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Free radical scavenging and immunomodulatory activities of *Ganoderma lucidum* polysaccharides derivatives

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ABSTRACT

Polysaccharides extracted from the fruit body of *Ganoderma lucidum* were sulfated and carboxymethylated as reported. Free radical scavenging and immunomodulatory effects of sulfated and carboxymethylated polysaccharides were studied. Generally, sulfated polysaccharides showed better bioactivities than that of carboxymethylated polysaccharides. The two derivatives were injected intraperitoneally with or without 5-fluorouracil over a period of 7 days in BALB/c female mice. The polysaccharide derivatives increased mouse thymus and spleen index, an indication of improved immunity in mice. At the same time, they improved superoxide dismutase and glutathione peroxidase contents in the mice body.

1. Introduction

Oxidation is essential to many organisms for the production of energy to fuel biological processes. However, reactive oxygen species are often over-produced under pathological conditions, resulting in oxidative stress (Guo et al., 2010; Wang, Chang, & Chen, 2010). There are many kinds of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), hydroxyl radical (•OH) and singlet oxygen (Hu, Liu, Chen, Wu, & Wang, 2010; Luo et al., 2010; Sun, Li, & Liu, 2010; Xie et al., 2010). Although ROS at physiological concentration may be required for normal cell function, excessive amount of ROS can damage cellular components such as lipids, protein and DNA. Many diseases such as cancer, cardiovascular diseases, rheumatoid arthritis, and atherosclerosis are believed to be related to production of excessive amounts of ROS in the body (Chen, Shen, & Chen, 2009; Sun, Zhang, Zhang, & Niu, 2009; Wei, Wang, Yao, Gao, & Yu, 2010). The free radical theory of aging suggests that the damage produced by the interactions of such free radicals with cellular macromolecules results in cellular senescence and aging. Although almost all organisms possess antioxidant and repair systems to protect them against oxidative damage, these systems are insufficient to prevent the damage entirely (Tommonaro et al., 2007; Tseng, Yang, & Mau, 2008).

Polysaccharides are distributed in many kinds of animals, plant, microorganisms and fungus, exhibiting varied bioactivities. *Ganoderma lucidum* is one '*Traditional Chinese Medicine*' and has long been used in China and many Asian countries. Its fruit body has been long used to promote health and longevity coded as "elixir of youth" by ancient emperors. Nowadays, polysaccharides extracted from *G. lucidum* are still used to treat conditions like gastric ulcer, chronic hepatitis, nephritis, hypertension, hyperlipemia, arthritis, insomnia, bronchitis, asthma, arteriosclerosis, leukopenia, diabetes and anorexia (Jong & Birmingham, 1992; Mizuno, 1992; Ricardo, David, & Guilherme, 2004; Tommonaro et al., 2007; Yang, Zhao, Yang, & Ruan, 2008; Zhu & Wu, 2009). In the previous works, we isolated a linear $(1 \rightarrow 3)$-β-D-glucan from *G. lucidum* and synthesized the sulfated and carboxymethylated derivatives, and proved that these biomacromolecules have antitumor activities (Wang & Zhang, 2009). In this work, the antioxidant activities of the two derivatives both in vitro and in vivo were analyzed.

2. Materials and methods

2.1. Reagents and materials

The fruit body of *G. lucidum* was obtained from *Wuping*, Fujian province, China. The sulfated and carboxymethylated derivatives were prepared as reported before (Wang & Zhang, 2009). Sulfated and carboxymethylated derivatives were coded as S-GL and CM-GL, respectively. 1,1-Diphenyl-2-picryldydrazyl (DPPH), D-glucuronic acid sodium salt monohydrate and glucosamine hydrochloride were purchased from Sigma Chemical Co. (St. Louis, USA). Assay kits for superoxide dismutase (SOD) and glutathione (GSH). The BALB/c (7 weeks old) female mice were purchased from animal

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experimental center of Wuhan University. All other chemicals used were ultra pure or analytical grade.

