

Sulfated polysaccharides with antioxidant and anticoagulant activity from the sea cucumber *Holothuria fuscogliva**

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Abstract Sea cucumber is a traditional nutritional food and medicinal resource with many bioactive components in China. *Holothuria fuscogliva* is a big sea cucumber with a rich of bioactive polysaccharides. To investigate the bioactivities of the polysaccharides from sea cucumber *H. fuscogliva*, we prepared the sulfated polysaccharides (HfP) from sea cucumber *H. fuscogliva* using a protease hydrolysis method. Antioxidant activities of HfP were investigated, including hydroxyl radical scavenging activity and superoxide radical scavenging activity. And, the anticoagulant activities of HfP were studied, including the activated partial thromboplastin time (APTT), prothrombin time (PT) and thrombin time (TT). The average molecular weight was 1 867.1 Da, with a sulfate content of 20.7%. In addition, the molar ratio of monosaccharide composition of HfP was Man: Rha: Glc A: Glc: Gal: Xyl: Fuc=0.083 6: 0.437: 0.134: 0: 1.182: 0.748: 1. It had a strong antioxidant activity, the hydroxyl and superoxide radical scavenging activity EC_{50} of HfP was 3.74 and 0.037 mg/mL, respectively. It also showed a good anticoagulant activity in our study. The APTT of HfP was much higher than that of heparin sodium, and the PT and TT of HfP was close to that of heparin sodium at a low concentration. Therefore, HfP shows a good antioxidant and anticoagulant activity and it may become a potential candidate of the natural antioxidant and anticoagulant and will have a good application future in health product or medicine industry.

Keyword: sulfated polysaccharides; sea cucumber; *Holothuria fuscogliva*; antioxidant activity; anticoagulant activity

1 INTRODUCTION

Polysaccharides from marine organisms have many good activities and can be used as foods, health products, and prodrugs. For example, chitosan that is mainly prepared from the shell of shrimp and crab, is a natural polysaccharide mainly composed of β -1,4 linked d-glucosamine, and partially of β -1,4 linked N-acetyl-d-glucosamine (Li et al., 2012a). Chitosan is widely applied in the fields of food, biomaterials, and drug delivery because of its various bioactivities, such as antitumor, antioxidant and antimicrobial activities (Li et al., 2013b; Younes et al., 2014). In addition, polysaccharides from seaweed were proved to have antioxidant, anticoagulant, neuroprotective, antitumor activities (Wang et al., 2011; Li et al., 2013a; Jin et al., 2013, 2014), and some were even

used as important prodrugs in the clinical medicine (Wang et al., 2011).

Sea cucumber is a traditional nutritional food and medicinal resource widely consumed in China, Japan, and Korea. Polysaccharide is one of the most important bioactive components in sea cucumber. A fucoidan from *Acaudina molpadioides* could reverse the changes in tissue oxidation and antioxidant activities, ease regulate the signal pathways of

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mitogen-activated protein kinases and matrix metalloproteinases, and the damage of ethanol-induced gastric (Wang et al., 2012). A sulfated polysaccharide from *Stichopus japonicus* could change the morphological and proliferation of astrocytes in vitro if it combined with a basic fibroblast growth factor 2 (Sheng et al., 2011). A fucosylated chondroitin sulphate from *Cucumaria frondosa* exhibited anti-hyperglycaemic effects and inhibited pancreatic islets apoptosis via inhibition mitochondrial pathway (Hu et al., 2014a, b). In addition, some other polysaccharides had anticoagulant activities and could be potentially applicable as antithrombotic agents with reduced bleeding risk (Mourão et al., 1996; Matsuihro et al., 2012; Ye et al., 2012; Luo et al., 2013; Wu et al., 2013).

Sea cucumber *Holothuria fuscogлива* (Phylum Echinodermata, Class Holothuroidea, order Aspidochirotida, Family of Holothuriidae) is a big sea cucumber that can reach about 50 cm long and 13 cm wide when matured (Liao, 1997). This sea cucumber is rich of bioactive polysaccharides. However, no studies have reported on the preparation and bioactivities of polysaccharides in *H. fuscogлива* at present. Therefore, we prepared polysaccharides from the sea cucumber in an enzyme hydrolysis method and investigated the antioxidant and anticoagulant activities. It will be significant to the development and utilization of the sea cucumber in food, health product, or even medicine industries in the future.

