



Characterization and bioactivities of a novel polysaccharide obtained from *Gracilariopsis lemaneiformis*

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ABSTRACT

Gracilariopsis lemaneiformis is a type of red alga that contains seaweed polysaccharide agar. In this study, a novel non-agar seaweed polysaccharide fraction named GCP (short of crude polysaccharide obtained from *Gracilariopsis lemaneiformis*) was isolated from *Gracilariopsis lemaneiformis*. Structural analysis showed that GCP shows triple helical chain conformation when dissolved in water and has many branches and long side chains. Also, 1→3 linkage is the major linkage and the sugar structures are galactopyranose configurations linked by β-type glycosidic linkages. Two macromolecular substance fractions (GCP-1 and GCP-2) were purified by DEAE Sepharose Fast Flow column chromatography. Moreover, a splenocyte damage assay and splenocyte proliferation assay were used to analyse the bioactivities of GCP, GCP-1 and GCP-2. It was demonstrated that polysaccharides could protect splenocyte damaged by H₂O₂; GCP-2 shows a greatest protection rate, that is, 92.8%, which significantly enhanced the splenocyte proliferation, and GCP showed the highest proliferation rate, 9.30%. The results suggested that this type of novel non-agar polysaccharide displayed remarkable antioxidant and immunomodulatory activities and early alkali treatment could decrease the activities. It may represent a potential material for health food and clinical medicines.

Key words: bioactivities, *Gracilariopsis lemaneiformis*, polysaccharide, structural characterization.

INTRODUCTION

Gracilariopsis lemaneiformis, belonging to the Rhodophyta family, is an economically important red alga of China that grows widely along the coast of Shandong province (Tseng 2001). During the last several decades, this alga has been extensively cultivated and used as the raw material of commercial agar. Numerous studies have focused on the extraction of agar (Freile-Pelegrín and Murano 2005, Pereira-Pacheco et al. 2007). Fan et al. (2012a, b) reported that polysaccharides from *Gracilariopsis lemaneiformis* have in vitro and in vivo immunomodulatory activities and are mainly composed of galactose. The polysaccharides significantly inhibited tumour growth, promoted splenocytes proliferation and macrophage phagocytosis,

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and increased the level of IL-2 and CD⁸⁺ T-cells, CD³⁺ and CD⁴⁺ cells in the blood of tumour-bearing mice. Yang et al. (2014) extracted crude polysaccharides from *Gracilariopsis lemaneiformis*, purified by DEAE-52 cellulose chromatography and Sephadex G-100 size-exclusion chromatography and analysed the partial characterizations. However, there is also a type of non-agar polysaccharide in *G. lemaneiformis*, which is always treated as waste during agar processing. This type of polysaccharide may show great activities, which have not been analysed to date.

It is well-known that the activities of polysaccharides are related to their chemical structure, such as monosaccharide composition, average molecular weight, degree and pattern of substituting groups, solution conformation, and chain conformation (Chen et al. 2009, Tao et al. 2007, Wu et al. 2012). It is reported that triple helical chain conformation exhibited a relatively high inhibition ratio against the proliferation of tumour cells, whereas the bioactivity of single flexible chains almost disappeared (Fan et al. 2012b). Alkali treatment of *Gracilaria* is well known as a technique for gelling in the extraction process. Because the amount of sulphate can be lowered using an appropriate alkali treatment before agar extraction. In spite of that, studies indicated that the sulfate-polysaccharides showed significant activities on anticoagulation, antioxidation, antitumor, antiviral, immunomodulation and so on.

In order to make full use of the waste generated during the agar processing and identify natural medicine resources and natural health food ingredients, a type of non-agar polysaccharide has been isolated from *Gracilariopsis lemaneiformis* in this study. The polysaccharide purified by DEAE-FF cellulose chromatography and structural characterization and bioactivities were subsequently analysed. In the present study, the authors compared the polysaccharide with the one which was treated by alkali early (Shi et al. 2016) to analyse the effect of alkali on structure and activity of polysaccharide and provide a theoretical basis for the comprehensive utilization of *Gracilariopsis lemaneiformis*.

MATERIALS AND METHODS

MATERIALS AND REAGENTS

Gracilariopsis lemaneiformis were collected from Qinhuangdao, China. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), concanavalin A (ConA) were provided by Sigma Chemical Co. Fetal calf serum (FCS) was from Hangzhou Sijiqing Co. RPMI-1640 medium was purchased from Solarbio Co. 1-Pheny-3-Methyl-5-Pyrazolone (PMP) was purchased from Aladdin Chemistry Co. Ltd. Standard monosaccharides, a series of Dextran standards (Narrow Molecular Weight Distribution), were from the National Institute of Metrology.

ISOLATION AND PURIFICATION OF GCP

Extraction method is same to the early study but without alkali treatment (Pretreated with 6% NaOH at 80°C for 2 h before extracted with water). Dried parts of *G. lemaneiformis* were ground to a powder. Polysaccharide was extracted with water at 90°C for 3 h, and the ratio of powder and water was 1:100. To obtain the crude polysaccharide, supernatant was collected and deposited by 80% (v/v) ethanol and centrifuged (3000 × g, 10 min) after left overnight at 4°C. The precipitate was re-dissolved in distilled water, stored at -20°C for 12 h, and then turned to 4°C condition until it melted. Non-agar polysaccharide was the supernatant after centrifuged at 10000 × g for 30 min.

The protein was removed using n-butanol and chloroform (1:5, v/v) according to the method of Seavage. Next, the mixture was concentrated, dialysed (Mw 3500) for three days and lyophilized to yield GCP. The

total sugar content was measured by the phenol–sulfuric acid method using glucose as the standard and the content of sulphate in the polysaccharide was detected by the barium chloride-gelatin method using potassium sulphate as the standard.

The extracted polysaccharide was purified with DEAE Sepharose FF cellulose ion exchange chromatography. GCP was re-dissolved in 4 mL distilled water, filtered through 0.45 μm filters and applied to a DEAE-FF cellulose column (1.6 cm \times 30 cm) equilibrated with distilled water (Yang et al. 2014). Polysaccharides were fractionated and eluted with different concentrations of stepwise NaCl solution (0 and 1.0 M NaCl) at a flow rate of 2.0 mL/min. Elutes were concentrated to obtain the main fractions. The fractions obtained were combined according to the total carbohydrate content method. Relevant fractions were collected, concentrated, dialysed and lyophilized for further study.