2.2. Antioxidant ability of S-GL and CM-GL in vitro

2.2.1. Ferric-reducing antioxidant power (FRAP) assay

The total antioxidant potential of sample was determined by FRAP assay as described by Benzie and Strain (1996). FRAP assay measures the change in absorbance at 593 nm due to the formation of a blue-colored Fe (II)–tripyridyltriazine compound from the colorless oxidized Fe (III) form by the action of electron-donating antioxidants. The working FRAP reagent was prepared by mixing 10 vol of 300 mmol/L acetate buffer, pH 3.6, with 1 vol of 10 mmol/L TPTZ (2,4,6-tripyridyl-s-triazine) in 40 mmol/L HCl and with 1 vol of 20 mmol/L $FeCl_3$. Freshly prepared FRAP reagent (1.5 mL) was warmed to 37 °C, and a reagent blank reading was taken at 593 nm. Subsequently, 50 μL of sample and 150 μL of deionized water were added to the FRAP reagent. The final dilution of the sample in the reaction mixture was 1:34. The sample was run in triplicate. After the addition of the sample to the FRAP reagent, a second reading at 593 nm was performed after 8 min. The initial blank reading with the FRAP reagent alone was subtracted from the final reading of the FRAP reagent with the sample to determine the FRAP value of the sample. A standard curve was prepared using different concentrations (100–1000 μmol/L) of $FeSO_4{\cdot}7H_2O$. The final result was expressed as the concentration of antioxidants having a ferric reducing ability equivalent to that of 1 μmol/L $FeSO_4{\cdot}7H_2O$.

2.2.2. Effect of scavenging 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals

The free-radical scavenging capacity of the S-GL and CM-GL was analyzed by using the DPPH test according to the method of Blois (1985) with some modifications. Vitamin C (Vc) was used as reference material. Briefly, 0.2 mL of methanol (MeOH) and 0.3 mL of various concentrations (50–800 μg/mL) of sample 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 mL. The solution was kept at room temperature for 30 min, and the absorbance at 517 nm (A_{517}) was measured. The DPPH scavenging effect (Φ%) was calculated as the following formula,

$$\Phi\% = 1 - \frac{A_{\text{sample}}}{A_{\text{control}}} \times 100 \quad (1)$$

where A_{control} is the absorption of DPPH without sample or Vc and A_{sample} is the absorption with sample or Vc.

2.2.3. Hydroxyl radical-scavenging activity

•OH-scavenging activity of the samples was determined according to the deoxyribose assay (Halliwell, Gutteridge, & Aruoma, 1987). One milliliter phosphate buffer (20 mM pH 7.4, containing 0.1 mM ferric chloride, 0.1 mM EDTA, 2.8 mM deoxyribose), 0.1 mL Vc (1 mM) and 0.5 mL H_2O_2 (20 mM) were added to 1 mL of appropriately diluted sample. Following incubation at 37 °C for 90 min, 1.0 mL of 1% (w/v) TCA and 0.3 mL of 2.8% (w/v) TBA were added, and then the reaction mixture was heated in a boiling-water bath for 15 min. The absorbance at 532 nm was measured against a blank. A control contained all the reaction reagents except the samples was prepared and measured, Vc was used for comparison, and the •OH-scavenging ratio was calculated as formula (1).

2.2.4. Superoxide anion-scavenging activity

The assay was based on the capacity of the sample to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) in the NADH–NBT–PMS system. The reaction mixture consisted 1.0 mL of NBT (78 lM in 20 mM potassium phosphate buffer pH 7.4), 1.0 mL of NADH (468 lM in 20 mM potassium phosphate buffer pH 7.4) and 1.0 mL of an appropriately diluted sample solution. The reaction was initiated by addition of 0.4 mL of PMS (60 μM in 20 mM potassium phosphate buffer pH 7.4) to the mixture. The tube was incubated at 25 °C for 5 min and the absorbance was measured at 560 nm against a blank. Decreased absorbance of the reaction mixture indicated increasing superoxide anion-scavenging activity. BHT was used as a positive control in the study, and a control contained all the reaction reagents except the samples or positive control was prepared. The percentage inhibition of superoxide anion generation was calculated using a formula similar to that for DPPH radical-scavenging activity.

