Carbohydrate Polymers 88 (2012) 1313–1318

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Partial characterization and anti-tumor activity of an acidic polysaccharide from *Gracilaria lemaneiformis*

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ARTICLE INFO

Article history:
Received 2 December 2011
Received in revised form 20 January 2012
Accepted 7 February 2012
Available online 14 February 2012

Keywords:
Gracilaria lemaneiformis
Polysaccharide
Anti-tumor
Immunomodulation

ABSTRACT

An acidic polysaccharide (GLSPs) with carbohydrate content of 72.06% and sulfate content of 6.13% was isolated from *Gracilaria lemaneiformis*. FTIR analysis of GLSPs showed that two typical absorptions appeared at 1259 and 804 cm^{-1} were assigned to S=O and C—O—S bonds stretching respectively. HPGPC showed the molecular weight of GLSPs was mainly distributed at 1.37×10^6 Da. GC analysis showed GLSPs was chiefly composed of galactose, and contained a small amount of rhamnose, arabinose, xylose and mannose. Moreover, the anti-tumor and immunomodulatory effects of GLSPs were evaluated in ICR mice transplanted H22 hepatoma cells. It was shown that GLSPs significantly inhibited the growth of tumor, promoted splenocytes proliferation and macrophage phagocytosis, and increased the level of IL-2 and $CD8^+$ T cells in blood of tumor-bearing mice. The results suggested that GLSPs displayed remarkable anti-tumor and immunomodulatory activities, and it may provide a potential material of healthy food and clinical medicines.

1. Introduction

During the last decades, a mass of polysaccharides have been obtained from multifarious sources such as mushroom, bacteria, animal cartilage and carapace, leaves and roots of plant (Cimino et al., 2001; Liu et al., 2011; Nergard et al., 2004; Rout & Banerjee, 2007). These polysaccharides with long-chain and high-molecule-mass have attracted a great deal of attention because of their prominent bioactivities and relatively low toxicity.

Gracilaria lemaneiformis distributed widely in marine environment belongs to the family *Gracilariaceae* (Rhodophyta), in which majority members are utilized as the main sources of the manufacture of agar. Numerous studies have focused on the extraction of this neutral polysaccharide (Freile-Pelegrín & Murano, 2005; Pereira-Pacheco, Robledo, Rodríguez-Carvajal, & Freile-Pelegín, 2007; Sousa, Alves, Morais, Delerue-Matos, & Goncalves, 2010), but there also is a sort of acidic polysaccharide-sulfated polysaccharide in *G. lemaneiformis*, which is always got rid of as waste during the agar processing for a long period. Actually, in order to obtain agar with good quality, the indicator of desulfuration efficiency which strongly affects the physical properties of the gel is necessarily evaluated in the extraction (Marinho-Soriano & Bourret, 2003). However, according to the previous studies, sulfated polysaccharides have shown much more beneficial activities, such as anticoagulant (Alban, Sxhauerte, & Franz, 2002), antioxidant (Zou et al., 2008), antiviral (De Sousa Cardozo, Camelini, Mascarello, Rossi, & Nunes, 2011), anti-inflammatory (De Araújo, De Sousa Oliveira Vanderlei, Rodrigues, Coura, & Quinderé, 2011), antihyperlipidemic (Yu et al., 2003), antibacterial (Venkatpurwar & Pokharkar, 2011) and anti-tumor activities (Leiro, Castro, Arranz, & Lamas, 2007). Nevertheless, there were few reports on the acidic *G. lemaneiformis* polysaccharides and their anti-tumor and potential immunomodulatory activities.

Furthermore, although the medical treatments against cancer, including chemotherapy, radiotherapy and surgery, have been greatly improved, it is hard to take effective measures for advanced stage cancer. Therefore, more attentions have been paid for some new strategies such as vaccination (Zhang, Tsai, Monie, Hung, & Wu, 2010) and antigen-specific immunotherapy (Borghaei, Smith, & Campbell, 2009). Though effective vaccination is a convenient approach to prevent tumor, the security is worth considering carefully, and it will be quite difficult to make it generally work on. It is necessary to seek some new therapies taken orally to protect us from cancer and some other serious conditions. In the current

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doi:10.1016/j.carbpol.2012.02.014

study, an acidic polysaccharide was isolated from the macroalgae *G. lemaneiformis*, and its potential anti–tumor and immunomodulatory activities *in vivo* were investigated.