2 MATERIAL AND METHOD

2.1 Materials and reagents

Sea cucumber *H. fuscogлива* was identified by Dr. N Xiao, Department of Marine Organism Taxonomy & Phylogeny, Institute of Oceanology, Chinese Academy of Sciences. The monosaccharide standards of D-galactose, L-fucose, D-mannose, L-rhamnose monohydrate, D-glucuronic acid, D-glucose, and D-xylose were purchased from Sigma (USA). The anticoagulant activity assay kits were purchased from Nanjing Jiancheng Bioengineering Institute. Other reagents used in this study were of the highest quality available from commercial vendors.

2.2 Preparation of polysaccharides

The sulfated polysaccharides of *H. fuscogлива* (HfP) were prepared according to the method described by Liu et al. (2012) and Zheng et al. (2007) with some modifications. Briefly, dry *H. fuscogлива* without viscus

was cut into small pieces and then immersed in 30 times (v/w) of pH 6.0, 0.1 mol/L NaAc buffer, containing 5% papain, 5 mmol/L EDTA and 5 mmol/L cysteine, and incubated at 60°C for 24 h. The mixture was centrifuged at 3 000×g for 15 min, and then added 2 times of ethanol into the supernatant, standing at 4°C for 12 h. Then, the precipitate was collected and washed by ethanol. After freeze-drying, the crude polysaccharides were dissolved in distilled water and adjusted to pH 10.0 with 0.1 mol/L KOH. The polysaccharides solution was added with 1/7 v: v 30% H₂O₂, and incubated at 60°C for 24 h. The solution was then centrifuged at 3 000×g for 15 min and the supernatant was adjusted to pH 2.0 with 1 mol/L HCl. After centrifugation at 3 000×g for 15 min, the supernatant was added with KAc to 1.5 mol/L, standing at 4°C for 12 h. After centrifugation at 3 000×g for 15 min, the precipitate was washed with ethanol and then freeze-dried to obtain polysaccharide of HfP.

2.3 Molecular weight assay

An Agilent 1260 HPLC system in high performance gel permeation chromatography of TSKgel G3000 PW_xL column (7.8 mm×30.0 cm) was employed to measure the molecular weight of HfP by eluting with 0.2 mol/L Na₂SO₄ at a flow rate of 1.0 mL/min. The eluate was monitored by a refractive index detector, and the column calibration was performed with standard dextrans Mw 670 000, 270 000, 80 000, 50 000, 25 000, 12 000, 5 000 and 1 000 Da (Sigma, USA).

2.4 Monosaccharide composition and sulfated content analysis

The molar ratio of monosaccharide composition of HfP was determined following Zhang et al. (2009). Briefly, 10 mg/mL sample was hydrolyzed with 2 mol/L trifluoroacetic acid in a 10-mL ampoule. It was then sealed in nitrogen atmosphere and hydrolyzed at 110°C for 4 h and adjusted to pH 7.0 with 0.1 mol/L NaOH. Later, it was converted into its 1-phenyl-3-methyl-5-pyrazolone derivatives and analyzed by a HPLC chromatography, using Ribose as the internal standard. Sulfate content was analyzed with barium chloride-gelatin method (Kawai et al., 1969).

2.5 FTIR and NMR spectroscopy

Fourier transform infrared (FTIR) spectra of HfP was measured in the 4 000–400/cm regions using a Thermo Scientific Nicolet iS10 FT-IR spectrometer in KBr discs. Twenty mg HfP was co-evaporated with

D₂O twice. Then it was dissolved in D₂O containing 0.1 μL deuterated acetone. A Bruker AVANCE III 600 MHz was employed to analyze its NMR spectra at 25°C and the internal standard of deuterated acetone was used as the chemical shifts.

2.6 Antioxidant activities assay

2.6.1 Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity of HfP was determined according to Guo et al. (2005). Briefly, the reaction mixture, total volume 4.5 mL, containing different concentrations of HfP, was incubated with 220 μmol/L EDTA-Fe²⁺, 0.23 μmol/L safranin O, 60 μmol/L H₂O₂ in 150 mmol/L, pH 7.4 PBS for 30 min at 37°C and then measured the absorbance at 520 nm. As the hydroxyl radicals can bleach the safranin O, the increased absorbance indicated the capability of the hydroxyl radical scavenging activity of the sample, which can be calculated by the follow equation:

$$\text{Scavenging effect (\%)} = \frac{(A_{\text{sample}} - A_{\text{blank}})}{(A_{\text{control}} - A_{\text{blank}})} \times 100,$$

A_{blank} is the absorbance of the blank, using double distilled water (ddH₂O) instead of the samples and A_{control} is the absorbance of the control, using dd H₂O instead of H₂O₂.

