Purification, physico-chemical properties and antioxidant activity of polysaccharides from *Sargassum fusiforme* by hydrogen peroxide/ascorbic acid-assisted extraction

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Abstract: The biological activities *of Sargassum fusiforme* polysaccharides (SFP) were affected significantly by the extraction method. In order to screen the optimum extraction technology for SFP with high yield and biological activities, six extraction methods, including hot water extraction (HWE), acid-assisted extraction (ACAE), alkali-assisted extraction (ALAE), ultrasonic-assisted extraction (UAE), microwaveassisted extraction (MAE) and hydrogen peroxide/ascorbic acid-assisted extraction (HAE) were compared for the preparation of SFP. Based on the yield and *in*

vitro antioxidant activity of the crude polysaccharides obtained by the six extraction methods, HAE was selected for the extraction of SFP. The SFP prepared by HAE (H-SFP) was purified by cellulose DEAE-52 ion-exchange chromatography, obtaining two purified fractions, namely H-SFP3 and H-SFP5. The analyses of their chemical composition, physico-chemical properties and the antioxidant capacity were performed. It was found that the crude SFP and the purified fractions possessed considerable ability to scavenge DPPH, hydroxyl and $ABTS^{\bullet+}$ radicals. These polysaccharide fractions were also found to effectively reduce the reactive oxygen species (ROS) level and increase the superoxide dismutase (SOD) activity in H_2O_2-induced oxidative stress RAW264.7 cells. The SFP prepared by the HAE has the potential as a natural non-toxic antioxidant and can be used as an ingredient in functional foods.

Keywords: *Sargassum fusiforme* polysaccharide; extraction method; physico-chemical properties

1. Introduction

Oxidative stress is defined as an increase in the level of intracellular reactive oxygen species (ROS), mainly including hydrogen peroxide, superoxide anion and hydroxyl radicals, which cause damage to DNA, lipids and proteins [1]. Oxidative stress has been shown to be involved in various pathogenic processes including carcinogenesis, aging, inflammation, atherosclerosis, and rheumatoid arthritis [2]. Therefore, antioxidants contribute significantly to overall health maintenance by

41 controlling the oxidative process [1,3]. Recently, there has been extensive interest to
42 develop the antioxidants from natural sources with no side effect and toxicity, such as
43 polysaccharides, in the food industry and pharmaceuticals [4].

44 *Sargassum fusiforme* (*S. fusiforme*), an edible brown algae belonging to
45 *Sargasaceae* family, is widely distributed in China, Korea and Japan, and has been
46 applied as a therapeutical agent for thousands of years [5,6]. The polysaccharides from
47 *S. fusiforme* (SFP) have attracted particular attention recently due to their important
48 biological properties, such as immunoregulatory [7], antioxidant, hypolipidemic [8],
49 antibacterial [5], and hypoglycemic activities [9].

50 The physicochemical properties, bioactivities and yield of polysaccharides have
51 been demonstrated to be significantly influenced by the extraction method [10]. Several
52 extraction methods have been used to extract polysaccharides from *S. fusiforme*,
53 including traditional hot water extraction (HWE) [11], acid-assisted extraction (ACAE)
54 [10], alkali-assisted extraction (ALAE) [8], ultrasonic-assisted extraction (UAE) [12],
55 and microwave-assisted extraction (MAE) [8]. These methods have disadvantages in
56 one way or another, such as relatively low yield and poor biological activities, use of
57 specific facilities, which limits the practical applications of SFP. Therfore, it is in
58 demand to develop a novel extraction method to improve the yield and bioactivities of
59 SFP. Hydrogen peroxide/ascorbic acid has been reported to degrade effectively
60 polysaccharides to yield relatively low molecular weight polysaccharides, which