2. Methods

2.1. Chemicals

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), concanavalin A (ConA) and lipopolysaccharide (LPS) were provided by Sigma Chemical Co. Fetal calf serum (FCS) was from Hangzhou Sijiqing Co. The RMPI-1640 medium was purchased from HyClone, Thermo Scientific Inc. All other reagents were of reagent grade.

2.2. GLSPs preparation

Semi-dried *G. lemaneiformis* purchased from the market were cut into small pieces and pretreated with 0.3 M NaOH at room temperature for 2 h before the product was extracted with cold water three times (2 h each time). The filtrate was collected and centrifuged to get the supernatant, and the value of pH was adjusted to 3.0 with 0.1 M HCl, then the precipitation was removed. The supernatant was deposited by 30% (v/v) ethanol and left overnight at 4 °C to obtain the crude polysaccharides by centrifugation (3500 × *g*, 20 min). The precipitation was redissolved in distilled water, and protein was removed using n-butanol and chloroform (1:4, v/v) according to the method of Sevag (Alam & Gupta, 1986). Then the mixture was concentrated, dialyzed (Mw 3500) three days and lyophilized to yield GLSPs.

2.3. Characterization of GLSPs

2.3.1. Chemical composition assay

Carbohydrate was quantified by the phenol-sulfuric acid method using glucose as the standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Uronic acid was determined according to the method of carbazole-sulfuric acid using galacturonic acid as the standard (Xia et al., 2011). Protein content was measured by the Coomassie brilliant blue method using bovine serum albumin as the standard (Barbosa, Slater, & Marcos, 2009). The content of sulfate in polysaccharide was detected by the method of barium chloride-gelatin using potassium sulfate as the standard (Lloyd & Dodgson, 1961).

2.3.2. Infrared spectrum analysis

FTIR spectrum of GLSPs was collected with a Fourier transformed infrared spectrometer (VECTOR-22, BRUKER) in the wave number range of 4000–400 cm^{-1} using the KBr-disk method.

2.3.3. Molecular weight distribution assay

The molecular weight distribution of GLSPs was determined by high performance gel-permeation chromatography (HPGPC) using HPLC (Agilent-1200) equipped with a TSK-gel G4000PWxl column (7.8 mm × 300 mm, column temperature 40 °C) and a differential refractive index detector (RID). The sample was eluted with the mobile phase (0.2 M NaCl) and run at a flow rate of 0.8 ml/min. The standard curve was established using T-series Dextran as the standards (T-10, T-40, T-70, T-500 and T-2000).

2.3.4. Analysis of monosaccharide composition

Gas chromatography (GC) was performed for the monosaccharide identification. The polysaccharide was hydrolyzed with 2 M trifluoroacetic acid (TFA) at 120 °C for 2 h. The hydrolyzed product was converted into the alditol acetates as described (Blakeney, Harris, Henry, & Stone, 1983) and analyzed by GC using a capillary column (DB-17, Agilent) and flame-ionization detector (FID).

2.4. Animal groups and tumor transplantation

Forty eight-week-old female ICR mice with an average weight of 20.0 ± 2.0 g were divided into four groups with ten mice each group. Blank control and model control groups were both applied saline solution orally throughout the experimental period. GLSPs groups were given GLSPs with 200 or 600 mg per kilogram of body weight per day respectively in the same period. On day 14, all the mice were transplanted with H22 hepatoma cells provided by the Cell Bank of Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China) in the right thigh of the lower limb with 0.2 ml of 5×10^6 cells except blank control group. All groups received the oral administration for 4 weeks.

2.5. Physiological index observation

The tumor size (volume, *V*) was calculated from the tumor length (*L*) and width (*W*) which were measured with a caliper, using the following formula: $V = LW^2/2$ (Huang et al., 2011). Following 4 weeks feeding, mice were weighed and the blood was collected by extirpating the eyeball of the mice. Then the animals were sacrificed by cervical dislocation. Thymus and spleen were collected and weighed, and apparatus indexes were expressed as thymus or spleen weight relative to body weight.

