Antitumor and Immunoregulatory Activities of Polysaccharides from Gracilaria Lemaneiformis on S180 Tumor-Bearing Mice

Abstract

Gracilaria Lemaneiformis polysaccharides (GLPs) were extracted with 1.5 M NaOH solution, their primary structural characterization and *in vivo* antitumor activity were evaluated in this study. Chemical analysis showed that GLPs were sulfated polysaccharides containing 76.49% of carbohydrate, 27.88% of uronic acid, and 11.23% of sulfate, results of FT-IR, GC, HPGPC indicated that GLPs were mainly composed of galactose with α-type glycosidic linkages and pyranose form. The *in vivo* antitumor tests revealed that GLPs could effectively suppress the growth of transplanted S180 tumors in Kunming mice. Besides, the spleen/thymus indexes, splenic lymphocytes proliferation, and NK cells activities, as well as the levels of serums IL-2, IFN-γ and TNF-α of S180-bearing mice were remarkably improved by GLPs oral administration. Our results suggested that the notable antitumor activity of GLPs were mediated by the activation of antitumor immune responses.

Keywords: *Gracilaria Lemaneiformis*; Polysaccharides; Antitumor; Immunomodulatory

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Abbreviations: GLPs: *Gracilaria Lemaneiformis* Polysaccharides; FTIR: Fourier Transform Infrared Spectroscopy; GC: Gas Chromatography; HPGPC: High Performance Gel Permeation Chromatography; NK: Natural Killer; IL-2: Interleukin-2; IFN-γ: Interferon-γ; TNF-α: Tumor Necrosis Factor-α; BRM: Biological Response Modifier; ConA: Concanavalin A; LPS: Lipo Poly Saccharide; DMSO: Dimethyl Sulfoxide; FBS: Fetal Bovine Serum; FID: Flame-Ionization Detector; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5 Diphenyltetrazolium bromide; 5-FU: 5-Fluorouracil.

Introduction

Cancer continues to be the leading cause of death and accounts for approximately 13% of deaths all over the world; however, more than 30% of cancer-induced deaths can be avoided by changing or avoiding key risk factors [1]. As we all know, chemotherapy or radiotherapy are conventional and effective approaches for the tumor treatment [2]. However, these traditional therapies are not only costly but also extremely easy to cause lots of undesirable side effects and influence the survival probability [3-5]. Therefore, further exploration of novel anti-cancer drugs will be crucial for human beings to prevent and treat cancer.

Immunosuppression can be detected well in both tumor-bearing animals and cancer patients [6,7], which indicates the importance of immune system in immunosurveillance against malignant cells. Thus, boosting the immunity of organism will be helpful for the prophylaxis and therapy of malignant tumors. Recently, polysaccharides as a kind of biological response modifier (BRM) have been extensively used as preventive and therapeutic agents for cancer because of their relatively low toxicity and effective antitumor activities via enhancing antitumor immune responses of an individual [8-11]. In view of this, further research should be undertaken to isolate the polysaccharides from natural sources, such as algae, plants, microorganisms, and so forth.

Gracilaria lemaneiformis, one of the red seaweed in *Gracilariaceae* family native to China and other eastern Asian countries, has been widely consumed as the sea vegetables and Chinese medicinal herb in oriental countries [12,13]. At present, *G. lemaneiformis* is widely used to produce agarose and other natural polysaccharides, which has been demonstrated to have the antitumor, immunoregulatory, and hypoglycemic activities, etc. [14,15]. However, the potential application of *G. lemaneiformis* bioactive polysaccharides is still not enough in food and pharmaceutical industries due to their poor solubility and high viscosity [16].

In this study, the residues of *G. lemaneiformis* pretreated with 0.3 M NaOH at room temperature for 4 h were used as raw materials, a novel acidic sulfated polysaccharide was extracted with 1.5 M NaOH at room temperature, and its potential antitumor and Immunomodulatory activities *in vivo* were investigated using S180-bearing mice model. These results will be valuable to provide the scientific basis for comprehensive utilization of *G. lemaneiformis* polysaccharides in functional food and pharmaceuticals industries.