possess improved biological activities [13,14]. Ma et al.[15] also found that polysaccharides from blue honeysuckle had better biological activity after degradation by hydrogen peroxide/ascorbic acid. Moreover, hydrogen peroxide/ascorbic acid system has the advantages of high reaction efficiency, mild reaction conditions and ecological friendliness [16]. In addition, polysaccharides with low molecular weight might have better water solubility, which could be favor to improve the extraction yield. In order to develop a novel extraction method for SFP with improved yield and biological actities, herein hydrogen peroxide/ascorbic acid assisted extraction (HAE) is proposed and compared with other commonly used extraction methods based on yield and 1,1-diphenyl-2-picrylhydrazine (DPPH) radical scavenging ability. Furthermore, the SFP prepared by HAE method was purified, and the physico-chemical properties and the antioxidant activity of the SFP fractions were investigated.

2. Materials and methods

2.1. Materials

S. fusiforme was collected in May 2019 from Wenzhou Yuanpeng Aquatic Products Co. Ltd., Dongtou, Wenzhou, Zhejiang province (China). The algae were cleaned in fresh water and air-dried before being dried in an oven at 60℃. The dried algae were blended, sieved (100 mesh), and stored in a tightly sealed bottle. RAW264.7 macrophages were provided by College of Pharmaceutical Science, Zhejiang University. The kits used for the determination of ROS level, SOD activity and

protein content were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). All other chemicals and solvents were of analytically pure grade.

2.2. Extraction of SFPs by different methods

The extractions of SFPs were performed according to previously reported methods. For HWE, the dried *S. fusiforme* powder (20.0 g) was extracted with a 20-fold volume of distilled water at 90℃ for 3 h [13]. For ACAE, 20.0 g of *S. fusiforme* powder was mixed with 400 mL of 0.1 mol/L HCl solution, and extraction was performed at 80℃ for 3 h [10]. For ALAE, 20.0 g of *S. fusiforme* powder was extracted with 400 mL of 0.5 mol/L NaOH solution at 70℃ for 3 h [8]. For UAE, 20.0 g of *S. fusiforme* powder was mixed with 400 mL of water and then extracted using ultrasonic method at a power of 300 W for 30 min then the mixture was heated at 90℃ for 2.5 h [12]. For MAE, 20.0 g of *S. fusiforme* powder was dispersed in 400 mL of water and heated in a microwave experiment equipment at microwave power of 600 W for 30 min, then the mixture was heated at 90℃ for 2.5 h [8]. In the case of HAE, the suspension of 20.0 g *S. fusiforme* powder with 400 mL of water was heated at 90 ℃ for 2.5 h, hydrogen peroxide/ascorbic acid were introduced to the solution (both final concentration 17 mM) at 50℃ and the

96 resulting mixture was heated at 50℃ for 30 min. In all the six cases, the extraction 97 supernatant obtained by centrifugation (8000 rpm, 10 min) was concentrated to one98 quarter of the original volume under vacuum, and then mixed with 95% ethanol (1:5,

99 V/V), and allowed to stand at 4℃ for 12 h. The resulting precipitate was collected by

100 centrifugation at 8000 rpm for 10 min, redissolved in distilled water and deproteinated

101 with papain (0.1%, W/V) and Sevage reagent (n-butanol:chloroform = 1:4, V/V). The

102 resulting aqueous solution was dialyzed (Mw cut-off 3500 Da) against ultrapure water

103 for 72 h. Finally, the solution was concentrated and lyophilized, yielding the crude

104 polysaccharide (SFP). The SFPs obtained by HWE, ACAE, ALAE, UAE, MAE and

105 HAE were named as HW-SFP, AC-SFP, AL-SFP, U-SFP, M-SFP and H-SFP,

106 respectively. The extraction yield of SFPs was calculated according to the following

107 formula:

108 $$\text{Extraction yield (\%)} = \frac{\text{weight of crude polysaccharide (g)}}{\text{weight of } S.\ fusiforme \text{ powder (g)}} \times 100$$

109 2.3. Isolation of SFP

110 H-SFP solution (15 mL, 20 mg/mL) was separated using DEAE-52 cellulose

111 column chromatography (2.6 cm x 60 cm), eluting with deionized water and various

concentrations of NaCl (0.1, 0.3, 0.5, 0.7, and 0.9 mol/L) at a flow rate of 1.0 mL/min.