MOLECULAR WEIGHT DISTRIBUTION ASSAY

The molecular weight distribution of the polysaccharides after purified were determined by high performance gel-permeation chromatography (HPGPC) using HPLC equipped with a SB-804 column (7.8 mm \times 300 mm, column temperature 40°C) and a differential refractive index detector (RID). The samples were eluted with the mobile phase (0.2 M Na_2SO_4) and run at a flow rate of 0.5 ml/min. The standard curve was established using Dextran as the standards (Mw= 4320Da, 1.26×10^4 Da, 6.06×10^4 Da, 1.1×10^5 Da, 2.89×10^5 Da, 6.7×10^5 Da).

CHARACTERIZATION OF GCP

ANALYSIS OF MONOSACCHARIDE COMPOSITION

High performance liquid chromatography with pre-column derivation was performed for the monosaccharide identification. The polysaccharide was hydrolysed with 4 M trifluoroacetic acid (TFA) at 120°C for 2 h (Seedevi et al. 2015, Zhang et al. 2013), derived by PMP and analysed by HPLC using a C18 column (Agilent) and DAD detector.

Congo-red test

Sample solutions (2 mg/mL) at various concentrations of NaOH (0-0.5 M) containing 0.08 mM Congo red were prepared. The shifts in the visible maximum absorption λ_{max} of the polysaccharide's complexes with Congo red were recorded by a UV–Vis spectrophotometer (Wang et al. 2014a).

I₂-KI test

Sample solution (1 mg/mL) of 2 mL were mixed with 1.2 mL I₂-KI (containing 0.02% I₂ and 0.2% KI solution), and the absorbance was measured by UV–Vis spectrometer in the range of 300 to 700 nm (Chen and Xiang 2013).

β -elimination reaction

Sample solutions (1 mg/mL) were dissolved in NaOH (0.2 M) at 45°C for 3 h. The absorbance was measured by a UV–Vis spectrometer (K5500, Beijing Kai-Ao Science and Technology Development Co., Ltd.) in the range of 200 to 800 nm (Yang et al. 2012, Liu et al. 2014). Sample solution without the addition of NaOH served as the control.

Periodate oxidation and Smith degradation

The polysaccharide fraction (20 mg) was added separately to 15 mM KIO₄ solution and stored in the dark. After completely reacting, the excess periodate was decomposed by the addition of ethylene glycol. The consumption of KIO₄ and the production of HCO₂H were evaluated by the spectrophotometric method and sodium hydroxide titration (Song and Du 2012), respectively. The periodate-oxidized material was reduced with NaBH₄, hydrolysed with 4 M TFA, acetylated and finally analysed by GC (Agilent 7890B).

Fourier-transform infrared spectra (FTIR) analysis

FTIR spectrum of GCP-1 and GCP-2 were recorded with an FTIR spectrometer (Spectrum 65, PerkinElmer, USA) in the wave-number range of 4000 to 400 cm⁻¹ using the potassium bromide (KBr) disc method.

Scanning electron microscopy (SEM)

The samples were mounted on a copper sample-holder using a double sided carbon tape and coated with gold to make the polysaccharide granules conductive. SEM studies were carried out using a scanning electron microscope (SU-8010, Hitachi High-Technologies Co., Japan) at an acceleration voltage of 5 kV.

NMR spectra

The ¹H NMR spectrum was recorded on an NMR spectrometer (Ultrashield 400, BRUKER). Samples (20 mg) were dissolved in DMSO-d₆.

BIOACTIVITIES OF GCP, GCP-1 AND GCP-2

Superoxide radical scavenging activity

Superoxide radicals were generated in the system of the pyrogallol's autoxidation in an alkalescent condition (Vijayabaskar et al. 2012). All experiments were performed at 25°C, and the reaction was performed in 2.9 mL Tris-HCl buffer (50 mM, pH8.2) that contained 0.1 mL samples with different concentrations (50–800 µg/mL); 0.2 mL pyrogallol solution (60 mM) and 0.2 mL VC (3%) was added before testing. The inhibition of superoxide radical production was calculated according to the following formula:

$$\text{Scavenging rate(\%)} = (A_{\text{control420}} - A_{\text{sample420}}) / A_{\text{control420}} \times 100 \quad (\text{Eq.1})$$

where A_{control420} is the absorbance obtained using water replacement for the sample solution.

Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activities of polysaccharides were determined through Fenton's reaction as previously described. The reaction system consisted of 1.5 mL of the sample solution (50–800 µg/mL), 0.5 mL of EDTA-FeSO₄ solution (25 mM), 0.5 mL of H₂O₂ solution (6.0 mM), and 0.8 mL of salicylic acid solution (2.0 mM). To determine the concentration of the hydroxyl radicals, the absorbance of this solution was detected at 510 nm after incubation for 60 min at 37°C. The following formula was used to calculate the hydroxyl radical scavenging activity:

$$\text{Scavenging rate(\%)} = (A_{\text{control510}} - A_{\text{sample510}}) / A_{\text{control510}} \times 100 \quad (\text{Eq.2})$$

where $A_{\text{control510}}$ is the absorbance obtained using water replacement for the sample solution.

Splenocyte damage assay

The spleen was passed through a steel mesh to obtain a homogeneous cell suspension under aseptic condition (Shi et al. 2015). The cells were washed three times with PBS and resuspended in complete medium (RPMI1640 supplemented with 100 IU/ml penicillin, 100 g/ml streptomycin and 10% FCS). Next, splenocytes cells were seeded into a 96-well flat-bottom microtiter plate with 40 μL each well at 2×10^5 cells/ml. The experimental group contained 40 μL samples with different concentrations (2.5–40 $\mu\text{g/mL}$), 40 μL H_2O_2 (the terminal concentration was 200 $\mu\text{mol/L}$) (Duan et al. 2007) and RPMI 1640 medium to give a final volume of 160 μL . Next, the plate was incubated at 37°C in a humid atmosphere with 5% CO_2 for 2 h. Then, 10 μL of MTT solution (5 mg/ml) was added to each well and incubated for another 4 h. 80 μL of SDS solution (14%SDS+50%DMF, pH4.7) was added to each well, and the absorbance was evaluated on an ELISA reader at 570 nm overnight. The protection rate was calculated based on the following formula:

$$\text{Protection rate(\%)} = (A_{\text{sample}} - A_{\text{injury}}) / (A_{\text{control}} - A_{\text{injury}}) \times 100 \quad (\text{Eq.3})$$

where A_{injury} is the absorbance obtained using RPMI1640 (supplemented with 100 IU/ml penicillin, 100 g/ml streptomycin) replacement for the sample solution; A_{control} is the absorbance obtained using PBS replacement for the H_2O_2 and RPMI1640 replacement for the sample solution.