2.6. Evaluation of interleukin 2 (IL-2) and lymphocyte subsets in peripheral blood

A small amount of blood was collected from the post-global veins of ICR mice alive, and lymphocytes were obtained *via* removal of the erythrocytes. 1×10^6 cells were incubated with either isotypic control or monoclonal antibodies (mAb) against $CD3^+$, $CD4^+$, $CD8^+$ or $CD19^+$ for 30 min on ice in the dark. After incubation, antigen expression was measured by flow cytometry (BD FACSCalibur, USA), and analyzed by CellQuest Pro. software. After the mice sacrificed, the serum of each mouse was prepared for IL-2 assessment using an ELISA kit (Dakewe Biotech. Co., Shenzhen, China). Under the manufacturer's instruction, the plate was read at 450 nm on a BD Microplate Reader.

2.7. Splenocyte proliferation assay

Proliferation of splenic lymphocytes was measured by MTT method as described previously (Cao et al., 2011). Briefly, spleen was passed through a steel mesh to obtain a homogeneous cell suspension under aseptic condition, and the erythrocytes were lysed with ammonium chloride (0.8%, w/v) subsequently. The cells were washed three times with PBS and resuspended in complete medium (RPMI 1640 supplemented with 12 mM HEPES (pH 7.1), 0.05 mM 2-mercaptoethanol, 100 IU/ml penicillin, 100 μg/ml streptomycin and 10% FCS). Then splenocytes were seeded into a 96-well flat-bottom microtiter plate with 100 μl each well at 1×10^7 cells/ml in the presence of ConA (5 μg/ml), LPS (10 μg/ml), or RPMI 1640 medium to give a final volume of 200 μl. Following the plate was incubated at 37 °C in a humid atmosphere with 5% CO_2 for 44 h, 20 μl of MTT solution (5 mg/ml) was added to each well and incubated for another 4 h. The plates were centrifuged (1400 × *g*, 5 min) and the untransformed MTT was removed carefully by a pipette. To each well 150 μl of DMSO was added, and the absorbance was evaluated on an ELISA reader at 570 nm with a 630 nm reference after 20 min. The stimulation index (SI) was calculated based on the following formula: SI = the absorbance value

for mitogen-cultures/the absorbance value for non-stimulated cultures.

2.8. Macrophage phagocytosis assay

Macrophage phagocytosis was assessed according to the previous method with a little modification (Zhao, Dong, Chen, & Hu, 2010). Stimulated by 4 ml of Hank's solution injected intraperitoneally, the mice abdomen was kneaded for 5 min. The macrophages were aseptically prepared from peritoneal exudates of mice as quickly as possible and washed three times, then adjusted to 5×10^6 cells/ml. 100 μl of macrophages each well were added into 96-well flat-bottom microtiter plate, and incubated for 5 h at 37 °C in a humid atmosphere with 5% CO_2. Following incubation, the supernatant was discarded and 50 μl of 0.073% neutral red was added each well. Incubation for another 30 min, the superfluous neutral red was abandoned. The plate was washed twice by PBS pre-heated at 37 °C and added 100 μl of cell lysis solution (100 mmol/l acetic acid: anhydrous ethanol = 1:1 v/v) each well overnight in 4 °C. The absorbance was obtained at 540 nm.

2.9. Statistical analysis

Data were presented as the mean ± standard deviation (S.D.) for all treatments. Student's *t*-test and ANOVA were performed for their statistic significance of difference with controls. The data was considered statistically different from controls at $p < 0.05$.

3. Results and discussion

3.1. Preparation

The acidic polysaccharides from *G. lemaneiformis*, primarily the sulfated polysaccharides, were isolated with a yield of 2.06% calculated as the weight of semi-dried *G. lemaneiformis* previous to the agar extraction. In order to avoid agarose being dissolved out in abundance and improve the gel strength and stability of agar, room temperature and cold alkali solution were used during the separation process of sulfated polysaccharide, which was different from other means (De Araújo et al., 2011; Dong, Hayashi, Mizukoshi, Lee, & Hayashi, 2011). Dilute alkali solution was considered in respect that agar was stable in alkalescent condition, but the sulfated component was dissolved out easily (Freile-Pelegrín & Murano, 2005). Besides, it is shown that a plenty of flocs appeared when ethanol concentration was up to 30% (v/v), and there was no obvious increase of product with the enhancement of alcohol concentration.

3.2. Chemical analysis and partial characterization

Carbohydrate, uronic acid, protein and sulfate contents of GLSPs were 72.06%, 4.50%, 0.28% and 6.13% respectively by chemical analysis. The results showed that protein content of GLSPs was much lower than that of the polysaccharides from other sources (Wang, Luo, Zha, & Feng, 2010; Xie et al., 2008).