2.6.2 Superoxide radical scavenging activity

The superoxide radical scavenging activity of HfP was determined according to the method of Nishikimi et al. (1972). Briefly, the reaction mixture, containing different concentrations of HfP, 30 μmol/L PMS, 338 μmol/L NADH, and 72 μmol/L NBT in 16 mmol/L, pH 8.0 Tris-HCl buffer, was incubated 5 min at room temperature and then measured at 560 nm. The capability of superoxide radical scavenging activity was calculated by the following equation:

$$\text{Scavenging effect (\%)} = (1 - A_{\text{sample}}/A_{\text{control}}) \times 100,$$

A_{control} is the absorbance of the control, using Tris-HCl instead of NADH.

2.7 Anticoagulant activities assay

2.7.1 Activated partial thromboplastin time (APTT)

The APTT of HfP was measured according to the manufacturer's instructions. Briefly, 40 μL platelet-poor plasma was mixed with 10 μL solutions of HfP at different concentrations in 0.9% NaCl, and added 50 μL preheated APTT into the mixture, followed by

an incubation at 37°C for 5 min. At the same time, 0.025 mol/L CaCl₂ was also preheated at 37°C. Then, these two preheated solutions were mixed, and the time for clot formation was recorded by an automatic coagulation analyzer (Healife HF6000-4, China). The controls were used 0.9% NaCl instead.

2.7.2 Prothrombin time (PT)

The PT of HfP was tested according to the manufacturer's instructions. Briefly, 100 μL PT reagent was incubated for 5 min. Then, 10 μL HfP with different concentrations in 0.9% NaCl was mixed with 40 μL platelet-poor plasma. The mixed solution was preheated for 3 min. Then, these two preheated solutions were mixed, and recorded the time for clot formation by an automatic coagulation analyzer (Healife HF6000-4, China). The controls were used 0.9% NaCl instead.

2.7.3 Thrombin time (TT)

The TT of HfP was tested following the manufacturer's instructions with some modifications. Briefly, 90 μL platelet-poor plasma was mixed with 10 μL HfP with different concentrations in 0.9% NaCl. After preheating for 3 min, the solution was then mixed with 100 μL TT reagent and recorded the time for clot formation was recorded by an automatic coagulation analyzer (Healife HF6000-4, China). The controls were used 0.9% NaCl instead.

2.8 Statistical analysis

All data in this study were presented as means ± standard deviation and the statistical differences between the experimental groups and controls with $P < 0.05$ was considered to be statistically significant.

3 RESULT AND DISCUSSION

3.1 General analysis

The average molecular weight of HfP was about 1 867.1 Da using an Agilent 1260 HPLC system with a TSKgel G3000 PW_xL column. The molar ratio of monosaccharide composition of HfP showed that Man: Rha: Glc A: Glc: Gal: Xyl: Fuc = 0.083 6: 0.437: 0.134: 0: 1.182: 0.748: 1 (Fig.1), and the sulfate content of HfP is about 20.7%.

The FTIR spectra of HfP and its methylated derivative were showed in Fig.2. In the 4 000–1 800/cm region, there was a peak at 3 431.96/cm, which was contributed by NH₂, NHAc, or OH group. We

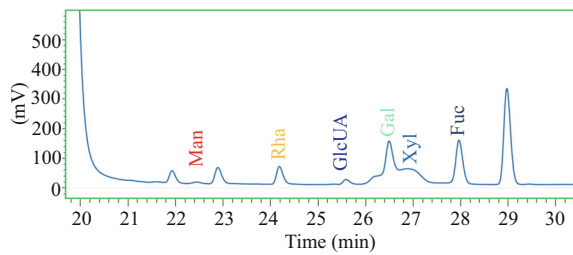


Fig.1 HPLC analysis of monosaccharide composition in HfP

Man: mannose; Rha: rhamnose; GlcUA: glucuronic acid; Gal: galactose; Xyl: xylose; Fuc: fucose.