2.2.5. Self-oxidation of 1,2,3-phentriol assay

The inhibitory capacity of the samples against self-oxidation of 1,2,3-phentriol of all different contents was investigated according to the method of Marklund (Marklund & Marklund, 1974) with a minor modification. Briefly, samples were dissolved in distilled water at 0 (control), 40, 60, or 80 mg/mL. The sample solution (0.1 mL) was mixed with 2.8 mL of 0.05 M Tris–HCl buffer (pH 8.0) containing 1 mM EDTA and 1,2,3-phentriol (0.2 mL, 6 mM), was shaken rapidly at room temperature. The absorbance of the mixture was measured at 325 nm per 30 s for 4 min against a blank, and a slope was calculated as absorbance per min. The ability of different inhibitory capacity of the samples against self-oxidation of 1,2,3-phentriol (φ%) of all fractions was calculated using the equation as follow:

$$\varphi\% = 1 - \frac{S_{\text{sample}}}{S_{\text{control}}} \times 100$$

where S_{sample} is the slope of sample and S_{control} is the slope of the control.

2.3. Antioxidant ability of S-GL and CM-GL in vivo

2.3.1. Spleen and thymus indices

A total 80 BALB/c (7 weeks old) mice, weighing 20.0 ± 2.0 g, were randomly divided into 10 groups (n = 8) and allowed free access to a standard laboratory diet and water. Group-1 was the control group which were injected with the same volume of 0.9% aqueous sodium chloride solution intraperitoneally daily as the other 9 groups. Group-2 was the reference group which were injected with 5-fluorouracil (5-Fu) at a dose of 50 mg/kg. The rest 8 groups were united-treated models with polysaccharide derivatives (S-GL or CM-GL) and 5-Fu of different doses comparatively. The injected dose of Group-3-1 was 50 mg/kg S-GL + 50 mg/kg 5-Fu, and Group-3-2 was 50 mg/kg CM-GL + 50 mg/kg 5-Fu. Group-4-1 was 100 mg/kg S-GL + 50 mg/kg 5-Fu, and Group-4-2 was 100 mg/kg CM-GL + 100 mg/kg 5-Fu. Group-5-1 was 150 mg/kg S-GL + 50 mg/kg 5-Fu, and Group-5-2 was 150 mg/kg CM-GL + 50 mg/kg 5-Fu. Group-6-1 was 200 mg/kg S-GL + 50 mg/kg 5-Fu, and Group-6-2 was 200 mg/kg CM-GL + 50 mg/kg 5-Fu. After 7 days administration, mice were killed by decapitation; spleen and thymus were removed and weighed immediately. The indices of spleen (I_s) and thymus (I_t) were calculated as follows,

$$I = \frac{W_a\,(\text{mg})}{W_b\,(\text{g})}$$

where I is the index of spleen or thymus, W_a is the average weight of spleen or thymus of each group and W_b is the average weight of the mice after administration.

2.3.2. Biochemical assay

A small portion of murine thymus or spleen was removed and homogenized with 10% trichloroacetic acid (TCA), the supernatant after centrifugation (10,000 × g, 30 min) was used for the GSH assay