The infrared spectrum of GLSPs shown in Fig. 1 displayed an intense peak at 3449 cm^{-1} due to the hydroxyl stretching vibration and a weak peak at 2948 cm^{-1} due to C-H stretching vibration. The broad band at 1640 cm^{-1} was assigned to the bound water (Majumder, Singh, & Goyal, 2009). The strong band distribution of 1200–950 cm^{-1} within the so-called fingerprint region was specific for each polysaccharide, which showed the possible characteristics of polysaccharide according to the position and intensity of the bands. The intense peaks at 1031 and 1072 cm^{-1} were attributed to the glycosidic linkage C—O—H and C—O—C stretching vibration (Ai et al., 2008). The peak at 932 cm^{-1} was assigned to C-O vibration

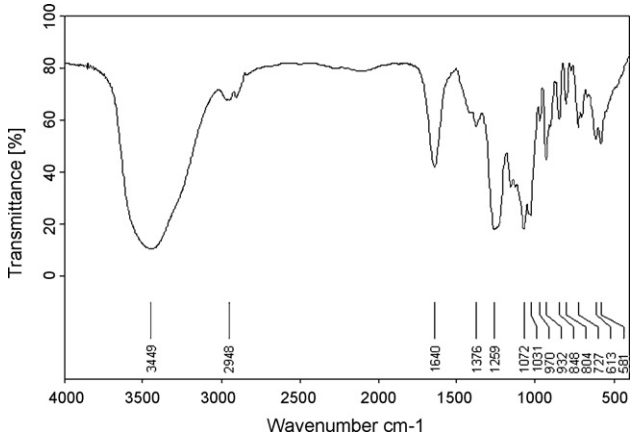

Fig. 1. FTIR spectrum in KBr disk of GLSPs in the wavenumber region between 4000 and 400 cm^{-1}.

of 3, 6-anhydro-α-L-galactose (Andriamanantoanina, Chambat, & Rinaudo, 2007). In particular, the strong characteristic absorptions at 1259 cm^{-1} and 804 cm^{-1}, due to the sulfo groups, were assigned to S=O and C—O—S bond stretching respectively (Xie et al., 2008).

The result of HPGPC analysis (Fig. 2) indicated that there were two main peaks in the illustration with the retention times of 9.547 and 13.140 min respectively, which might be two different average molecular weights of GLSPs. Probably it still included a small amount of other constituents that were removed incompletely like proteins, pigments, lipids combined with polysaccharide and so forth. These two peaks were both much symmetrical, and the area proportions were 76.13% and 14.60% respectively. It was drawn to a conclusion that the principal constituent of GLSPs accounted for 76.13% of the product was mainly distributed at 1.37×10^6 Da calculated in accordance with the retention time of standard dextrans.

Analysis of monosaccharide composition of GLSPs using GC (Fig. 3) revealed that GLSPs was mainly composed of galactose, and additionally contained a small amount of rhamnose, arabinose, xylose and mannose. The molar ratio of them was 29.64:0.19:0.21:0.59:1.00 in turn.

3.3. Anti-tumor activity of GLSPs in vivo

Two doses (200 and 600 mg/kg) of GLSPs conferred significantly preventative and inhibitory activities against H22 solid tumor formation in *vivo*. The differences in tumor size between the groups were noted at the end of experiment period. As the results presented in Table 1, the mean tumor sizes in model control group were relatively different from GLSPs groups ($p < 0.05$). All mice in

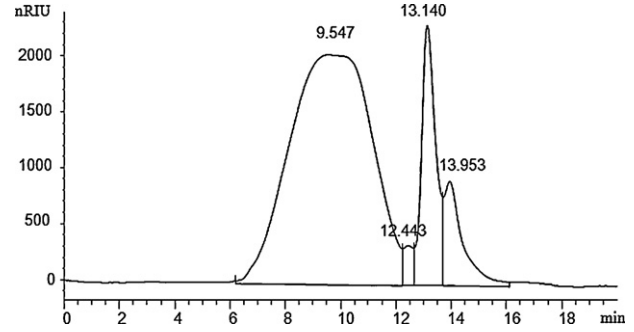

Fig. 2. HPGPC profiles of GLSPs. The molecular weight distribution of GLSPs was determined by high performance gel-permeation chromatography (HPGPC) equipped with a TSK-gel G4000PWxl column and a differential refractive index detector. The sample was eluted with the mobile phase (0.2 M NaCl) and run at a flow rate of 0.8 ml/min.