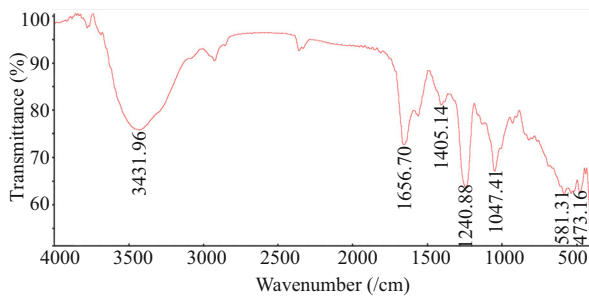


Fig.2 FTIR spectra of the HfP

FTIR was measured in the 4 000–400/cm regions using a Thermo Scientific Nicolet iS10 FT-IR spectrometer in KBr disc.

also observed two small peaks at 2 926.50/cm and 2 854.87/cm, which should be assigned to CHO, CH₃, CH₂, and CH group. In the 1 800–400/cm region, we can find a peak at 1 656.70/cm, which was obviously contributed by COOH group. Moreover, the peaks at 590/cm and 1 240.88/cm were assigned to the SO₄ group. As FTIR spectra could provide only primary information about the functional groups, more information was confirmed by ¹H NMR (Fig.3). Similar spectra containing characteristic signals, the main peaks were assigned. Specifically, signals at 1.30×10^{-6} was readily assigned to the methyl protons of Fuc (CH₃) (Wu et al., 2013). Signals at 1.98×10^{-6} was assigned to the Acetyl galactosamine protons of GalNAc (CH₃), which was coincidence with the previous results (Dong et al., 2014).

3.2 Antioxidant activities assay

Hydroxyl radical scavenging activity and superoxide anion radical scavenging activity are the most important indicators for the antioxidant activities of the bioactive substances. Hydroxyl radical is responsible for oxidative injury to biomolecules generated by reaction of Fe (II) complex with H₂O₂ in the presence of acid. As shown in Fig.4, both HfP and Vc have obvious hydroxyl radical scavenging activity and the EC₅₀ of HfP and Vc was 3.74 and 2.72 mg/mL,

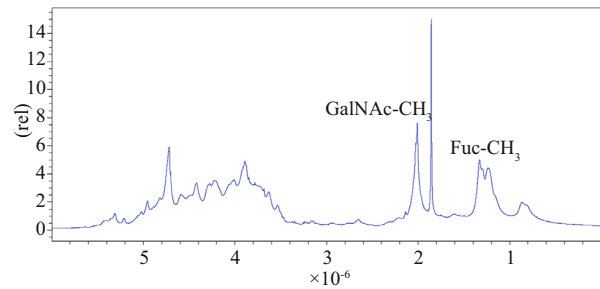


Fig.3 ¹H NMR spectrum of HfP

The ¹H NMR spectra were recorded using a Bruker AVANCE III 600 MHz at 25°C.

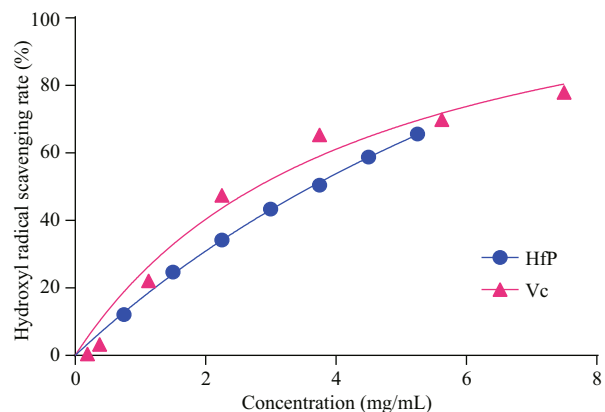


Fig.4 Hydroxyl radicals scavenging activity of HfP

HfP: polysaccharides of *H. fuscoglivia*; Vc: vitamin C. All results were representative of three parallel experiments and expressed as mean±SD.

respectively. The hydroxyl radical scavenging activity of HfP is close to that of Vc, which means that HfP and Vc has a similar scavenging ability for hydroxyl radical. In the PMS/NADH-NBT system, superoxide anion radicals derived from dissolved oxygen by the PMS-NADH coupling reaction reduce NBT. At 560 nm, the decrease of absorbance with antioxidants indicates the consumption of superoxide anion radicals in the reaction mixture. Figure 5 shows that the % scavenging effect on superoxide anion radicals of HfP and the EC₅₀ values, defined as the concentration for 50% of maximal scavenging radical, were usually used to express the capacity of radical scavenging. In present study, the EC₅₀ of HfP and Vc was about 0.037 8 and 0.93 mg/mL, respectively.