Splenocyte proliferation assay

Proliferation of splenic lymphocytes was measured by the MTT method as described previously (Shi et al. 2015). Briefly, the spleen was obtained according to the method described in the *I-KI test*. Then splenocytes were seeded into a 96-well flat-bottom microtiter plate with 40 μL in each well at 2×10^5 cells/ml in the presence 8 μL ConA (5 $\mu\text{g/mL}$); the experimental group contained 40 μL samples with different concentrations (50–800 $\mu\text{g/mL}$) and RPMI 1640 medium to give a final volume of 160 μL . Next, the plate was incubated at 37°C in a humid atmosphere with 5% CO_2 for 44 h, 10 μL of MTT solution (5 mg/ml) was added to each well and incubated for another 4 h. 80 μL of SDS solution (14%SDS+50%DMF, pH4.7) was added to each well and the absorbance was evaluated on an ELISA reader at 570 nm overnight. The stimulation rate was calculated based on the following formula:

$$\text{Stimulation rate(\%)} = (A_{\text{sample}} - A_{\text{control}}) / A_{\text{control}} \times 100 \quad (\text{Eq.4})$$

where A_{control} is the absorbance obtained using RPMI1640 (supplemented with 100 IU/ml penicillin, 100 g/ml streptomycin) replacement for the sample solution.

RESULTS AND DISCUSSION

ISOLATION, PURIFICATION AND MOLECULAR WEIGHT OF THE POLYSACCHARIDES

The yield of non-agar polysaccharide GCP was approximately 1.63% calculated as the weight of semi-dried *G. lemaneiformis*. Sulphate content was 15.00% which indicated that GCP is an acidic polysaccharide. The

polysaccharide was first fractionated through an anion-exchange chromatography of cellulose DEAE-FF to obtain two independent elution fractions of GCP-1 and GCP-2 (Fig. 1); the yields were 29.82% and 38.18%, respectively. Two fractions were collected, dialysed and dried. The single symmetrical elution peak of GCP-1 by HPGPC showed that GCP-1 was a homogeneous component and no other polysaccharides were present. According to the calibration curve of the elution times of the standards, Fig. 2 shows the molecular weight of GCP-1 was estimated to be 3.75×10^6 Da. GCP-2 was estimated to be 3.82×10^6 and 4.92×10^5 Da. GCP-1 has a wide peak and GCP-2 has two peaks on HPGPC, so GCP-1 and GCP-2 are not homogeneous. DEAE-FF Column could not purify the polysaccharide completely.

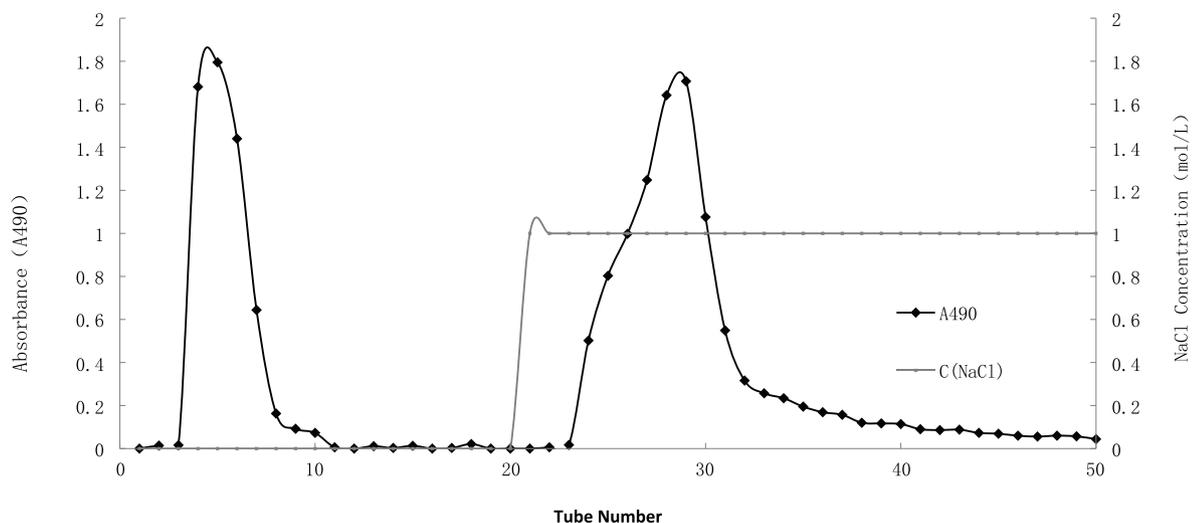


Figure 1 - Chromatography of GCP on Cellulose DEAE-FF Column.

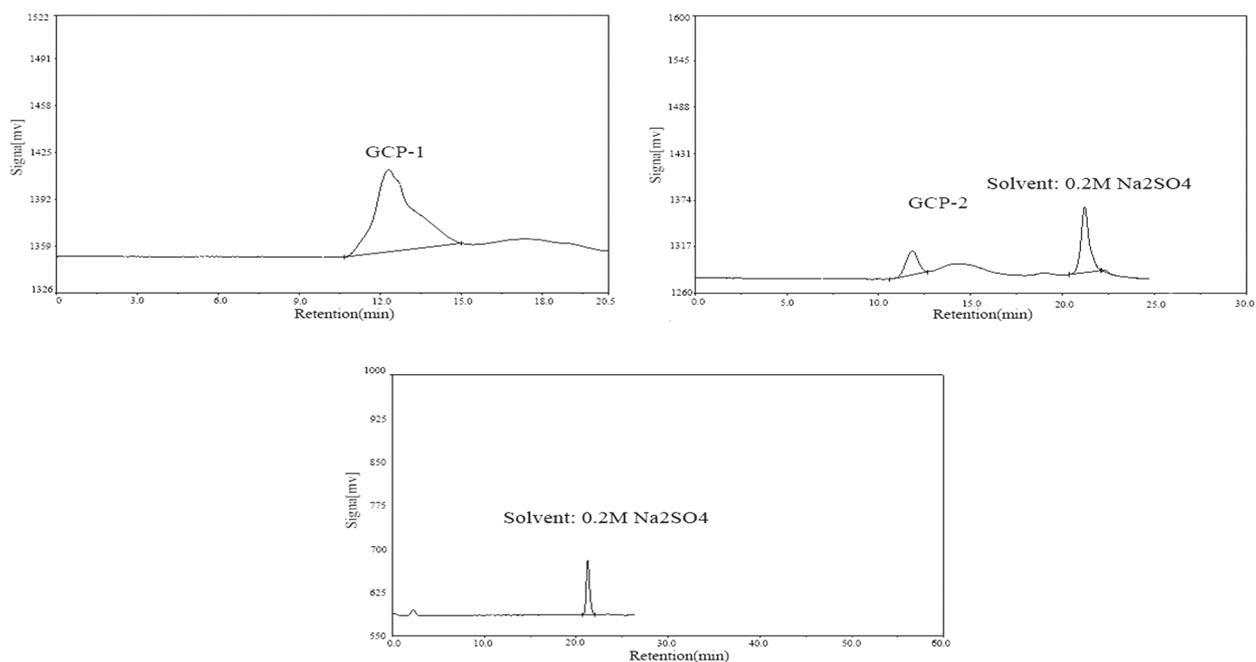


Figure 2 - Molecular weight determined by HPGPC.