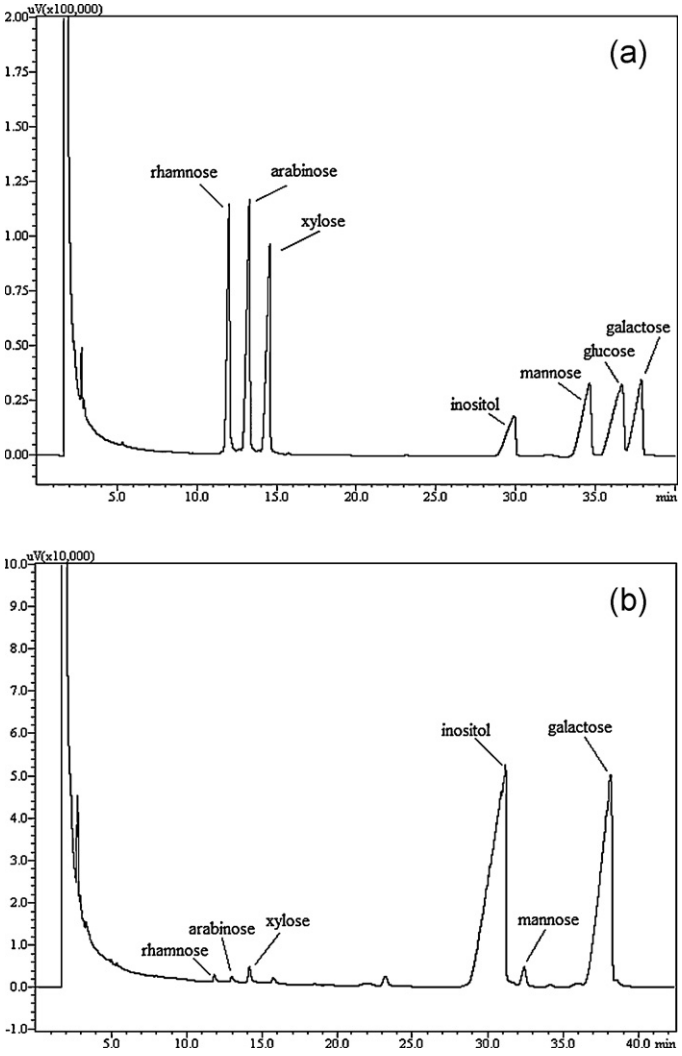

Fig. 3. GC chromatography of alditol acetate derivatives of standard monosaccharide (a) and GLSPs (b).

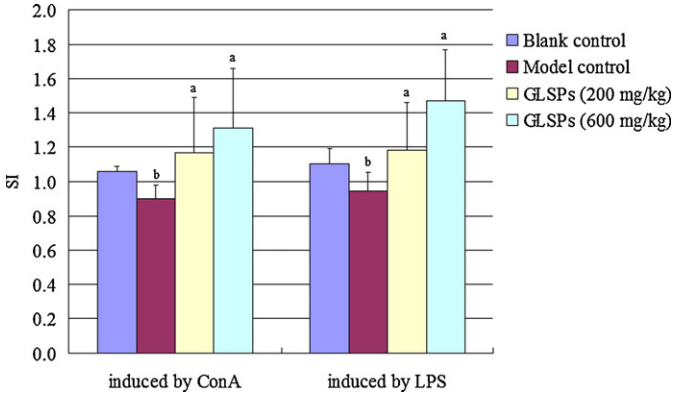

Fig. 4. Effect of GLSPs on ConA- and LPS-induced splenocyte proliferation in tumor-bearing mice. Splenocyte proliferation was measured by the MTT method as described in the text and shown as a stimulation index (SI). Each value represents the mean ± S.D. ($n = 8$). Significant differences compared to model control group are designated as $^{a}p < 0.05$. Significant differences compared to blank control group are designated as $^{b}p < 0.05$.

model control group were implanted tumor successfully and began to develop visible tumors at one week after transplantation. In contrast, the mice in GLSPs groups were noticed the appearance of tumor after approximately 10 days of transplantation with obviously smaller tumor size. However, due to the two or three mice implanted unsuccessfully, the relative standard deviation of tumor size in GLSPs groups was much greater than the model control. It was also shown that the antitumor effect of GLSPs at 600 mg/kg was superior to the dose of 200 mg/kg, indicating that the beneficial effect was acted in a dose-dependent manner.