The eluates were collected in tubes of 10 mL each. The phenol-sulfuric acid method 114 [17] was used to determine the amount of SFPs in the eluates.

2.4. Determination of physicochemical properties of the SFPs

2.4.1. Chemical component and molecular weight analysis

The total sugar content was determined by the phenol-sulfuric acid method using glucose as a standard [17]. The protein, uronic acid and sulfate contents of the samples were analyzed using Bradford's method, turbidimetric method, and Bitter's method, respectively [18-20]. The monosaccharide composition of each component was determined using high performance anion exchange chromatography (HPAEC-PAD) [21]. The molecular weight of the polysaccharide samples was measured using high performance gel-filtration chromatography (HPGFC) [22].

2.4.2. UV, FT-IR and NMR analysis

The UV–Vis spectra of SFP solutions (1 mg/mL in ultrapure water) were recorded on a UV spectrophotometer (UV-2550, Shimadzu) scanning from 200 to 600 nm [21]. The FT-IR spectra of the samples were recorded on a Nicolet 380 infrared spectrometer using the KBr pellet method [23]. A Bruker Avance 400 spectrometer was used to record the ^{1}H NMR (20 mg, dissolved in 600 μL D_2O, 400 MHz) and ^{13}C NMR (40 mg, dissolved in 600 μL D_2O, 100 MHz) spectra of the samples [21].

2.4.3. Triple-helical conformation analysis

Congo red test was conducted to analyze the triple-helical conformation of polysaccharides according to a previously reported method [21].

2.4.4. The atomic force microscopic (AFM) analysis

AFM analysis of the samples was performed to investigate the surface conformation of the polysaccharides using a scanning probe microscope (Dimension

Icon; Bruker, Germany) referring to a reported method [21].

2.4.5. Scanning electron microscope (SEM) analysis

The surface morphology of lyophilized SFP powder was studied by a scanning electron microscope (SEM) (Nano Nova 450, FEI, USA) according to a reported method [21].

2.4.6. Measurement of rheological properties

The steady state shear and dynamic-viscoelastic analyses of the samples (10 mg/mL) were performed at 25 ± 0.1°C using a HR-2 rheometer (TA Discovery,USA) with a slit distance of 0.5 mm and a plate diameter of 40 mm [21]. The viscosity of SFP was determined using steady rate shear tests with shear rates ranging from 0.01 to 100 s^{-1}. The dynamic viscoelastic analysis was conducted by determining the change in the storage modulus (G′) and loss modulus (G″) of SFPs with an angular frequency range of 0.1–100 rad/s.

2.5. *In vitro* antioxidant activities

The DPPH• scavenging activity of SFPs was measured according to our previous report [24]. The Hydroxyl radical (•OH) scavenging activity was determined based on a previously reported method [13]. The $ABTS^{\bullet+}$ scavenging activity of SFPs was measured as described in a previous report [24].

2.6. Antioxidant assays in the oxidative stress RAW264.7 cells induced by H_2O_2

2.6.1. Cell culture

157 The RAW 264.7 macrophages were cultured in DMEM medium containing 10%
158 fetal bovine serum at 37°C in a 5% CO_2 incubator to exponential proliferation stage [2].