CHEMICAL STRUCTURE AND CONFORMATIONAL CHARACTERIZATION OF GCP

Analysis of the monosaccharide composition

The HPLC analysis of the monosaccharide revealed that the major sugar component in the polysaccharide was galactose (Fig. 3); the peak at the retention time of 11 min was PMP. This monosaccharide type is the major building block of red algae-derived sulphated polysaccharides. Sulphated galactans (agars, carageenans or others) are the only types of sulphated polysaccharides in red algae, and these polysaccharides are all galactose-composed.

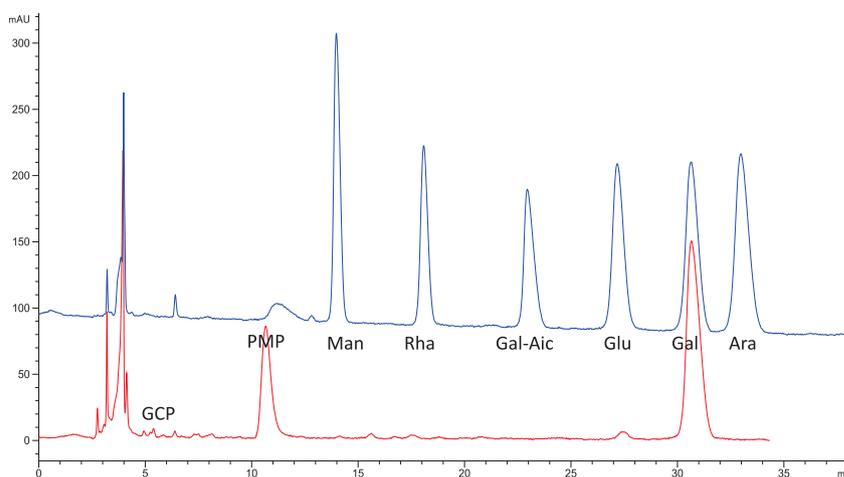


Figure 3 - HPLC chromatogram of monosaccharide composition of GCP.

Conformational characterization of GCP

Congo red can form special complexes with triple-helical polysaccharides in alkaline solution and the maximum absorption wavelength (λ_{max}) will increase. It has been reported that (1 \rightarrow 3)- β -D-glucans with a helical conformation can form complexes with Congo red in a dilute alkaline solution as indicated by a shift in the maximum absorption wavelength (max) of Congo red (Dong et al. 2006). A strong alkaline environment can destroy the ordered helical structure into a disordered structure by breaking the intra- and inter-molecular hydrogen bonds. The greater the effect in the max shift, the higher the helical structure content is (Rout et al. 2008). In this way, a shift in the maximum visible absorption of Congo red induced by the presence of a polysaccharide was used for conformational studies. The max Congo red at various concentrations of sodium hydroxide solution is shown in Fig. 4a. As shown in Fig. 4a, when the concentration of NaOH increased, the polysaccharide formed complexes with Congo red in the dilute alkaline solution and the maximum absorption wavelength increased. The max absorption of GCP increased at the NaOH concentration of 0-0.3 mol/L because of the complexes with Congo-red. The strong alkaline environments destroy the ordered helical structure into a disordered structure by breaking the intra- and inter-molecular hydrogen bonds lead to the absorption decreased at the 0.5 mol/L. It could be concluded that GCP has a triple helical chain conformation, which is a feature of high bioactivities. But studies have shown that alkali-treated polysaccharide exhibit single helix conformation, and this may affect the activities of polysaccharide (Shi et al. 2016).