As Table 1 shown, there was no significant difference of body weight in 0-2 week between the groups, while a considerable increase of body weight in model control group appeared after 4 week's administration compared to the other groups ($p < 0.05$), which might be due to the rapidly growing tumor in model control group.

3.4. Immune functions of GLSPs in tumor-bearing mice

The immunomodulatory potential of GLSPs *in vivo* was evaluated by thymus and spleen indexes, splenocyte proliferation, macrophage phagocytosis as well as the level of IL-2 and lymphocyte subsets in blood of tumor-bearing mice. Compared to the blank control, there was a significant increase of the spleen index in the model control group ($p < 0.05$), while the thymus index decreased obviously (Table 1), suggesting that the spleen of mice in model control group developed a severe inflammation and enlargement, and the thymus was probably damaged and diminished owing to the tumor transplantation. However, the thymus index of GLSPs groups also reduced to some extent compared to the blank control ($p < 0.05$), but it was superior to the model control ($p < 0.05$). With the same situation, the spleen index ascended significantly compared to the blank control ($p < 0.05$), but it was superior to the model control ($p < 0.05$). It was indicated that the innate immunity of tumor-bearing mice was activated by GLSPs, and the immune organs were protected well from the grievous damage. To this point, GLSPs at the dose of 600 mg/kg performed more preferably than the dose of 200 mg/kg.

The effect assay of splenocyte proliferation stimulated by different mitogens in H22-bearing mice was performed to evaluate the immune modulatory potential of GLSPs (Fig. 4). As shown, GLSPs at the two doses significantly promoted the splenocyte proliferation ability induced by both ConA and LPS compared to the model control ($p < 0.05$) in a dose-dependent manner. It is generally known

Table 1
Effects of GLSPs on body weight, tumor size, thymus and spleen indexes of tumor-bearing mice.

Groups	Dose (mg/kg)	Weight (g)			Tumor size (cm^3)	Thymus index (mg/g)	Spleen index (mg/g)
		0 week	2 week	4 week			
Blank control	–	23.09 ± 1.62	28.47 ± 1.91	31.39 ± 2.09	–	4.16 ± 0.45	3.86 ± 0.52
Model control	–	23.19 ± 0.89	27.80 ± 0.99	35.91 ± 2.54	1.12 ± 0.37	0.63 ± 0.03	9.80 ± 0.51
GLSPs	200	23.29 ± 1.66	27.30 ± 0.92	33.80 ± 2.71[a]	0.17 ± 0.13[a]	1.49 ± 0.20[a,b]	7.11 ± 0.41[a,b]
GLSPs	600	23.94 ± 1.13	28.38 ± 2.37	33.84 ± 1.83[a]	0.06 ± 0.05[a]	1.76 ± 0.36[a,b]	5.15 ± 0.64[a,b]

Tumor size was measured at the end of experiment period and calculated using the equation: $V = LW^2/2$. Each value represents the mean ± S.D. ($n = 8$).
[a] $p < 0.05$ compared to model control.
[b] $p < 0.05$ compared to blank control.

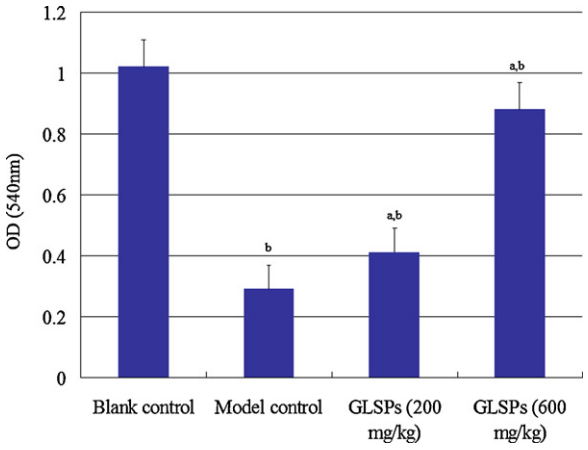

Fig. 5. Effect of GLSPs on macrophages phagocytosis in tumor-bearing mice. Macrophage phagocytosis was evaluated by the OD value (540 nm) of neutral red ingested by macrophage. Each value represents the mean ± S.D. ($n = 8$). Significant differences compared to model control are designated as $^{a}p < 0.05$. Significant differences compared to blank control are designated as $^{b}p < 0.05$.

that ConA stimulates T-cells as well as LPS stimulates B-cells proliferation, and T-cells specifically regulate the cell-mediated immune, additionally B-cells are responsible for the specific antigen antibody reaction related to the humoral immune. The stimulation effect of GLSPs was even superior to the blank control, probably because the immunity of mice in GLSPs groups was caused more activation by the polysaccharide so as to promote the body defense and resist the tumor cells.