Reactive oxygen species, such as the superoxide anion radical, hydroxyl radical, can be generated inevitably in aerobic organisms during respiration. It is necessary for the normal cell function at physiological concentrations. However, it can cause a number of pathological events such as aging, cellular injury, lipids damage, proteins and DNA degradation when excessively or the antioxidant defense systems are depressed (Söğüt et al., 2003). Therefore, the body

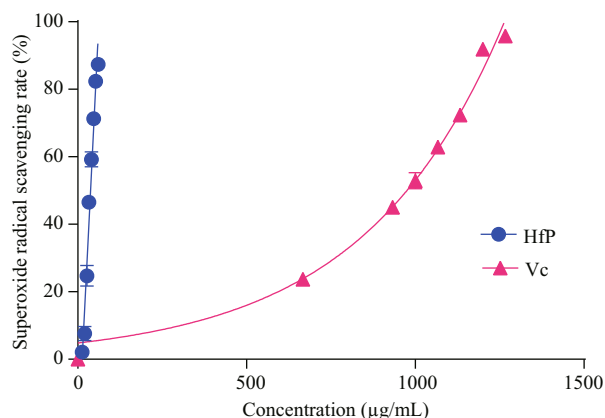


Fig.5 Superoxide radicals scavenging activity of HfP

HfP: polysaccharides of *H. fuscogliva*; Vc: vitamin C. All results were representative of three parallel experiments and expressed as mean±SD.

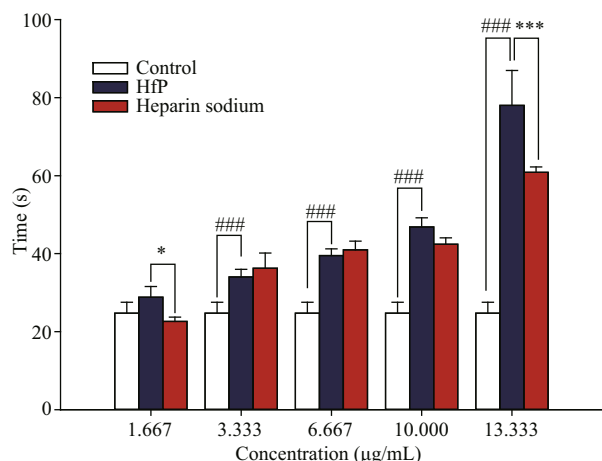


Fig.6 Activated partial thromboplastin time (APTT) analysis of HfP

Control: the blank control; HfP: polysaccharides of *H. fuscogliva*; Heparine sodium: the positive control with heparine sodium ($n=4$, $*/# P<0.05$, $**/## P<0.01$, $***/### P<0.001$).

needs some antioxidant to help to scavenge them in vivo. Although, the synthetic antioxidants, such as butylated hydroxytoluene, butylated hydroxyanisole, and tert-butylhydroquinone, have a strong capacity of scavenging reactive oxygen species nowadays. They were used under strict regulation because of their toxic effects on human’s enzyme systems (Hatate et al., 1990; Chen et al., 1996; Yu et al., 2005; Li et al., 2012b). Therefore, HfP as a good natural antioxidant from sea cucumber *H. fuscogliva* can be applied to enrich health products.

3.3 Anticoagulant activities assay

Anticoagulant activities often involve three indicators of APTT, PT, and TT. The coagulation factors VIII, IX, XI, XII and prekallikrein in the intrinsic blood coagulation pathway were often used

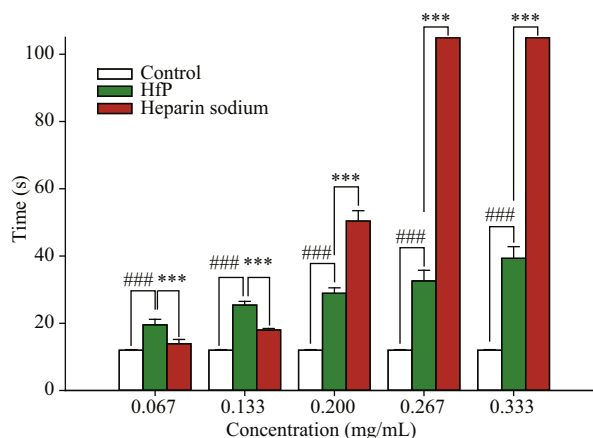


Fig.7 Prothrombin time (PT) analysis of HfP

Control: the blank control; HfP: polysaccharides of *H. fuscogliva*; Heparine sodium: the positive control with heparine sodium ($n=4$, $*/# P<0.05$, $**/## P<0.01$, $***/### P<0.001$).