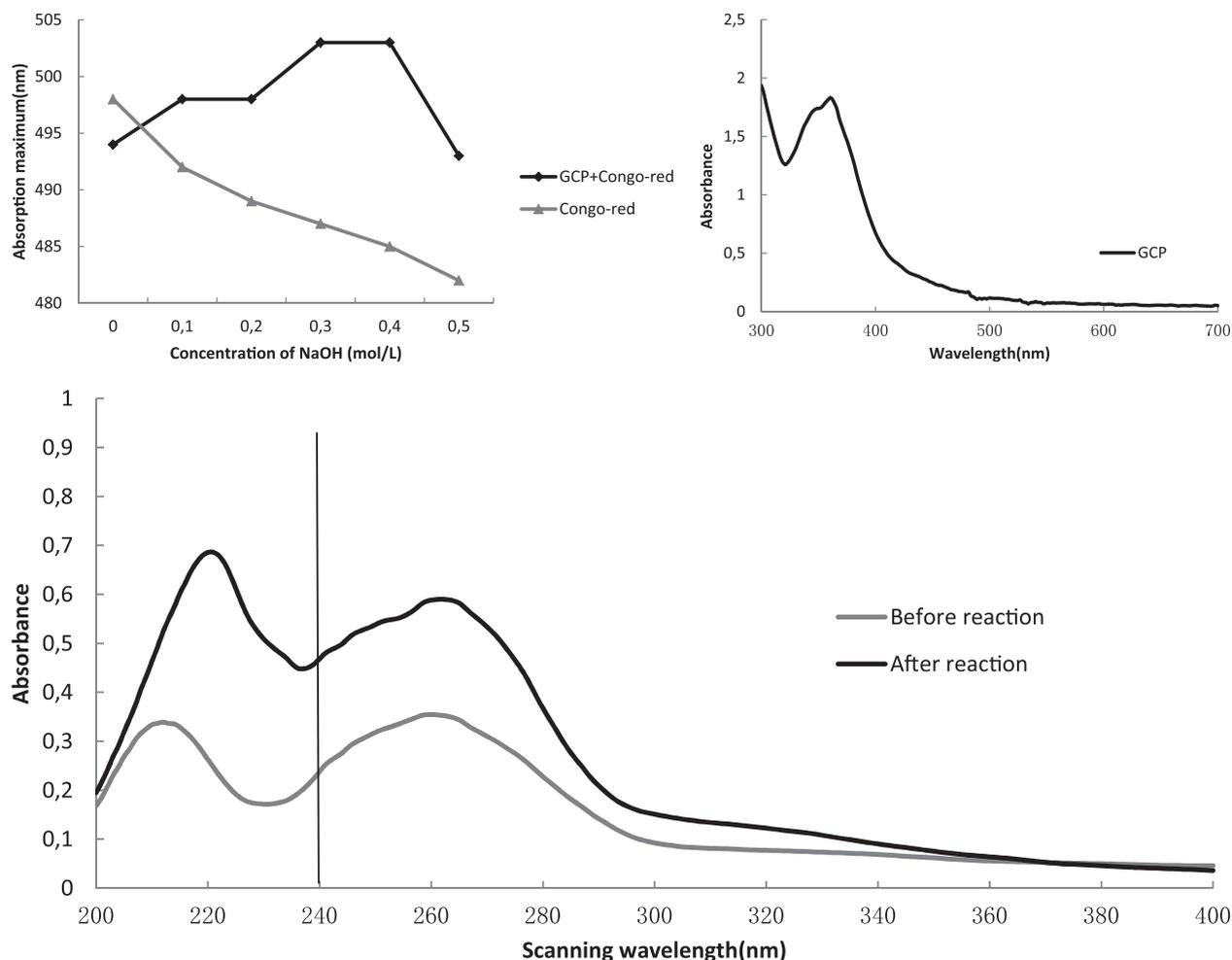


Figure 4 - Changes in the absorption maximum of the Congo red-GCP complex at various concentrations of sodium hydroxide solution (a); I_2 -KI test: the UV spectra of GCP in the range of 300–700nm (b); β -elimination reaction: Changes in the absorption maximum of the GCP complex with hydroxide solution (c).

I_2 can form special complexes with polysaccharides and the maximum absorption in wavelength of 565 nm will increase. This type of polysaccharide had more branches and longer side chains. Fig. 4b shows there was no increase in the absorption in the wavelength of 565 nm, which indicated that GCP has more branches and longer side chains. This feature was similar to the previous study (Ma 2009, Zhu 2006).

β -elimination is the reaction which indicated the linkage type of the glycoprotein is O-glycosidic linkage or not. By increasing the concentration of NaOH, Serine and Threonine in O-glycosidic linkage turn to α -aminoacrylic and α -aminocrotonic acid, which leads to an increase of absorption in the wavelengths of 240 nm. Fig. 4c shows that the absorption at 240 nm increases, indicating that the linkage type of the glycoprotein is O-glycosidic linkage.

Periodate can be used to destroy the O-dihydroxy structure. This experiment revealed that 1 mol of $NaIO_4$ was consumed and 2 mol of HCO_2H were produced per mol of sugar residue containing 1 \rightarrow 6 (or non-reducing terminal) linkages. $NaIO_4$ was consumed but no HCO_2H was produced containing 1 \rightarrow 2 or 1 \rightarrow 4 linkages. Whereas no $NaIO_4$ was consumed or no HCO_2H was produced when the sugar residue contained 1 \rightarrow 3 linkages (Wang et al. 2014b). Subsequently, GC analysis revealed the presence of erythritol

and glycerol to distinguish the linkages of 1→2 and 1→4 linkage. The periodate oxidation revealed that GCP consume 0.03 mmol of NaIO_4 and 0.01 mmol of formic acid was produced, indicating the possible presence of 1→6 linked, 1→2 linked and 1→4 linked or 1→3 linked; after Smith degradation, glucose, galactose, glycerol and erythritol were found in the GC analysis. A small amount of glycerol was detected and little NaIO_4 was consumed, which revealed that there were many 1→3 linkages in GCP. The results from the periodate oxidation–Smith degradation showed that 1→3, 1→2, 1→6 (or no-reducing terminal) linkages might exist in GCP; 1→3 accounted for the major part. It is well-know that agar is made of alternating 1→3 and 1→4 linkages in disaccharide units, so the novel polysaccharide is a type of agar with high solubility.

The FT-IR spectrum of GCP-1 and GCP-2 are shown in Fig. 5. The absorption bands within the range of 3600 to 3000 cm^{-1} , 3000 to 2800 cm^{-1} , 1400 to 1200 cm^{-1} and 1200 to 700 cm^{-1} are the characteristic absorption peaks of polysaccharides (Zhao et al. 2014). The absorption at 2927.38 cm^{-1} of GCP-2 corresponds to the stretching vibration of $-\text{CH}_3$. The absorption at 1251.82 cm^{-1} corresponds to the stretching vibration of $\text{S}=\text{O}$, which indicated that GPC-2 is a sulphated polysaccharide. The absorptions at $1156.17/1153.89$

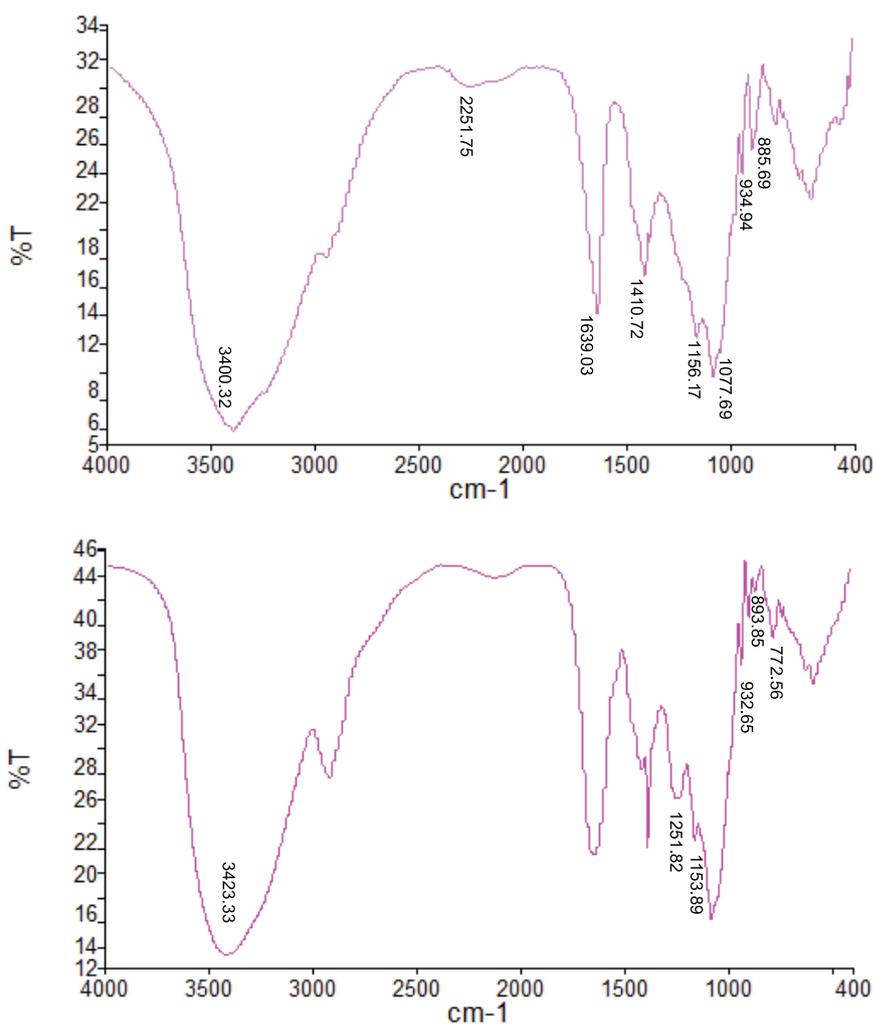


Figure 5 - FT-IR spectrum of GCP-1 and GCP-2.