Experimental

Materials and chemicals

The *G. lemaneiformis* was purchased from a local supermarket in Tianjin. Concanavalin A (ConA), lipopolysaccharide (LPS), dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-

2,5diphenyltetrazolium bromide (MTT) were provided by Sigma Chemical Co. (St. Louis, MO, USA). Medium RPMI-1640 was obtained from Hyclone (Thermo Scientific Inc, USA). Fetal bovine serum (FBS) was purchased from Hangzhou Sijiqing Corp (Hangzhou, China). Cytokine detecting ELISA kits were provided by Lufeng Bio-Technique Co. Ltd (Shanghai, China). All the chemicals and agents were of analytical grade.

Preparation of GLPs

The residues, obtained according to previous method [14], which was residual after the extraction of GLSPs, were extracted with 1.5 M NaOH solution three times at room temperature. The supernatant was collected by centrifugation (5000 rpm, 20 min) and neutralized with 1.0 M HCl. The filtrate was concentrated under reduced pressure and precipitated with four volumes of anhydrous ethanol overnight at 4˚C. The precipitate was dissolved in the distilled water and the protein was removed by the Sevag method [17]. After the removal of Sevag reagents, the crude sample was concentrated, dialyzed (Mw 3500), and finally lyophilized to yield the GLPs.

Chemical characterization

The total carbohydrate was determined using the phenol-sulphuric method with glucose as the standard [18]. Uronic acid was quantified by the method of carbazole-sulfuric acid using galacturonic acid as the standard [19]. The content of sulfate was measured by barium chloride-gelatin method using potassium sulfate as the standard [20].

Determination of homogeneity and molecular weight

The average molecular weight of the polysaccharide fraction was evaluated by high performance gel permeation chromatography (HPGPC) (Agilent-1200) equipped with a TSK-gel G4000PWxl column (7.8 mm×300 mm, column temperature 30˚C) and Refractive Index Detector (RID, detector temperature 35˚C) (Schambeck SFD GmbH, German). The samples (20 μL) were performed and run with the ultrapure water as their mobile phase at 0.6 mL/min. The average molecular weight of the polysaccharide fraction was calculated by the standard curve established using T-series Dextran (T-10, T-40, T-70, T-110, T-500 and T-2000) as standards.

Monosaccharide composition analysis

Monosaccharide composition of GLPs was determined by gas chromatography (GC) equipped with a capillary column (DB-17, Agilent) and flame-ionization detector (FID). The polysaccharide sample was dissolved in 2 M TFA and hydrolyzed at 120˚C for 3 h. After the TFA was removed, the hydrolysates were acetylated according to previous method [21]. The myo-inositol was used as an internal standard. The monosaccharides, including D-rhamnose, L-arabinose, D-galactose, D-glucose, L-xylose and D-mannose, were also derivatized for GC analysis (GC2010, Shimadzu, Japan).

IR spectrum analysis

FT-IR spectrum was performed with a Fourier-transform infrared spectrophotometer (Bruker VECTOR-22, German) in the wavelength range of 4000-400 cm^{-1} using the KBr pellet method.

Cell lines and culture

Mouse Sarcoma S180 cells were purchased from Shanghai Institute for Biological Sciences (Chinese Academy of Sciences, Shanghai, China) and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and then cultured at 37˚C in 5% CO_2.

Animals

Female Kunming mice (20±2 g) were purchased from the Center of Experimental Animals of the Academy of Military Science (Beijing, China). These animals were housed under pathogen-free conditions at a controlled temperature (20-25˚C) and relative humidity (50±5%). All mice were allowed to acclimate for a week in animal barrier system prior to experiments. All animal experimental procedures were conducted according to the principles of Laboratory Animal Care as approved by the Local Ethics Committee for Animal Care and Use at Tianjin University of Science and Technology.