It was reported that macrophage which is directly involved in the antigen presentation and killing of tumor cells is one of the target cells of polysaccharides (Liu et al., 2008). As shown in Fig. 5, the macrophage phagocytosis towards neutral red of tumor-bearing mice reduced observably compared to the blank control ($p < 0.05$), and it was perhaps due to the sacrifice of macrophages in the counterwork against tumor cells. In spite of that, the GLSPs relatively enhanced the macrophage phagocytosis compared to the model control ($p < 0.05$) in a dose-relevant manner.

The different proportions of lymphocyte subsets in mice peripheral blood were shown in Table 2. As the results, the percentage of $CD19^+$ cells in model control and GLSPs groups was notably descended relative to the blank control ($p < 0.05$), while $CD3^+$ cells was notably ascended. The percentage of $CD4^+$ cells in the GLSPs groups was significantly reduced compared to the blank control ($p < 0.05$), while $CD8^+$ cells increased significantly in GLSPs group at the dose of 600 mg/kg ($p < 0.05$) (Fig. 6). However, the percentages of all the lymphocyte subsets ($CD3^+$, $CD19^+$, $CD4^+$ and $CD8^+$ cells) in GLSPs groups did not significantly differ from model control group except the $CD3^+$ cells of GLSPs group at the dose of 600 mg/kg, which was significantly lower than the model control ($p < 0.05$). Actually, although the statistical differences between GLSPs groups and model control group were obscure, there still was visible increasing or decreasing trend according to the results. Moreover, the concentration of IL-2 in serum was assayed to assess the immune function of GLSPs. As Table 2 shown, the amounts of IL-2 in GLSPs groups were significantly higher than the blank control and model control ($p < 0.05$), but there was no manifest dose-dependence.

In view of the facts, there was a distinct increase of $CD8^+$ cells and IL-2 in the peripheral blood of tumor-bearing mice in the GLSPs group at 600 mg/kg. $CD8^+$ cells quantify the cytotoxicity T cells, and IL-2 could enhance the proliferation of T cells, B cells and monocytes and increase the cytotoxicity of T cells. These results suggested

