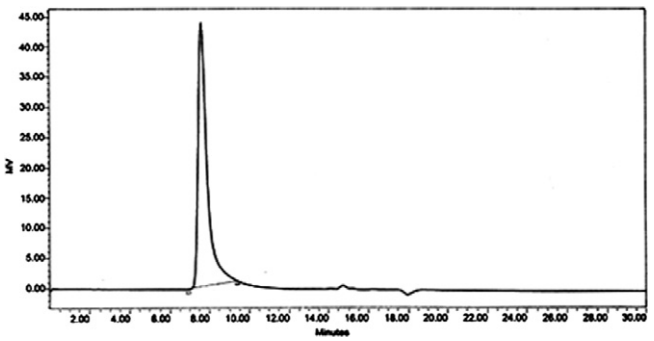

Fig. 1. Profile of SEP in HPGPC, with 0.05 mol/L NaCl at 0.8 ml/min.

positive value of optical rotation (+210.0°) suggested the dominating presence of α-form glycosidic linkages in SEP (Zhao, Kan, Li, & Chen, 2005). The optical rotation of the SEP hydrolyzate obtained was shown to be $[\alpha]_D^{20} + 52°$ (*c* 0.2, H_2O) [compare authentic D-glucose $[\alpha]_D^{20} + 52.6°$ (*c* 0.2, H_2O)], also indicating the D-configuration of the glucosyl residues (Hara, Kiho, & Ukai, 1983).

The FTIR spectrum of SEP showed a strong band at 3384.54 cm^{-1} attributed to the hydroxyl stretching vibration of the polysaccharide. The band at 2929.43 cm^{-1} was due to C–H stretching vibration. The broad band at 1646.88 cm^{-1} was due to the bound water (Park, 1971). The band at 852.41 cm^{-1} was ascribed to α-type glycosidic linkages in the polysaccharide(Barker, Bourne, Stacey, & Whiffen, 1954). The bands at 852.41 and 932.72 cm^{-1} was characteristic of (1 → 4)-α-glucan. The IR spectrum, together with the high positive value of the specific rotation indicated the presence of α-glycosidic linkages in the SEP (Bao, Duan, Fang, & Fang, 2001; Tsumuraya & Misaki, 1979). The absorptions at 1022.51, 1080.17 and 1155.13 cm^{-1} also indicated α-pyranose form of the glucosyl residue.

Periodate oxidation of SEP resulted in the values of 0.90 mol periodate consumed and 0.23 mol formic acid produced per sugar residue. After further Smith degradation of the periodate-oxidized SEP, the glycerol and erythritol were found with molar ratio 1.0:4.0 by GLC after conversion to the corresponding alditol acetates. It was thus deduced that 1 →, (1 → 6)-, and (1 → 4,6)- amounted to 20.0%, with (1 → 4)-linked glycosyl bonds amounting to 80%, respectively. SEP was partially hydrolyzed with 0.3 M TFA. After periodate oxidation and Smith degradation, only erythritol was found, indicating SEP was a polysaccharide with (1 → 4)-linked backbone.

The fully methylated product of SEP was hydrolyzed with acid, converted into alditol acetates, and analyzed by GLC and GLC–MS (Table 1). SEP furnished three types of glucose derivatives in a relative molar ratio of 1.0:7.8:1.15 according to the peak areas. The overall results suggested that the polysaccharide SEP was a glucan with a (1 → 4)-linked backbone and (1 → 6)-linked branches. This was also in accordance with the mode of linkage of glucose present in the polysaccharide by periodate oxidation and Smith degradation.

The 500-MHz ^1H NMR spectrum of SEP (Fig. 2) showed two anomeric protons at δ5.3857 and 4.9713, which were assigned as (1 → 4)-α-D-Glc*p* (Residue 1) and

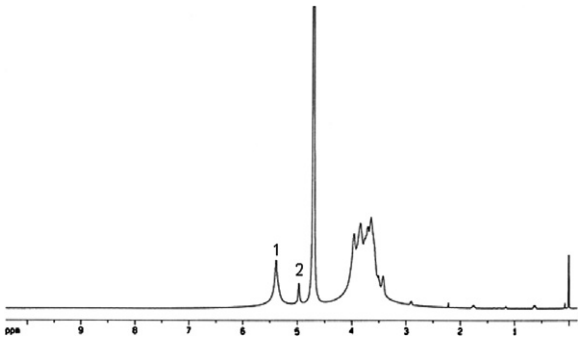

Fig. 2. ^1H NMR (500 M) spectrum of polysaccharide SEP isolated from *Strongylocentrotus nudus*.