cm^{-1} are attributed to the ether bond on the pyranose ring (Kacurakova et al. 2000). The absorptions at $1077.69/1075.17 \text{ cm}^{-1}$ and $885.69/893.85 \text{ cm}^{-1}$ show that the sugar structures are galactopyranose configurations (Shao et al. 2014, Sun et al. 2014) and their linkage types are β -type glycosidic linkages (Dong et al. 2006). The absorptions at $934.94/932.65 \text{ cm}^{-1}$ indicated that 3,6-Anhydro- β -D-glucopyranose exists in GCP-1 and GCP-2. The FT-IR spectrum showed that GPC was a type of sulphated polysaccharide, the sugar structure was in the galactopyranose configuration, and their linkage type was β -type glycosidic linkage. But FT-IR spectrum of alkali-treated polysaccharide showed almost none S=O, and this could affect the activities of polysaccharides.

The microstructure of GCP was examined by scanning electron microscopy. As shown in Fig. 6, GCP-1 has a rough surface similar to laminated rock; GCP-2 has a rough surface resembling a dense blade. A possible reason is that these polysaccharides have different chemical properties such as differences in molecular weights.

Figure 7 shows $^1\text{H-NMR}$ of GCP-1 and GCP-2. The chemical shifts of most of the protons on the sugar moieties were at 3-4 ppm; these signals overlapped seriously and made the analysis difficult. The numbers of proton signals at 4.3-5.9 ppm indicated several types of monosaccharides. Moreover, the H^1 proton chemical shifts of the α -pyranose configuration are greater than 4.95 ppm while β -pyranose

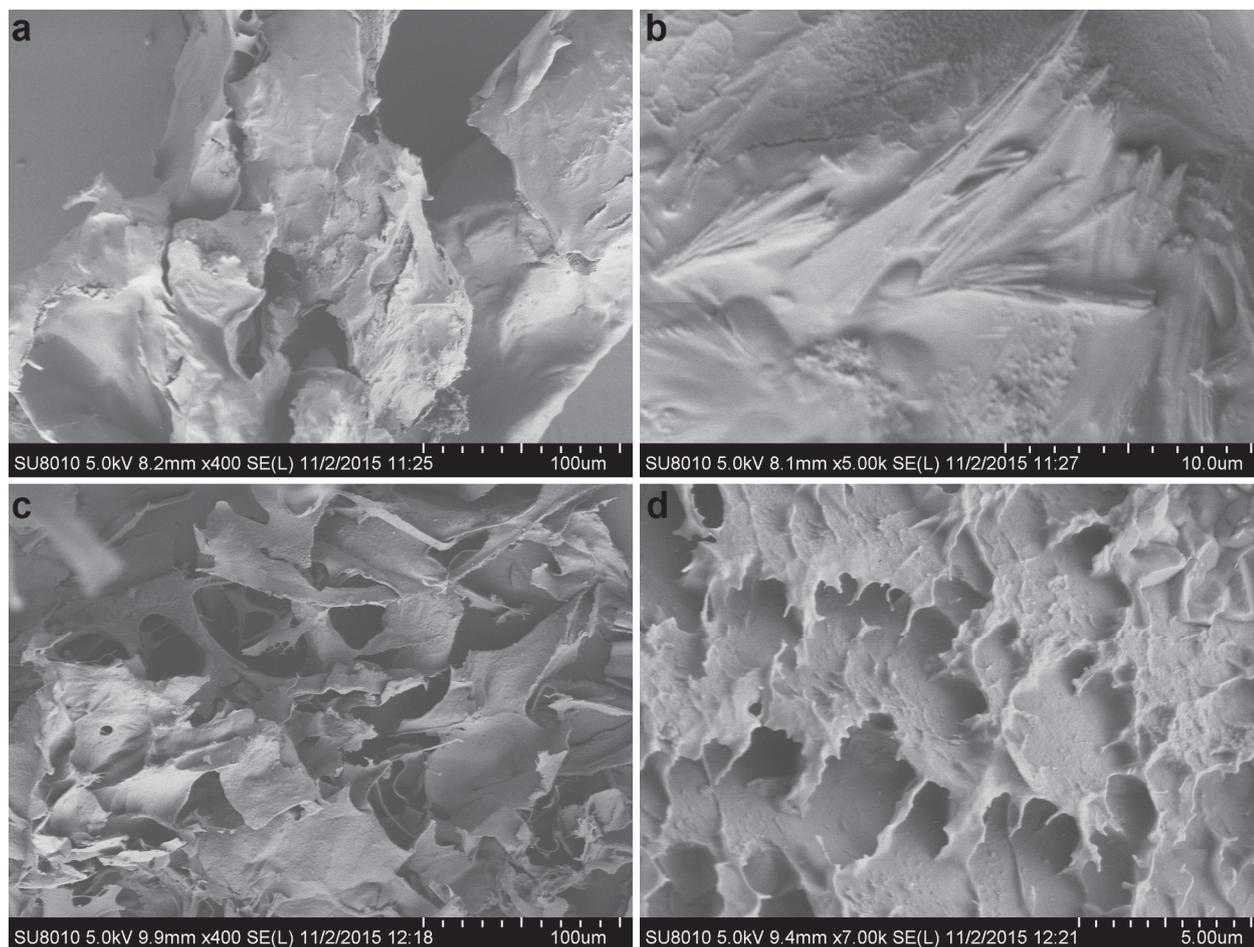


Figure 6 - Scanning electron micrographs of GCP-1(a, b) and GCP-2(c, d). Magnification: (a) $\times 400$, (b) $\times 5000$, (c) $\times 400$ and (d) $\times 7000$.

configuration is less than 4.95ppm. Three major peaks were observed in GCP-1 and GCP-2; the anomeric proton signals $\delta=5.197$ ppm, 5.057 ppm, 4.474/4.487 ppm, which were arbitrarily labelled A, B, and C, respectively, showing GCP consists of three monosaccharide compositions. According to the analysis of the monosaccharide composition by HPLC and FTIR, the monosaccharide compositions of GCP were β -galactose and α -glucose.