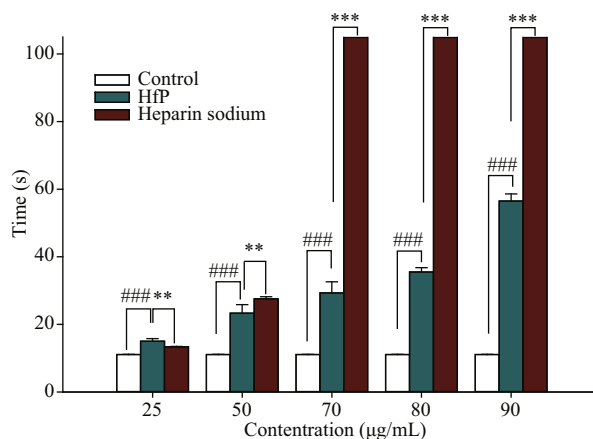


Fig.8 Thrombin time (TT) analysis of HfP

Control: the blank control; HfP: polysaccharides of *H. fuscogliva*; Heparine sodium: the positive control with heparine sodium ($n=4$, $*/# P<0.05$, $**/## P<0.01$, $***/### P<0.001$).

to evaluate the anticoagulant activity of bioactive substances in the APTT assay. In addition, PT indicated the extrinsic coagulation factors to assess the capacity of the anticoagulant activity. TT represents blood coagulation status that transforming fibrinogen into fibrin (Lin et al., 2007).

As shown in Fig.6, APPT of HfP is longer than that of heparin sodium at most concentration. Especially, the APPT of HfP and heparin sodium is 78.022 5 s and 60.866 7 s, respectively. Figure 7 shows that the PT of HfP is higher than that of heparin sodium when the concentration is lower than 0.133 mg/mL, while the PT of heparin sodium is much higher than that of HfP when the concentration is above 0.133 mg/mL. Therefore, the anticoagulant activity evaluated by PT of HfP is also higher than that of heparin sodium, with the concentration lower than 0.133 mg/mL. Figure 8

shows that the TT of HfP and heparin sodium. The TT of HfP is higher than that of heparin sodium when the concentration is lower than 50 µg/mL. But, the TT of heparin sodium is much higher than that of HfP when the concentration is higher than 50 µg/mL. It may be related to the sulfate content and the molecular weight. The sulfated group plays an important role in anticoagulant biological activity (Borsig et al., 2007; Ye et al., 2012). But, the molecular weight may be more important when the concentration is lower than 50 µg/mL. Therefore, the anticoagulant activity evaluated by TT of HfP is also higher than that of heparin sodium, with the concentration lower than 50 µg/mL.

At present, cardiovascular and cerebrovascular disease is threatening human health seriously for a long time and become one of the major causes for the death. Most patients, who have thromboembolism problems, require anticoagulation therapy, and heparin is a currently available anticoagulant polysaccharide drug (Moll and Roberts, 2002). However, heparin also has many limitations clinically, including short half-life, osteoporosis, and continuous injection. Sea cucumber was used as a traditional Chinese medicine for many years. Polysaccharides are one of the most important bioactive components in sea cucumber and showed good anticoagulant activities in present study. Especially, APTT of HfP is much higher than that of heparin sodium. In addition, PT and TT of HfP are very close to that of heparin sodium at a low concentration. Therefore, HfP will have a good potential to be a drug for the prevention or therapy of thromboembolism in the future.

4 CONCLUSION

In present study, we prepared sulfated polysaccharides HfP from sea cucumber *H. fuscogлива* in protease hydrolysis method. The mean molecular weight was 1 867.1 Da with sulfate content of about 20.7%. The molar ratio of monosaccharide composition of HfP was Man: Rha: Glc A: Glc: Gal: Xyl: Fuc=0.083 6: 0.437: 0.134: 0: 1.182: 0.748: 1. HfP showed notable antioxidant activities including hydroxyl radical scavenging activity and superoxide radical scavenging activity in vitro. In addition, HfP had strong anticoagulant activities. Especially, the APTT of HfP was much higher than that of heparin sodium, and the PT and TT of HfP was close to that of heparin sodium at a low concentration. Therefore, HfP will have a good application future in health product or medicine industry.

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