Experimental design and drug treatment

Fifty healthy mice were randomly divided into five groups, including blank group, model control group, positive control group (5-FU, 20 mg/kg), and two doses of GLPs groups (125 and 250 mg/kg GLPs). All mice were provided with tap water and standard pellet diet on a 12 h light/dark cycle throughout the experimental period. The mice in GLPs groups were intragastrically administrated with GLPs, whereas the normal control and model control mice were treated with the equal volume of saline solution once a day. After 15 days of gavage, all mice except the blank group were transplanted with S180 tumor cells (1×10^7 cells/mL, 0.2 mL) in armpit of right hind limbs. All groups were orally administrated once daily for another 15 days, whereas 5-FU was intraperitoneally administered in the positive group once daily for 10 days.

Tumor inhibition rate and immune organ indexes

24 hours after the last dose, all the mice were sacrificed and the tumors, thymuses, and spleens were dissected carefully and weighed immediately. The tumor inhibitory rate was calculated as follows: inhibitory rate (%) = [(M−T) / M]×100, where M is the average tumor weight of the model group and T is that of the treated groups. The thymus and spleen indexes were evaluated by the following formula: thymus/spleen indexes = the average weight of thymus/spleen (mg) / the average body weight (g).

Apoptosis detection of tumor tissues

The tumor cells suspension were prepared and fixed with cold 70% ethanol for at least 18 h at 4˚C. Subsequently, the fixed cells were subjected to PI/RNase Staining Buffer for PI labeling and analyzed by flow cytometer (FACS Cablibur, Becton Dickinson, USA).

Lymphocytes proliferation assay

Lymphocytes proliferation assay was measured as described previously, with a slight modification [22]. The homogeneous cell suspension was obtained from spleens through sterilized meshes (200 meshes). The prepared splenocytes (1×10^6 cells/mL)

were seeded into a 96-well plate and cultured with ConA (final concentration is 5 μg/mL), LPS (final concentration is 10 μg/mL) or RPMI-1640 medium with a final volume of 200 μL at 37°C with 5% CO_2. After 48 h of incubation, the number of cells was determined by MTT assay at 570 nm using a microplate reader (Model 680, Bio-Rad, Hercules, CA, USA).

Natural killer (NK) cells cytotoxicity

The splenocytes (2×10^6 cells/mL) obtained above were used as effector cells and S180 cells were used as target cells. Splenocytes and S180 cells were incubated at the ratio of 20:1 in 96-well plates. After incubation for 48 h at 37°C in a humidified 5% CO_2 incubator, the absorbance values were measured at 570 nm by MTT assay using a microplate reader (Model 680, Bio-Rad, and Hercules, CA, USA). The percentage of NK cells was calculated using the following equation: NK cytotoxic activity (%)= $1-(OD_\alpha-OD_\beta)/OD_0\times100$, where OD_α is the OD value of effector-to-target cells, OD_β is the OD value of effector cells, and OD_0 is the OD value of target cells.

Evaluation of cytokines in serum

After mice were sacrificed, the blood was immediately transferred into test tubes and settled at room temperature for 30 min. The serum was obtained from blood samples by centrifugation (5000×g, 15 min) for IL-2, IFN-γ, and TNF-α assessment using ELISA kits according to the manufacture's protocol.

Statistical analysis

All values were presented as the mean±standard deviation (S.D.) and the statistical significance of differences was determined using the student's t-test and one-way analysis of variance.

Results

Primary characteristics of GLPs

The total sugar, uronic acid and sulfate contents of GLPs were 76.49%, 27.88% and 11.23%, respectively, based on our chemical analysis. As shown in Figure 1, HPGPC analysis showed that the average molecular weight of GLPs was 20, 958 Da, which was calculated based on the standard dextran curve.