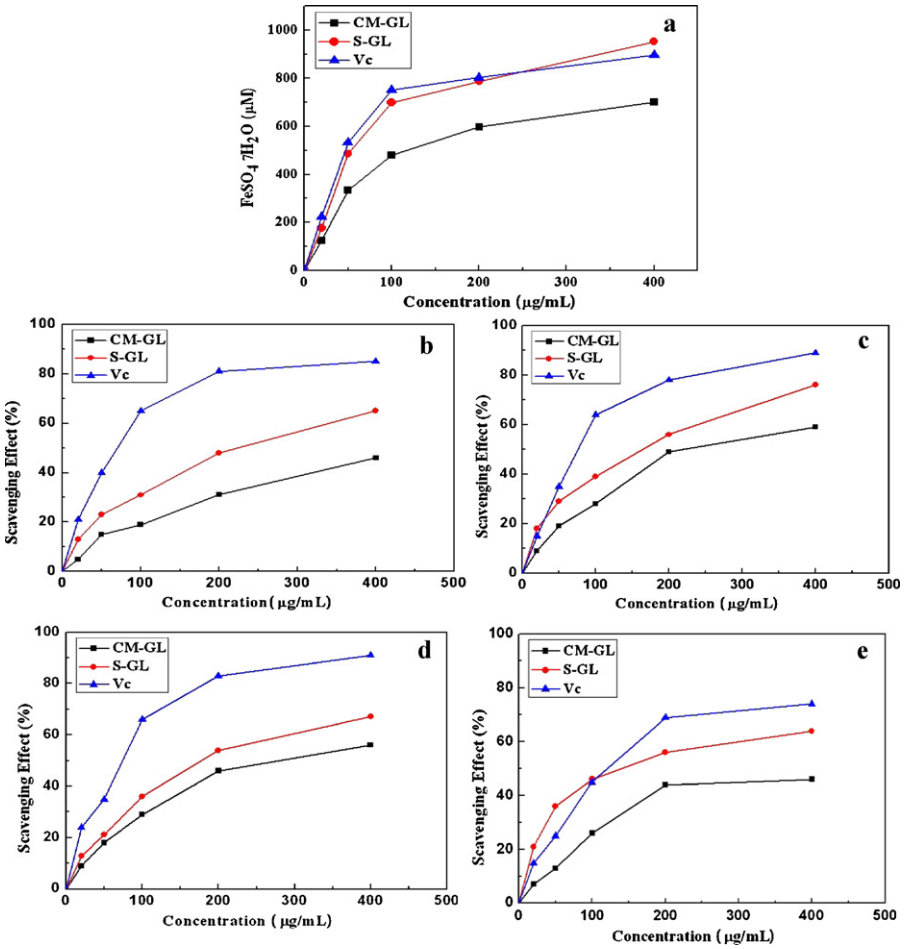

Fig. 1. FRAP assay (a); effects of scavenging DPPH radicals (b), hydroxyl radical (c), superoxide radical (d) and scavenging abilities for the self-oxidation of 1,2,3-phentriol (e) of S-GL and CM-GL. Data are presented as mean values ($n = 3$).

with DTNB (Chen, Hu, & Zheng, 2007). The other parts of both thymus and spleen were kept on ice and homogenates of them were kept on ice and homogenized in 0.2 g/mL isotonic physiological saline for biochemical assays. The homogenates were centrifuged to collect supernatants that were used to measure the activity of SOD. The activity of SOD was analyzed by the auto-oxidation of 1,2,3-phentriol (Marklund & Marklund, 1974).

2.4. Statistical analysis

Results were expressed as the means ± SD of data obtained from triplicate experiments. Statistical analysis was performed by a paired *t*-test. p values < 0.05 were considered statistically significant.

3. Results and discussion

3.1. Antioxidant activity analysis

3.1.1. Ferric-reducing antioxidant power

The FRAP assay treats the antioxidants contained in the samples as reductants in a redox-linked colorimetric reaction, and the value reflects the reducing power of the antioxidants (Pulido, Bravo, & Saura-Calixto, 2000; Schlesier, Harwat, Böhm, & Bitsch, 2002). The procedure is relatively simple and easy to standardize. This assay is also commonly used for the routine analysis of single antioxidants and total antioxidant activity of complex to the TPTZ–Fe (II) complex. The antioxidant capacities of the purified polysaccharide are shown in Fig. 1a and compared with Vc as a control standard. Lower FRAP values could be a response to the lower production of reactive oxygen species (ROSs) or the result of an increase in ROSs that react with the antioxidants. Based on the FRAP value, S-GL and CM-GL show good reducing power in a dose-dependent manner. The results indicate that the S-GL and CM-GL have a strong potential antioxidant activity, which could bear comparison with that of Vc.

3.1.2. Scavenging effect on DPPH radicals

The model of scavenging the stable DPPH radical is a widely used method to evaluate the free radical scavenging ability of natural compounds (Amarowicz, Pegg, Rahimi-Moghaddam, Barl, & Weil, 2004; Leong & Shui, 2002). DPPH is a stable free radical that shows maximum absorption at 517 nm in methanol. When DPPH encounters a proton-donating substance, for example, an antioxidant, the radical would be scavenged and the absorbance at 517 nm is reduced (Espin, Rivas, & Wichers, 2000). It is visually noticeable as a color change from purple to yellow. Based on this principle, the antioxidative activity of a substance can be expressed as its ability in scavenging the DPPH free radical. In this experiment, the DPPH free-radical scavenging effect of each sample was measured, and the results are shown in Fig. 1b. Both S-GL and CM-GL showed good scavenging effect against DPPH radical in a dose-dependent manner in all concentration studied, the scavenging ratio at high concentration of S-GL and CM-GL are 65% and 46%, respectively.