159 2.6.2. Cell viability assay

160 The cell viability was measured in 96-well plates by MTT assay referring to a
161 published method with minor modification [25]. RAW 264.7 cells (100 μL, 3×10^4
162 cells/mL) were seeded into each well of 96-well plate and incubated at 37°C in a 5%
163 CO_2 incubator for 24 h. The supernatant of cells was removed and replaced by a
164 complete medium containing various concentrations of sample (100 μL; 100, 200 and
165 400 μg/mL in DMEM medium), incubated at 37°C for 24 h, the medium was then
166 replaced by MTT (10 μL; 5 mg/mL) and incubated for 4 h at 37°C. After removal of
167 the culture media, the resulting insoluble formazan derivative was dissolved in DMSO
168 (100 μL), and the absorbance of the resulting solution was measured at 570 nm. All
169 treatments were performed in quintuplicate.

170 2.6.3. Protective effect assay

171 Prior to the treatment with SFPs, RAW264.7 cells were pre-incubated as described
172 in Section 2.6.1. Protective effect assay of polysaccharides was performed according to
173 a reported method [25]. A group of normal cells without H_2O_2 or polysaccharide
174 treatments was used as control group, and a group of H_2O_2-induced cells was used as
175 oxidative stress model. After incubation, supernatant removal, and washing with PBS,
176 the RAW264.7 cell was subsequently incubated for 24 h with polysaccharide sample

177 (100 μL, 100, 200 and 400 μg/mL). Apart from the control group, the cells in the other
178 groups were exposed to DMEM media containing H_2O_2 (100 μL, 400 μmol/L) for 1 h
179 at 37℃ and cell viability was determined using the MTT kit.

180 2.6.4. Determination of intracellular SOD activity

181 The SOD activity in the cells was determined according to previously reported
182 methods with slight modification [2, 26]. Cells (2mL, 1×10^5 cells/mL) was cultured in
183 6-well plates, the culture medium was removed after 24 h. The cells were treated with
184 sample solution (2 mL; 100, 200 and 400 μg/mL) at 37℃ for 24 h. After removing the
185 supernatant, the cells were incubated with H_2O_2 solution (2 mL, 400 μmol/L), and the
186 medium was removed after 1 h of treatment. After rinsing each well with pre-cooled
187 PBS, the lysis solution (150 μL) was added. The supernatant from the culture medium
188 was then collected for SOD activity assay. The activity of SOD in the cell lysate was
189 determined based on protein content, which was analyzed using BCA kits.

190 2.6.5. Analysis of intracellular ROS level

191 The ROS level in the cells was determined according to a literature [26].
192 RAW264.7 cells were seeded in fluorescent 96-well plates (100 μL, 3×10^4 cells/mL)
193 and cultured for 24 h. After removal of the medium, sample solutions (100 μL; 100,
194 200 and 400 μg/mL) were added. After 24 h incubation, the medium was removed, the
195 cells were washed with PBS, and then 2′, 7′-dichlorofluorescin diacetate (DCFH-DA)
196 probe (100 μL, 25 μmol/L) was added. After incubation for 1 h, the cell culture medium

was removed, H_2O_2 (100 μL, 400 μmol/L) was added and incubated for 1 h. The culture medium was removed and rinsed twice carefully with PBS. Then, the liquid in the wells was discarded, the cells were washed with PBS, and the fluorescence intensity of each well was measured by microplate spectrophotometer (excitation wavelength 485 nm, emission wavelength 525 nm).

2.7. Statistical analysis

Data were expressed as mean ± standard deviation (SD). Origin 2018 64Bit software was used to analyse the experimental results. The statistical significance was analysed by one-way analysis of variance (ANOVA) and Tukey's test with SPSS21.0 software [10]. $P<0.05$ was considered statistically significant.