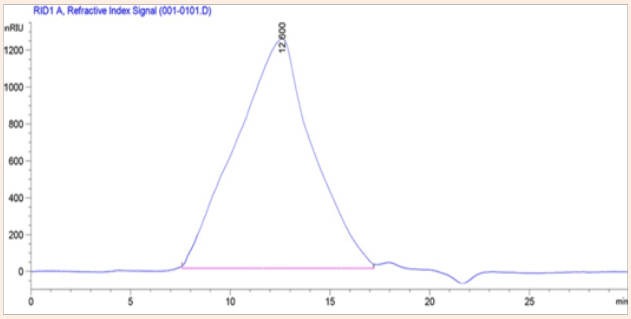

Figure 1: HPGPC profiles of GLPs.

The results of GC analysis (Figure 2) displayed that GLPs was a heteropolysaccharide which was composed of arabinose, xylose, mannose, glucose, and galactose, with molar ratios of 0.22: 0.23: 0.11: 0.17: 1.00, respectively. Our results suggested that galactose was the predominant sugar in GLPs.

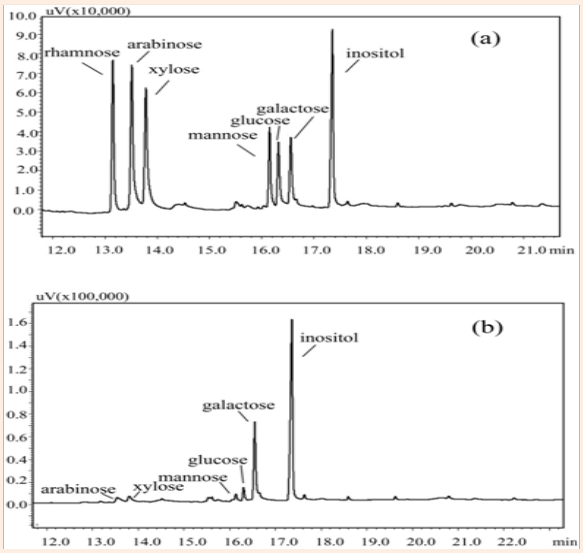

Figure 2: GC chromatograms of standard monosaccharide (a) and GLPs (b).

The IR spectrum of GLPs (Figure 3) revealed that the polysaccharide have three characteristic absorption peaks: an intense peak at 3430.8 cm^{-1}, which we can attribute to the -OH stretching vibration, a weak peak at 2921.2 cm^{-1} due to the C-H stretching vibration, and a peak at 1635.7 cm^{-1} due to the C=O stretching vibration. Furthermore, the three absorption peaks at 1158.3 cm^{-1}, 1065.2 cm^{-1} and 1025.0 cm^{-1} indicated the existence of pyranose, and a characteristic peak at 845.9 cm^{-1} was attributed to the presence of α-type glycosidic linkages. In particularly, the IR spectrum showed a strong absorption at 1227.3 cm^{-1} due to S=O band stretching, indicating the presence of sulfates in GLPs. The peak at 845.9 cm^{-1} suggested that most sulfated groups of GLPs were at axial position [16]. The absorption at 930.9 cm^{-1} was characteristic of 3, 6-anhydro-α-L-galactose [14].

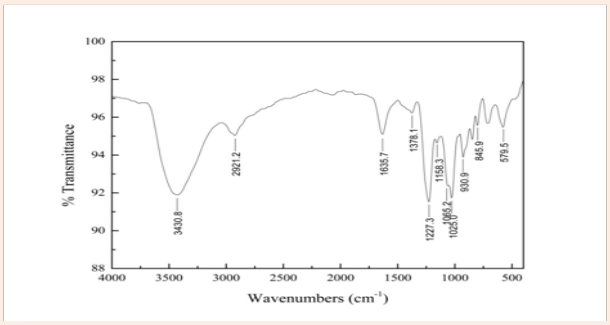

Figure 3: IR spectrums of GLPs.