3.1.3. Hydrogen peroxide scavenging activity

•OH can easily cross cell membranes, and can readily react with most biomolecules including carbohydrates, proteins, lipids, and DNA in cells, and cause tissue damage or cell death. Thus, removing •OH is important for the protection of living systems. Fig. 1c shows the percentage •OH scavenging effects of S-GL, CM-GL and Vc at the dose of 20, 50, 100, 200 and 400 μg/mL. At the test concentrations, S-GL and CM-GL exhibited scavenging activity on hydroxyl radicals in a dose-dependent manner. At 400 μg/mL, the inhibiting abilities of S-GL, CM-GL, and Vc were 76%, 56%, and 92%, respectively.

3.1.4. Scavenging activity to superoxide radicals

Superoxide anion radicals are known to indirectly initiate lipid peroxidation as a result of H_2O_2 formation, creating precursors of hydroxyl radicals (Meyer & Isaksen, 1995). So its scavenging is extremely important to evaluate the activity of antioxidant. In the PMS/NADH–NBT system, superoxide anion derived from dissolved oxygen by the PMS/NADH coupling reaction reduces NBT. The decrease of absorbance at 560 nm with antioxidants thus indicates the consumption of superoxide anion in the reaction mixture (Gülcn, Sat, Beydemir, Elmastas, & Küfrevioğlu, 2004). Fig. 1d shows the inhibitory effects of S-GL and CM-GL in a dose-dependent manner by comparison with the same doses of Vc. The scavenging ratio at high concentration of S-GL, CM-GL and Vc are 56%, 67% and 90%, respectively. These results indicate that the two derivatives have a good superoxide radical-scavenging activity.

3.1.5. Scavenging activity of self-oxidation of 1,2,3-phentriol

Inhibition of pyrogallol auto-oxidation was performed according to the method of Marklund's (Marklund & Marklund, 1974) with minor modifications. Superoxide anion radical can be generated by pyrogallol auto oxidation and it can produce a colored compound. Resulting from a color change from purple to yellow, the absorbance at 320 nm increased when the superoxide anion was scavenged by an antioxidant, which can represent the content of superoxide radicals and indicate the antioxidant activity of the sample. As we can see in Fig. 1e, the scavenging powers of samples and standards both correlated well with increasing concentrations, the scavenging ratio of S-GL and CM-GL in high concentration (400 μg/mL) is 64% and 46%, respectively. S-GL showed stronger antioxidant ability which is comparable to Vc (74%).

3.2. Effect of S-GL and CM-GL on mouse spleen and thymus indices

Fig. 2 shows the thymus and spleen indices of the animals treated with 5-Fu at dose of 50 mg/kg bodyweight decreased significantly when compared with the normal control. The thymus and spleen indices of the animals treated with both S-GL and CM-GL of 50, 100, 150 and 200 mg/kg united 5-Fu (50 mg/kg bodyweight) increased in a dose-dependent manner as compared with the animals treated with 5-Fu alone. This result indicated that the immune function was suppressed when the animals were treated with 5-Fu. The thymus index of the 5-Fu treated group is 0.9, which is much lower than that of normal controlled group (5.1). The spleen index also decreased from 6.3 to 3.9. Treating mice with S-GL and CM-GL at different doses united 5-Fu produced a significant and dose-dependent increase in both thymus and spleen indices. The thymus index of the 6-1 (S-GL + 5Fu (200 + 50 mg/kg)) and 6-2 (CM-GL + 5Fu (200 + 50 mg/kg)) group rose to 9.8 and 8.6, respectively. The spleen index of the 6-1 (S-GL + 5Fu (200 + 50 mg/kg)) and 6-2 (CM-GL + 5Fu (200 + 50 mg/kg)) group rose to 13.1 and 11.8, respectively. The findings suggested that *G. lucidum* polysaccharides derivatives overcame the immune-suppressed action of 5-Fu.