3. Results and discussion

3.1. Screening of extraction method of SFP

As shown in Fig. 1a, the yields of SFPs varied significantly with the extraction method ($P < 0.05$). Among the six extraction methods, HAE had the highest yield of SFP, being 11.43 ± 0.21%, followed by UAE (9.21 ± 0.24%), MAE (8.78 ± 0.31%), ACAE (8.09 ± 0.19%) and HWE (7.34 ± 0.13%). ALAE had the lowest SFPs yield of 6.55 ± 0.16%. In general, plant cell walls are complex and tough, and may not be readily disrupted by the hot water treatment, resulting in a low yield [27]. However, physical or chemical approaches can significantly increase the extraction rate of polysaccharides. For instance, the cavitation effect of UAE accelerates the cell wall disintegration and

enhances polysaccharide dissolution and diffusion from cells. In the case of HAE, ascorbic acid, as a reducing agent, is prone to react with hydrogen peroxide (a strong oxidant) to generate hydroxyl radical (HO•) in the hydrogen peroxide/ascorbic acid system [15,28]. HO• is very reactive and reacts with the hydrogen atoms of the polysaccharides, resulting in the rupture of the glycosidic bond [29], and consequently leading to the degradation of polysaccharides, which increases the solubility and yield of SFPs; in addition, HO• could also destroy cell wall of the algae, which facilitates the release of polysaccharides. The low yield of ALAE is possibly due to the overdegradation of polysaccharides [8].

As illustrated in Fig. 1b, the SFPs prepared by the six methods all exhibited marked DPPH• scavenging ability. The IC_{50} value of HW-SFP, AC-SFP, AL-SFP, USFP, M-SFP and H-SFP for DPPH• scavenging were 1.87 ± 0.01, 3.43 ± 0.08, 0.74 ± 0.02 , 1.15 ± 0.04, 1.33 ± 0.04 and 0.85 ± 0.04 mg/mL, respectively. The DPPH• scavenging activity of all the six polysaccharides was significantly weaker than Vitamin C (V_C). At a concentration of 5 mg/mL, the scavenging rates of H-SFP, AL-SFP, U-SFP, M-SFP, HW-SFP and AC-SFP were 96.58 ± 1.07% , 92.80 ± 1.27%, 91.57 ± 1.17%, 88.65 ± 0.94%, 78.21 ± 1.55% and 62.57 ± 1.76%, respectively. These results indicate that the extraction method significantly affects the antioxidant activity and yield of polysaccharides. Among them, H-SFP shows higher yield and better antioxidant activity. Therefore, H-SFP was selected for the subsequent investigation.

3.2. Isolation of SFPs

The elution curve of H-SFP on cellulose DEAE-52 is illustrated in Fig. 2. Due to the low polysaccharide content in the deionized water, 0.1, 0.7 and 0.9 mol/L NaCl eluents, they were not collected for further investigation. The two fractions eluted with 0.3 and 0.5 mol/L NaCl, named as H-SFP3 and H-SFP5, were obtained with a yield of 29.44 ± 0.37 % and 17.16 ± 0.43%, respectively.

3.3. Physicochemical properties

3.3.1. Chemical component

As presented in Table 1, the total sugar contents of H-SFP, H-SFP3 and H-SFP5 were 44.52 ± 1.98%, 42.86 ± 1.38% and 46.59 ± 1.59%, respectively, which were close to the results of previous studies [29]. All of these polysaccharide fractions contained low content of protein. Both the sulfate and uronic acid contents followed the order: HSFP > H-SFP5 > H-SFP3. The molecular weights of the these polysaccharides were measured using HPGFC (Fig. S1). The weight average molecular weight (Mw) of HSFP, H-SFP3 and H-SFP5 were determined to be 51.8, 23.7 and 58.5 kDa, respectively, with a polydispersity index (PDI) value of 3.84, 2.39 and 2.48. The monosaccharide composition analysis showed that the SFPs were mainly composed of fucose (Fuc), mannuronic acid (ManA), guluronic acid (GulA), galactose (Gal) and xylose (Xyl), together with a small amount of mannose (Man), glucuronic acid (GlcA)

and Glucose (Glc) (Fig. S2, Table 1). This finding basically agrees with the previous studies [8].