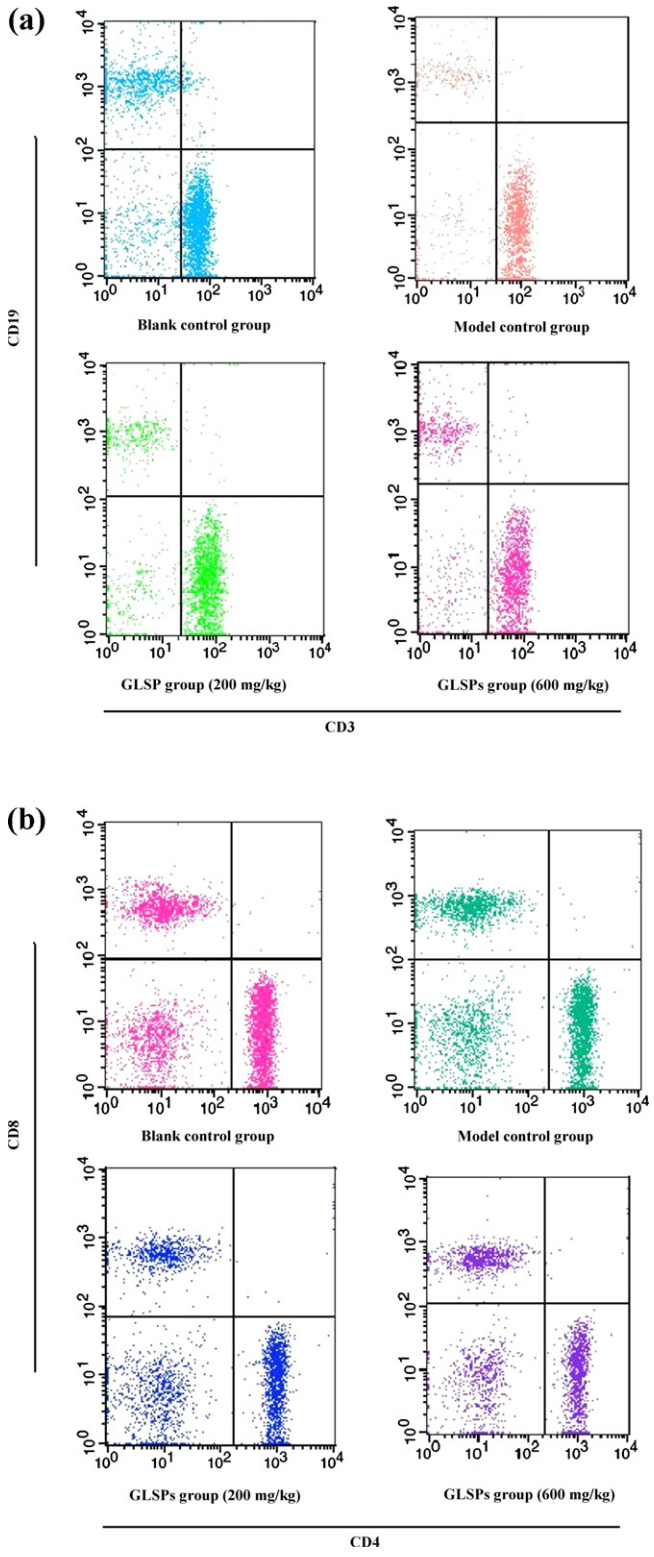

Fig. 6. Analysis of $CD3^+$, $CD4^+$, $CD8^+$ and $CD19^+$ cells in peripheral blood of tumor-bearing mice in blank control group, model control group, GLSPs group (200 mg/kg) and GLSPs group (600 mg/kg). The percentages of $CD3^+$ and $CD19^+$ cells were detected by flow cytometry using $CD3^+$ or $CD19^+$ monoclonal antibody (a). The percentages of $CD4^+$ and $CD8^+$ cells were detected by flow cytometry using $CD4^+$ or $CD8^+$ monoclonal antibody (b).

Table 2
Effects of GLSPs on the serum IL-2 concentration and percentages of lymphocyte subsets in peripheral blood of tumor-bearing mice.

Groups	Dose (mg/kg)	IL-2 (pg/ml)	$CD3^+$ (%)	$CD19^+$ (%)	$CD4^+$ (%)	$CD8^+$ (%)
Blank control	–	4.31 ± 0.90	65.62 ± 3.59	23.28 ± 3.90	50.56 ± 1.31	24.43 ± 1.69
Model control	–	5.10 ± 0.54	74.76 ± 4.51[b]	13.18 ± 1.98[b]	47.17 ± 3.04	27.89 ± 3.72
GLSPs	200	6.83 ± 0.81[a,b]	72.82 ± 4.29[b]	15.58 ± 4.76[b]	46.88 ± 2.54[b]	24.36 ± 4.20
GLSPs	600	6.37 ± 0.82[a,b]	68.60 ± 2.71[a]	16.99 ± 3.08[b]	43.97 ± 3.85[b]	29.28 ± 2.85[b]

Each value represents the mean ± S.D. ($n = 5$).
[a] $p < 0.05$ compared to model control.
[b] $p < 0.05$ compared to blank control.

that GLSPs enhanced the innate immunity in H22 tumor-bearing mice.

4. Conclusions

In this study, an acidic polysaccharide with the main molecular weight distribution of 1.37×10^6 Da and the sulfate content of 6.13%, which mainly composed of galactose, was obtained from *Gracilaria lemaneifor*. Treated with GLSPs could significantly inhibit the growth of transplanted tumor in mice and enhance splenocytes proliferation, macrophage phagocytosis, the level of IL-2 and the percentage of $CD8^+$ T cells in blood of tumor-bearing mice, which indicated that the GLSPs could improve both specific and non-specific cellular immune response and suggested the anti-tumor activity of GLSPs might be acted through immune-regulating mechanism.

Acknowledgements

The present work was supported by grants from Scientific and Technical Supporting Programs of Tianjin (No. 10ZXCXSH02600), Innovation Fund for Small and Medium Technology Based Corporations of Chinese Ministry of Science and Technology (No. 10C26211200196), National Natural Science Foundation of China (No. 31000755) and Key Project of Chinese Ministry of Education (No. 211008).

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