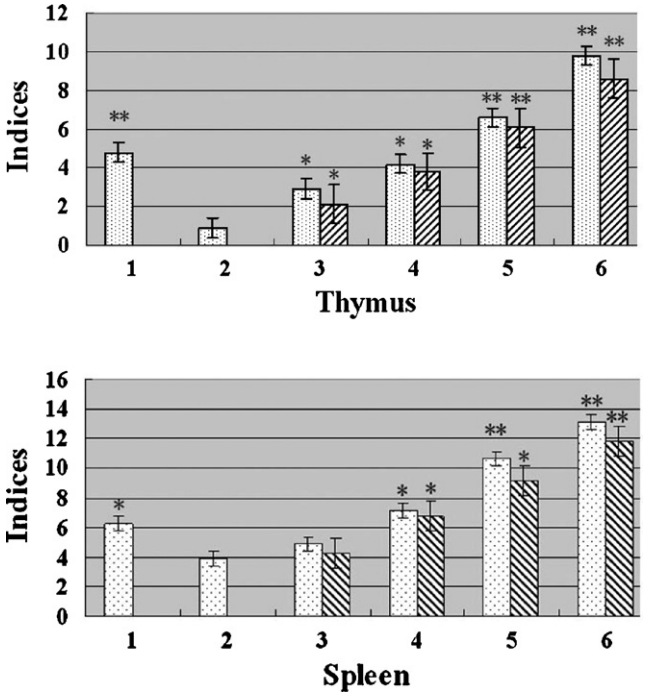

Fig. 2. Effect of S-GL and CM-GL on thymus and spleen indices in mice. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to 5-Fu treatment group.

3.3. Effects of S-GL and CM-GL on SOD in spleen and thymus

SOD is an intracellular compound that protects against oxidative processes initiated by the superoxide anion. Fig. 3 shows the contents of SOD in thymus of mice treated with 0.9% NaCl, 5-Fu alone and polysaccharides derivatives united 5-Fu in different doses. In the 5-Fu group, content of SOD sharply decreased from 5.8 U/mg to 1.9 U/mg. With the united treatment with S-GL and CM-GL, the SOD contents values rose in a dose-dependent manner. The SOD contents values of the 6-1 (S-GL + 5Fu (200 + 50 mg/kg)) and 6-2 (CM-GL + 5Fu (200 + 50 mg/kg)) group rose to 13.9 and 11.7 U/mg, respectively. Fig. 4 shows the contents of SOD in spleen of mice treated with 0.9% NaCl, 5-Fu alone and polysaccharides derivatives united 5-Fu in different doses. In the 5-Fu group, content of SOD sharply decreased from 7.1 U/mg to 3.9 U/mg. With the united treatment with S-GL and CM-GL, the SOD contents values rose in a dose-dependent manner. The SOD contents values of the 6-1 (S-GL + 5Fu (200 + 50 mg/kg)) and 6-2 (CM-GL + 5Fu (200 + 50 mg/kg)) group significantly rose to 19.8 and 17.8 U/mg, respectively.

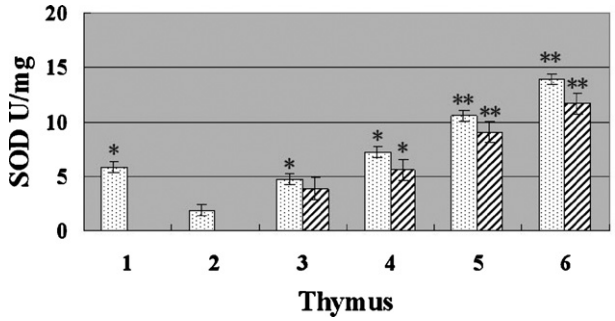

Fig. 3. S-GL and CM-GL raised activity of SOD in thymus of 5-Fu treated mice. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to 5-Fu treatment group.

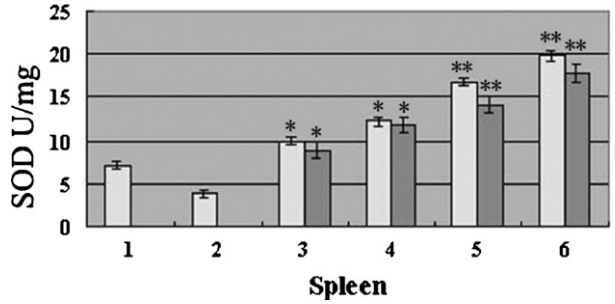

Fig. 4. S-GL and CM-GL raised activity of SOD in spleen of 5-Fu treated mice. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to 5-Fu treatment group.