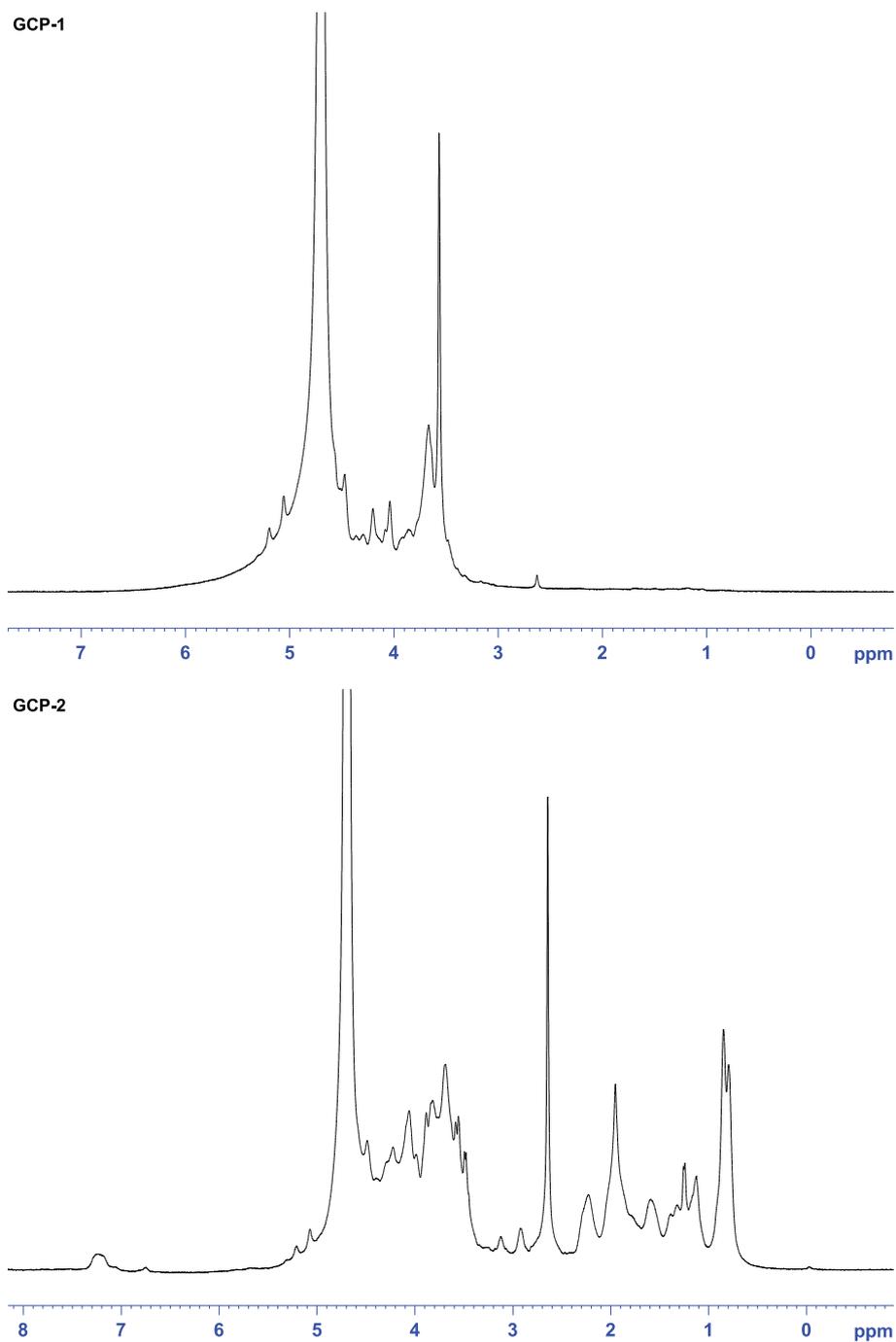


Figure 7 - ¹H-NMR spectrums of GCP-1 and GCP-2.

BIOACTIVITIES ANALYSIS OF GCP, GCP-1 AND GCP-2

Superoxide anion radicals were considered as a precursor of the hydroxyl radicals. In this study, the superoxide anion radical was generated by auto oxidation of pyrogallol in an alkalinescent condition. Fig. 8a shows that the inhibitory effect of GCP, GCP-1 and GCP-2 on superoxide radical was significant at all tested concentrations in a concentration dependent manner. GCP-1 and GCP-2 showed no significant different scavenging effects and the scavenging rate was almost 50%. GCP showed a lower scavenging effect and the scavenging rate was almost 40%, which may be due to GCP obtaining another low active substance.

The hydroxyl radical is considered as one of the most reactive oxygen radicals, which can easily cross cell membranes, readily react with most biomolecules and lead to tissue damage or cell death (Gao et al. 2013). As shown in Fig. 8b, all of the samples were found to have greater ability to scavenge hydroxyl radicals and in a concentration-dependent manner. GCP-2 showed the best scavenging ability with the scavenging rate, 60.24%. GCP and GCP-1 showed lower scavenging effects and the scavenging rates were 42.49% and 47.46%, respectively. Crude polysaccharide showed a lower scavenging effect than the purified polysaccharide. This may due to the crude polysaccharide containing other low active substances. Sulphated derivatives were found to have scavenging ability in a DS-dependent fashion; higher sulphate content showed greater scavenging effects of superoxide radicals (Qi et al. 2005). In this study, GCP-2 was an acid fraction with higher sulphate content that showed greater scavenging effect (GCP-2> GCP-1).