3.3.2. UV spectra, FT-IR and NMR analysis

There is a weak absorption peak at 280 nm in the UV–Vis spectra of H-SFP, HSFP3 and H-SFP5 (Fig. S3), indicating the presence of small amount of protein. This result is consistent with that of the protein content measurement (Table 1).

As shown in Fig. 3, the IR spectra of the three polysaccharide samples are very similar, indicating that the functional groups on the sugar chains are essentially the same.

A strong absorption peak appearing at around 3400 cm^{-1} is due to the stretching vibration of O–H in the sugar molecule [30]. The peak at 2930 cm^{-1} is assigned to the stretching vibration of saturated C-H [31]. The absorption peaks at 1613 cm^{-1} and 1425 cm^{-1} are assigned to the asymmetrical and symmetrical stretching vibrations of $-COO^-$, respectively, confirming the presence of uronic acid in polysaccharides [1]. The peak at 1255 cm^{-1} is attributed to the O=S=O stretching vibrations, confirming the presence of the sulphate group. The peak at 1035 cm^{-1} is the characteristic band of the polysaccharide, caused by the stretching vibration of the C-O-C in the pyranose ring [32]. The absorption peaks at 819 cm^{-1} and 890 cm^{-1} are attributed to α-type and β-type glycosidic bonds, respectively [33].

Both ^{1}H NMR and ^{13}C NMR spectra can be used to analyze the configuration of glycosidic bonds in polysaccharides. In general, chemical shifts of anomeric proton in α-pyranose appear at δ 4.95-5.5 ppm, while those in β-pyranose are at δ 4.5-4.95 ppm [14]. The chemical shifts at δ 90–102 and 103–110 ppm are assigned to the anomeric carbons in α- and β-pyranoses, respectively [34]. As shown in Fig. S4, H-SFP3 and HSFP5 contain both α-type and β- type glycoside bonds. This result agrees with that of FT-IR analysis. The peak at δ 5.27 ppm corresponds to the H-1 of α-L-Fuc*p* [35]. The peak at δ 1.15 is attributed to H-6 of →3)-α-L-Fuc*p*4OAc-(1→ [35]. The peaks at δ 3.79, 4.55 and 4.96 ppm can be attributed to the H-4 of β-D-Xyl, H-1 of β-D-ManA*p* and H-1 of α-L-GulA*p*, respectively [35,36]. The peak at δ 4.45 was correlated with H5 of guluronic acid [37]. The signals at chemical shifts δ 98-102 and 103-110 ppm in the ^{13}C NMR spectra of H-SFP3 and H-SFP5 (Fig. S5) indicate the presence of both α and β-glycosidic configurations in these two polysaccharides [14]. The peak at δ 69.96 ppm is attributed to C-6 of 3,6)-α-D-Man*p*(1→ [34]. The signal at δ 103.51 ppm (C1) indicates the presence of α-L-Fuc [35]. At low field, the carboxyl signal of uronic acid appears between 173 and 176 ppm [2,37].

3.3.3. Scanning electron microscopic imaging

The SEM analysis of sample was performed at ×100 and ×10,000 magnification. As shown in Fig. 4, there are a large number of lamellae and pore-like network columns on the surface of H-SFP, indicating that the strong attractions between the functional

groups exposed on the polysaccharide surface aggregate the polysaccharide chains [38]. The surfaces of H-SFP3 and H-SFP5 were relatively smooth, with less pores and lamellae. H-SFP3 presented a flat surface, while H-SFP5 showed a dense floc layered structure. The difference in the morphology of three polysaccharide samples could be attributed to the difference in their polysaccharide chains and electrical charges that cause the different entanglement and aggregation of polysaccharides [39].

3.3.4. AFM analysis

As shown in Fig. 5, H-SFP3 possessed smaller size and more uniform structure than H-SFP and H-SFP5, which could be related to its lower molecular weight. H-SFP and H-SFP5 possessed both linear structure and irregular spherical aggregates, indicating the branching, entanglement and aggregation of the polysaccharides, which is probably due to the strong intermolecular and intramolecular hydrogen bonds formed by hydroxyl groups on polysaccharide molecules [40].