(1 → 6)-α-D-Glc*p* (Residue 2), respectively. This confirmed that the sugar residues were linked α-glycosidically, which is consistent with presence of an IR band 852.41 cm^{-1}. The chemical shifts from 3.4 to 4.0 ppm were assigned to protons of carbons C-2 to C-6 of glycodidic ring (Chauveau, Talaga, Wieruszeski, Strecker, & Chavant, 1996). The α-configuration of the D-glucosyl groups was clearly conformed by the presence of two anomeric peaks in the regions δ102.495 and 100.363 ppm from ^{13}C NMR experiments(Fig. 3). The ^1H and ^{13}C NMR spectra of SEP were assigned according to 2D ^1H, ^1H COSY, NOESY and ^{13}C HSQC experiments (Table 2).

The NOESY spectrum of SEP provided information on the intra- and inter-residue connectivities based on dipole correlation (through space) (Cui, Eskin, & Biliaderis, 1995; Dabrowski, 1994). Strong correlation was observed between H-1 and H-4 in residue 1. This was an inter-residue correlation which confirmed that the α-D-glucose was 1,4-linked in the polysaccharide (Cui et al., 1995). From the NOESY spectrum correlations were also observed between H-1 and H-4, H-6 in residue 2. This observation

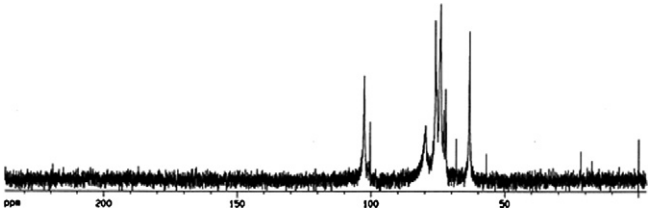

Fig. 3. ^{13}C NMR (125 M) spectrum of polysaccharide SEP isolated from *Strongylocentrotus nudus*.

Table 1
GC–MS of alditol acetate derivatives from the methylated product of SEP

Methylated sugars (as alditol acetates)[a]	Type of linkage	Relative retention time[b]	Molar (%)	Mass fragments (*m*/*z*)
2,3,4,6-Me4-Glc	Terminal Glc*p*	1.00	1.0	43,45,59,71,87,101,117,129,145,161,205
2,3,6-Me3-Glc	1,4-Linked Glc*p*	1.31	7.8	43,45,71,87,99,101,113,117,129,131,143,161,173,233
2,3-Me2-Glc	1,4,6-Linked Glc*p*	1.69	1.15	43,45,85,87,99,101,117,142,159,201,261

[a] 2,3,4,6-Me4-Glc = 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl- glucose, etc.
[b] Relative retention times of the corresponding alditol acetate derivatives compared to 2,3,4,6-tetra-*O*-methy l-D-glucose.

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

Residue	$\delta^{13}C/^{1}H$ (ppm)[a]						
	1	2	3	4	5	6(6a)	6b
(1 → 4)-α-D-Glcp, residue 1	102.495	74.080	72.863	79.683	75.801	63.243	–
	5.386	3.546	3.889	3.915	3.7115	3.767	3.843
(1 → 6)-α-D-Glcp, residue 2	100.363	72.220	72.863	74.495	76.076	68.282	–
	4.971	3.421	3.889	3.737	3.656	3.947	3.750

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

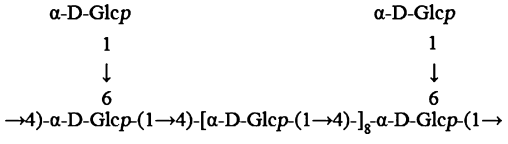

Fig. 4. Predicted structure of polysaccharide SEP isolated from *Strongylocentrotus nudus*.

suggested that the branches of SEP were linked at positions 6, which was consistent with the analysis above.

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

3.2. Antitumor activity

3.2.1. Tumor weight and relative spleen and thymus weight

A significant tumor inhibition was observed at three doses of the polysaccharide SEP while compared with model control (Fig. 5). At the doses of 16, 8 and 4 mg/kg, the inhibitory rate was 46.9%, 41.7% and 40.7%, respectively. SEP could also remarkably increase spleen and thymus index in S180-bearing mice (Table 3). Cyclophosphamide decreased considerably spleen and thymus index in S180-bearing mice, whereas it had a high inhibitory rate (55.4%) at a dose of 20 mg/kg.