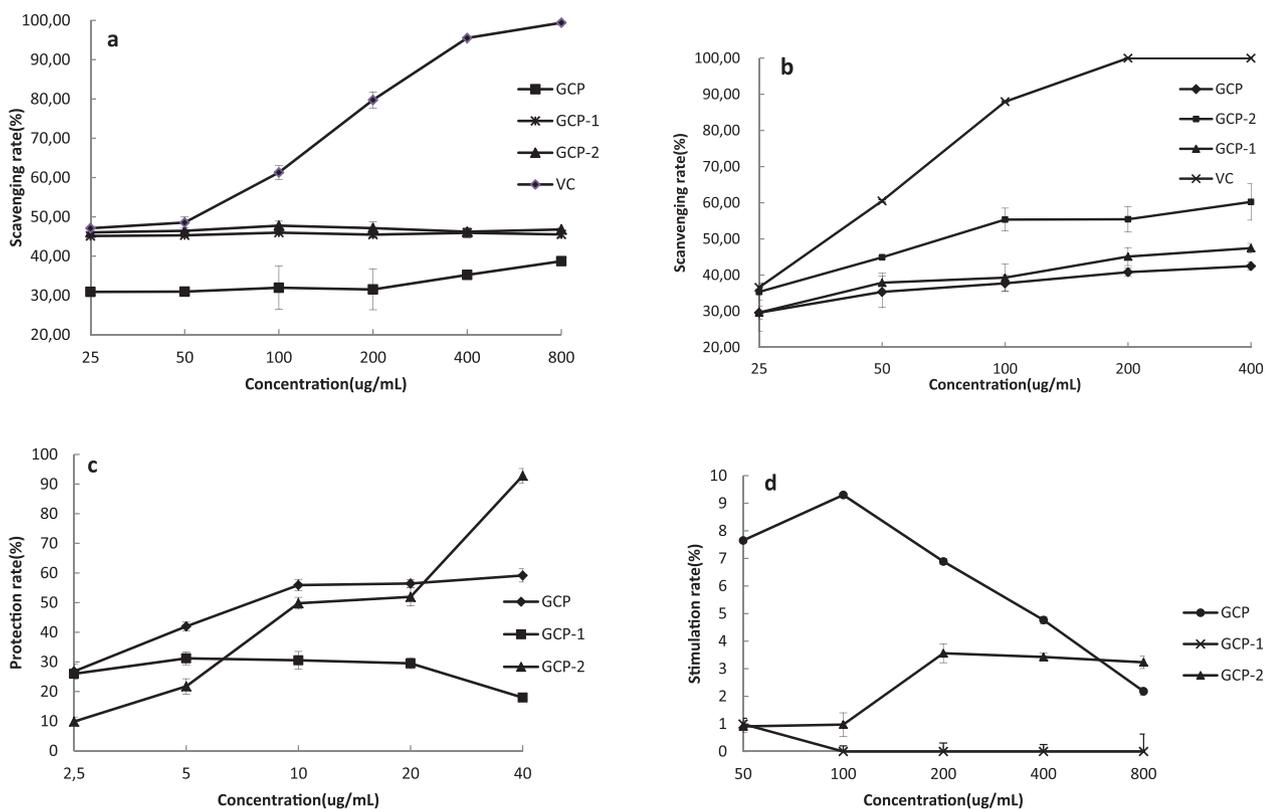


Figure 8 - Samples with different concentration were selected to evaluate antioxidant activities and immunological activity: scavenging activity of superoxide radicals (a); scavenging activity of hydroxyl radicals (b); protection of damaged splenocyte (c); effect on splenocyte proliferation (d).

Hydroxyl radicals can easily cross cell membranes react with most biomolecules such as carbohydrates, lipids, proteins and DNA, and give rise to cell death, even tissue damage (Leong and Shui 2012, Yuan et al. 2008). Recent studies have shown that polysaccharide could protect PC12 cells (Shi et al. 2012), myocardial cells (Zhou et al. 2011) and hepatocyte (Song et al. 2012) damaged by H_2O_2 . In this study, for the first time, spleen cells damaged by H_2O_2 were used to evaluate the protective effect of polysaccharide. Successfully, we found that polysaccharides from *Gracilariopsis lemaneiformis* showed strong protective effects. As shown in Fig. 8c, GCP showed a strong protective effect and in a concentration dependent manner. In the concentration range from 2.5 to 40 $\mu\text{g/mL}$, the maximum protection rate was 59.16%. GCP-1 and GCP-2 showed similar trends. GCP-2 showed greater protective effects than GCP-1, in a concentration dependent manner. The maximum protection rates of GCP-1 and GCP-2 were 31.21% and 92.8%, respectively. The sulphate group played an important role in the scavenging of hydroxyl radicals. This was in agreement with the results of Qi et al. (2005) which showed in the molecules of high DS. Parts of the OH groups were substituted by OSO_3H groups, meaning that the scavenging effect was enhanced. But polysaccharide with early alkali treatment showed lower protective effect with the maximum protection rate, 51.50% (Shi et al. 2016).

The immunomodulatory potential of GCP, GCP-1 and GCP-2 in vivo was evaluated by splenocyte proliferation. The effects on splenocyte proliferation stimulated with mitogens (ConA) or without mitogens are shown in Fig. 8D. It was demonstrated that GCPs significantly enhanced the splenocyte proliferation. At the concentration of 100 $\mu\text{g/mL}$, GCP showed the highest proliferation effect; the proliferation rate was 9.30%. GCP-1 and GCP-2 showed lower proliferation effects than GCP. Perhaps certain active ingredients disappeared after purification by DEAE-FF or GCP-1 and GCP-2 have synergistic effects. Polysaccharide with early alkali treatment showed lower proliferation effect and the proliferation rate was 7.45% (Shi et al. 2016). Immunostimulation itself is regarded as one of the important strategies to improve the body's defence mechanisms in elderly individuals as well as in cancer patients. There is a significant amount of experimental evidence suggesting that polysaccharides from *Gracilariopsis lemaneiformis* enhance the host immune system by stimulating T-cell system responses (Dalmo and Boqwald 2008).

CONCLUSIONS

In this study, a novel non-agar seaweed polysaccharide fraction, GCP, was prepared from *Gracilariopsis lemaneiformis*. The major monosaccharide composition was galactose. GCP showed a triple helical chain conformation in aqueous solution, had more branches and longer side chains, composed of O-glycosidic linkages, 1 \rightarrow 3 linkages were accounted for the major part, the sugar structures are galactopyranose configurations and their linkage types are β -type glycosidic linkages. This means the polysaccharide show higher antioxidant activity. In this study GCP showed great radical-scavenging ability and splenocyte proliferation effects. Two polysaccharide fractions were purified with DEAE Sepharose FF cellulose: GCP-1 and GCP-2, these fractions showed greater antioxidant activity but lower splenocyte proliferation effect than GCP, which means that some active ingredients disappear after purification by DEAE-FF cellulose or GCP-1 and GCP-2 have synergistic effect in immune function.

Comparing the present study with previous ones, we found that early alkali treatment could reduce activities by changing the structure of polysaccharide, such as reducing helical conformation, reducing the sulfate content.

Consequently, the results indicated that this novel non-agar seaweed polysaccharide without alkali treatment could be used as good natural antioxidants applied to the production of anti-aging medicine, prevent chronic diseases and enhance the immunity. Future research should focus on in vivo immunomodulatory and antitumor activity.

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