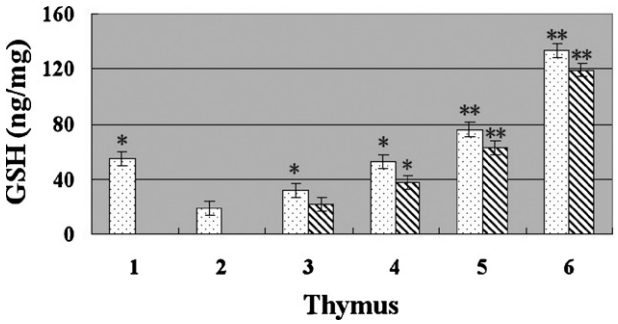

Fig. 5. S-GL and CM-GL increased the levels of GSH in thymus of mice treated with 5-Fu. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to 5-Fu treatment group.

3.4. Effects of S-GL and CM-GL on GSH in spleen and thymus

GSH is a tripeptide composed of glutamic acid, cysteine and glycine, participating in the circulation of tricarboxylic acid and glucose metabolism in the body. GSH can activate a variety of enzymes, thereby promoting carbohydrates, fat and protein metabolism and can affect cell metabolism. GSH is the most important biomolecule protecting against chemically caused cytotoxicity, can participate in the elimination of reactive intermediates by conjugation and hydroperoxide reduction, or of free radicals by direct quenching. It can protect the thiol-containing protein in the cell membrane and thiol-containing enzymes from destroying, thereby protecting organs. Fig. 5 shows the contents of GSH in thymus of mice treated with 0.9% NaCl, 5-Fu alone and polysaccharides derivatives united 5-Fu in different doses. In the 5-Fu group, content of GSH sharply decreased from 55 ng/mg to 19 ng/mg. With the united treatment with S-GL and CM-GL, the GSH contents values rose in a dose-dependent manner. The SOD contents values of the 6-1 (S-GL + 5Fu (200 + 50 mg/kg)) and 6-2 (CM-GL + 5Fu (200 + 50 mg/kg)) group significantly rose to 133 and 119 ng/mg, respectively. Fig. 6 shows the contents of GSH in spleen of mice treated with 0.9% NaCl, 5-Fu alone and polysaccharides derivatives united 5-Fu in different doses. In the 5-Fu group, content of GSH decreased from 78 to 49 ng/mg. With the united treatment with S-GL and CM-GL, the GSH contents values rose in a dose-dependent manner. The GSH contents values of the 6-1 (S-GL + 5Fu (200 + 50 mg/kg)) and 6-2 (CM-GL + 5Fu (200 + 50 mg/kg)) group significantly rose to 164 and 156 ng/mg, respectively.

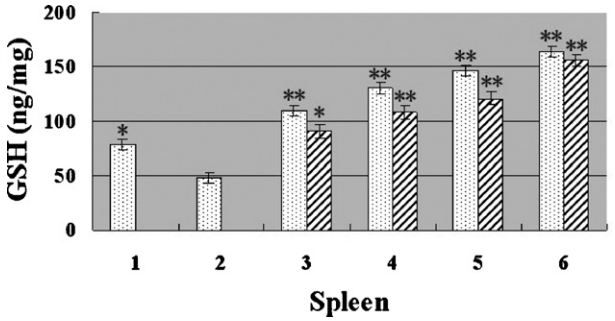

Fig. 6. S-GL and CM-GL increased the levels of GSH in spleen of mice treated with 5-Fu. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to 5-Fu treatment group.

4. Conclusion

S-GL and CM-GL, both have good antioxidant activity in vitro and in vivo. The antioxidant activity results of S-GL are superior to that of CM-GL. Meanwhile, S-GL and CM-GL can eliminate the immune inhibition effect of traditional anti-cancer drug, increase the mouse thymus and spleen indices and improve the immunity of mice. At the same time, they can effectively improve the content of SOD and GSH content in mice body and inhibit free radical to damage the spleen and thymus organ. *G. lucidum* polysaccharide derivatives are expected to develop new anti-oxidant drugs.

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