GLPs suppressed S180 cells growth in vivo

To investigate whether GLPs had inhibitory effects against S180 solid tumor growth *in vivo*, the S180 tumor-bearing mice were orally administrated with different dosages (125 and 250

mg/kg) of GLPs. As shown in Table 1, the average tumor weight in the GLPs groups dramatically shrank compared with that of the model group, with a tumor growth inhibitory ratio of 39.74 % for GLPs (125 mg/kg) and 59.35 % for GLPs (250 mg/kg), respectively. The inhibitory rate of high-dose GLPs group was comparable to that of 5-FU group (52.77%). In addition, the spleens and thymuses indexes of the GLPs-treated groups were significantly improved compared to those of the model group (p<0.05 or p<0.01), dose-dependently. Although, the 5-FU treatment exhibited a relatively high tumor inhibitory rate, the immune organ indexes of the S180-bearing mice in this group was also lower than those of the blank group (p<0.01), indicating the toxic effect of 5-FU on immune system as previously described. In terms of the body weight, a significant decrease of the average body weight was observed in the model group compared to the blank group (p<0.05). In contrast, the body weights in the GLPs groups, especially in the high-dose group (250 mg/kg) were higher than those of the model control (p<0.05), whereas those in 5-FU group was lower (p<0.05), indicating the side effects of 5-FU on the body weights of tumor-bearing mice. More importantly, the GLPs treatment could remarkably increase the survival rate of tumor-bearing mice compared to the 5-FU-treated group.

Table 1: Effects of GLPs on immune organ indexes and tumor growth in S180-bearing mice.

Groups	Dose (mg/kg)	Body Weight (g)		Numbers Start/End	Tumor Weight (g)	Inhibitory Rate (%)	Spleen Index (mg/g)	Thymus Index (mg/kg)
		Start	End					
Blank	-	23.05±1.17	27.95±1.04	10/10	-	-	4.67±0.42	2.44±0.59
Model	-	22.64±1.76	26.08±1.19*	10/10	2.47±0.63	-	7.54±0.56**	1.12±0.39**
5-FU	20	22.93±1.57	23.82±2.06$^{**\#}$	10/8	1.17±0.36$^{\#\#}$	52.77	3.06±0.68$^{**\#\#}$	0.89±0.41**
GLPs	125	23.23±2.01	27.02±1.93	10/10	1.95±0.47$^{\#}$	32.58	6.96±0.42**	1.62±0.44$^{**\#}$
	250	23.21±1.67	27.60±1.22$^{\#}$	10/10	1.23±0.56$^{\#\#}$	50.15	4.77±0.70$^{\#\#}$	2.54±0.58$^{\#\#}$

Note: *p<0.05 compared with the blank group. **p<0.01 compared with the blank group. #p<0.05 compared with the model group. ##p<0.01 compared with the model group.

Effects of GLPs on tumor cells apoptosis

To identify whether the tumor growth inhibitory effect of GLPs was due to tumor cells apoptosis, we investigated cell cycle arrest of S180 tumor cells in tumor-bearing mice by flow cytometry. As shown in Figure 4, GLPs treatment resulted in a dose-related accumulation of cell numbers in G_0/G_1 phase with an increase from 37.81 % to 54.28 %, and a corresponding decline in the S phase. In addition, the percentages of apoptotic DNA in sub-G1 phase were dramatically elevated in the positive control group (5-FU), which was up to 44.11%, whereas those of the GLPs-treated groups were 21.75 % and 39.75 %, respectively, at the dosages of 125 and 250 mg/kg. Our results indicated that GLPs could induce S180 tumor cells apoptosis by blocking the cell cycle in G_0/G_1 phase.

Effects of GLPs on splenic lymphocyte proliferation and NK cells activity in tumor-bearing mice

MTT assay was carried out to evaluate the effect of GLPs oral administration on splenic lymphocyte proliferation and NK cells activities in S180-bearing mice (Table 2). As shown, the proliferation of T cells and B cells of model group, induced by ConA and LPS, respectively, were significantly decreased compared to the blank group (p<0.01). In contrast, the splenocyte proliferation of the GLPs-treated groups was remarkably increased compared with the model group (p<0.05 or p<0.01), suggesting that GLPs could activate cellular and humoral immune responses to strengthen body function of tumor-bearing mice in a dose-dependent manner.