Table 3
Effect of the polysaccharide SEP on spleen index and thymus index of tumor-bearing mice

Group	Dose (mg/kg)	Spleen index (mg/g)	Thymus index (mg/g)
Normal control	–	5.68 ± 1.52	2.67 ± 1.26
Model control	–	5.75 ± 1.41	2.52 ± 0.58
SEP	16	5.79 ± 1.32[a]	2.76 ± 0.61[a]
	8	6.93 ± 1.81[a]	2.68 ± 1.39[a]
	4	6.79 ± 2.09[a]	2.59 ± 0.88[a]
Positive control	20	2.75 ± 1.00[b]	0.78 ± 0.31[b]

Thymus index was measured in the ratio of the thymus weight (mg) to body weight (g). Spleen index was measured in the ratio of the spleen weight (mg) to body weight (g). Values are means ± SD of 10 mice.
[a] $P < 0.05$.
[b] $P < 0.01$ vs. Model control.

3.2.2. Splenocyte proliferation

To confirm the effect of the polysaccharide SEP on the cellular immune response, we evaluated the proliferation of splenocytes from mice in response to ConA. The results, shown in Fig. 6, indicated that the proliferation index was suppressed in model group mice, whereas in the polysaccharide SEP groups, those responses were restored to normal level. The polysaccharide SEP groups were significantly different from that of the model control group, suggesting immunomodulation may be the mechanism of the antitumor activity of SEP.

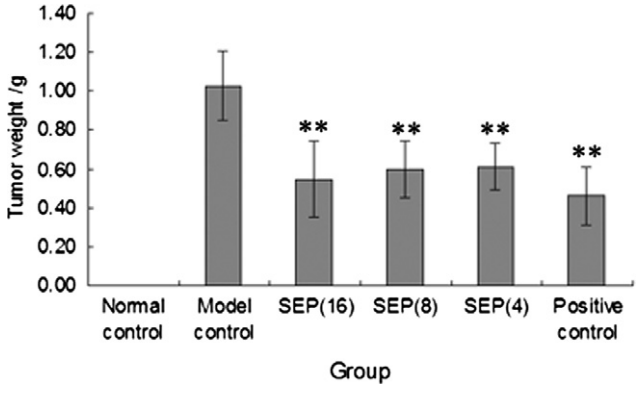

Fig. 5. Effect of the polysaccharide SEP on tumor regression of tumor-bearing mice. Model and positive control and SEP groups were inoculated with sarcoma 180, while normal control was not. Model control received saline intraperitoneally, while positive control was administered cyclophosphamide. The polysaccharide SEP was dissolved in saline and was administered intraperitoneally. The dose volume was 0.2 ml. Values are means ± SD of 10 mice; $^{**}P < 0.01$ *vs.* model control.

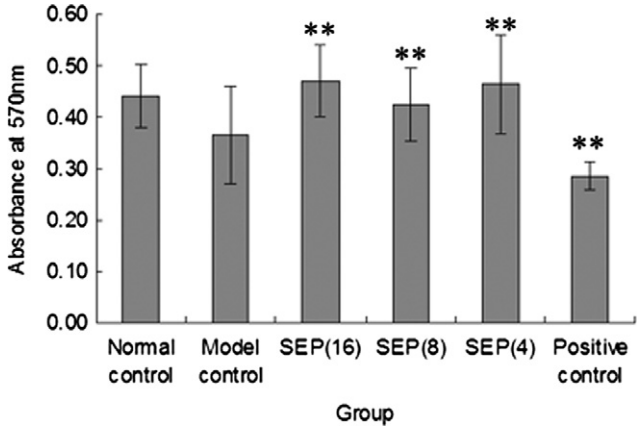

Fig. 6. Effect of the polysaccharide SEP on splenocyte proliferation. Proliferation activity was expressed as the absorption at 570 nm. Values are means ± SD of 10 mice; $^{**}P < 0.01$ *vs.* model control.

4. Conclusion

The results of the present investigation showed that the polysaccharide of *S. nudus* was a D-glucan containing α-(1 → 4)-linked backbone, branched α-(1 → 6)-linkage. Preliminary pharmacological tests suggested that SEP could significantly inhibit the growth of Sarcoma 180 tumor *in vivo*. The spleen lymphocyte proliferation assay indicated the antitumor effect of polysaccharide SEP might be related to immunomodulation.

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