As shown in Table 2, the killing activity of NK cells in the model group was lower than that of the blank group (p<0.01), whereas the 5-FU-treated S180-bearing mice exhibited lower killing activity by NK cells than the model group (p<0.01). However, the NK cells activity of GLPs groups were significantly enhanced in comparison with the model mice (p<0.05 or p<0.01), dose-dependently.

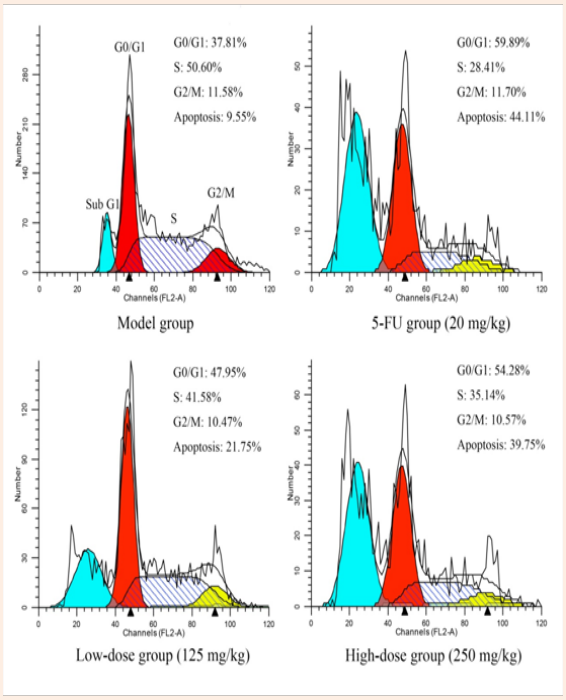

Figure 4: Effects of GLPs on cell cycle phase distribution of S180 tumor cells in vivo. The blue, red, yellow colored and shaded areas on the graphs showed the percentages of cell numbers in Sub-G1, G0/G1, G2/M, and S phase, respectively.

Table 2: Effect of GLPs on the spleen lymphocyte proliferation, and NK cells activities in S180-bearing mice.

Groups	Dose (mg/kg)	Stimulation Index		Activity of NK Cells (%)
		ConA	LPS	
Blank	-	3.72±1.08	6.36±0.98	63.30±5.48
Model	-	2.36±0.35**	4.11±0.91**	46.99±6.28**
5-FU	20	1.49±0.46**#	2.94±0.56**#	36.64±6.53**##
GLPs	125	4.31±0.80##	5.37±0.82*#	54.64±5.93*#
	250	5.12±0.61**##	7.71±0.73**##	68.29±3.59##

Note: *p<0.05 compared with the blank group. **p<0.01 compared with the blank group. #p<0.05 compared with the model group. ##p<0.01 compared with the model group.

Effects of GLPs on cytokines levels in tumor-bearing mice

ELISA kits were employed to determine the effects of GLPs on the expressions of serums IL-2, IFN-γ and TNF-α in S180-bearing mice. As seen in Table 3, the expressions of serum IL-2, TNF-α, and IFN-γ in model group were notably reduced compared to the blank group on account of the transplanted tumor (p<0.01). The levels of IL-2, TNF-α, and IFN-γ in serums of 5-FU group were lower than those of the model group (p<0.01), indicating that 5-FU treatment could restrain the expression of cytokines. After the 250-mg/kg GLPs intragastric administration, the expressions of serum IL-2 and TNF-α were significantly increased (p<0.01) in comparison with the model group. In particular, the level of serum IFN-γ in the high-dose GLPs group (250 mg/kg) was higher than the normal mice (p<0.01). All these data suggested that tumor growth could suppress the production and secretion of serum IL-2, TNF-α and IFN-γ, while GLPs could enhance the immune function by stimulating the expression of serum cytokines in S180-bearing mice, dose-dependently.

Table 3: Effects of polysaccharides on cytokine production in mice.

Groups	Dose (mg/kg)	IL-2 (pg/mL)	IFN-g (pg/mL)	TNF-a (pg/mL)
Blank	-	4.03±0.90	99.54±6.43	49.46±2.61
Model	-	1.98±0.34**	83.52±8.79**	40.18±2.84**
5-FU	20	0.73±0.20**##	58.40±5.34**##	25.68±4.64**##
GLPs	125	2.40±0.57**	96.37±10.31##	43.79±1.69**
	250	3.86±0.40##	116.55±7.42**##	52.13±3.48##

Note: *p < 0.05 compared with the blank group. **p<0.01 compared with the blank group. #p<0.05 compared with the model group. ##p<0.01 compared with the model group.

Discussion

Cancer threatens human's health and life continuously as a result of the increase of cancer-causing behaviors in economically developing countries [23]. Drugs with fewer side effects always kill off chemotherapy-sensitive cancer cells and leave chemo resistant cells behind, ultimately leading to cancer recurrence [24]. It is urgent to research and develop new antitumor substance which can enhance our antitumor immune system with relatively low toxicity [25].

Marine algae, as the major sources of sulfated polysaccharides, have shown various biological activities, especially antitumor and immunoregulatory activities [26]. The biological activities of polysaccharides were related to their monosaccharide composition, molecular weight, uronic acid content and functional groups and so on [27]. In general, polysaccharides with higher molecular mass and good solubility will possess higher bioactivities, and the presence of carboxyl can enhance the antitumor activities of polysaccharides [28]. Moreover, it was reported that α-configuration glycosidic bond in polysaccharides contributed to the antitumor activities [29]. In this study, GLPs possess α-type glycosidic linkages and pyranose form with total sugar (76.49 %), uronic acid (27.88 %), and sulfate (11.23 %), and exhibit strong antitumor effects on S180 tumor-bearing mice.

Thymus, as a specialized primary lymphoid organ of the immune system, is located in the thoracic cavity and essential for the differentiation and maturation of T cells [30]. Spleen exists as the largest filter and peripheral organ and plays an important role in immune reaction of human body [31]. T cells exert cellular immunity [32], whereas B cells are in charge of humoral immunity [33]. NK cells are a subset of cytotoxic lymphocytes [34], which could directly attack tumor cells *in vivo*. In the *in vivo* antitumor experiments, GLPs showed a strong enhancement on immune system against tumors cells. Results suggested that GLPs could effectively protect thymus and spleen from solid tumor cells, and significantly enhance the proliferation ability of splenic lymphocyte and killing activity of NK cells, and ultimately induce solid tumors cells apoptosis.

IL-2 is an important member of cytokine signaling molecule in the immune system that can participate in differentiation, clonal expansion and activation of cytotoxic and helper T lymphocytes, and stimulation of antibodies production [35]. TNF-α is a cell signaling protein (cytokine) derived from activated monocytes, which can increase tumor cell apoptosis, as well as inhibit tumorigenesis and viral replication [36]. IFN-γ plays a central role in the recognition and elimination of transformed cells [37]. This study showed that GLPs could obviously improve the levels of these cytokines in mice serums, which would be beneficial for antitumor immune response.

Conclusion

The sulfated polysaccharide (GLPs) mainly composed of galactose was obtained from *Gracilaria Lemaneiformis*, which possessed α-type glycosidic linkages and pyranose form with total sugar (76.49%), uronic acid (27.88%), and sulfate (11.23%). The *in vivo* antitumor experiments showed that GLPs could effectively inhibit the malignant proliferation of solid S180 tumor in mice via enhancing the proliferation of splenocytes, killing activity of NK cells, and improving the levels of IL-2, IFN-γ, TNF-α in serum of S180 tumor-bearing mice. Our findings would contribute to developing an effective anticancer agent and provide a new evidence for the immunotherapy of tumors.

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Conflict of Interest

No conflict of interest associated with this work.

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