3.3.5. Triple-helical conformation analysis

As can be seen in Fig. S6, compared with Congo red solution, λ_{max} of all the Congo red-SFPs complexes exhibited no significant shift, indicating the absence of triplehelical conformation in these SFPs [14]. This result agrees with that of Zheng et al. [41].

3.3.6. Rheological properties

As shown in Fig. 6a, increasing the shear rate (0.1 – 100 s^{-1}) reduced the apparent viscosity of the SFPs and caused shear thinning, indicating that the SFPs were shear thinned pseudoplastic fluids [42]. When the shear rate increased to 1 s^{-1}, the viscosity of the sample solutions was basically stable. Several other seaweed polysaccharides have revealed similar results [43]. The cause of this phenomenon may be attributed to the increase in the space between molecules, the decrease in the entanglement of molecular chains and the decrease in the viscosity of the solution at a higher shear rate [21]. As demonstrated in Fig.6 (b-d), with the increase of the angular frequency (0.1100 rad/s), the energy storage modulus (G') and loss modulus (G") also increasd, but the increasing rate of G' of all the three polysaccharides was larger than G", intersecting at about 1.0 rad/s. The intersection of G" and G' implies that these polysaccharides have good viscoelasticity. The larger the contribution of elasticity, the lower the intersection point value [21]. This may be due to the enhanced intermolecular and intramolecular interactions and the formation of entanglement networks in SFPs solutions [21,43].

3.4. *In vitro* antioxidant activities

3.4.1. DPPH• scavenging ability

As presented in Fig. 7a, the DPPH• scavenging effects of H-SFP, H-SFP3 and HSFP5 exhibited a concentration-dependent manner at a range of 0.5–5 mg/mL. At 4 mg/mL, the DPPH• scavenging effect of H-SFP (95.35 ± 0.01%) was found to be close to that of Vc (P >0.05), being significantly stronger than that of H-SFP3 (69.28 ± 0.01%)

and H-SFP5 (69.33 ± 0.01%) (p<0.05), which could be due to its higher sulfate content [13]. In general, H-SFP showed stronger DPPH• scavenging activity than the two separated fractions. This result is consistent with previous reports by Yuan et al. [44] and Wang et al. [24], who reported that crude polysaccharides exhibited relatively high DPPH• scavenging activity compared with purified fractions. The IC_{50} values of H-SFP, H-SFP3, and H-SFP5 were 0.88 ± 0.02, 2.15 ± 0.07 and 2.14 ± 0.04 mg/mL, respectively. The similar DPPH• scavenging activity of H-SFP3 and H-SFP5 could be attributed to the two opposite affecting factors, namely sulfate content and molecular weight. Although the sulfate content of H-SFP3 is lower than H-SFP5, it possesses lower molecular weight. It was reported that the IC_{50} value of SFPs prepared by cellulase-assisted extraction for DPPH• scavenging was 0.81 ± 0.02 mg/mL [45], which was close to that of H-SFP in this work.

3.4.2. Hydroxyl radical scavenging ability

The hydroxyl radical (•OH) is one of the most dangerous ROS and can cause injury to the body by damaging biomacromolecules [46]. All the three samples were found to possess potent •OH scavenging capacity (Fig. 7b). The IC_{50} values of H-SFP, H-SFP3 and H-SFP5 were found as 2.03 ± 0.02, 2.22 ± 0.04, and 2.45 ± 0.04 mg/mL, respectively, which are superior to the SFP (6.82 mg/mL) and ESFP (4.78 mg/mL) reported by Qian et al. [29]. Uronic acid content is an important factor affecting the

352 •OH scavenging capacity of polysaccharides due to its metal chelating ability [6,47]. At
353 5mg/mL, the three components were found to possess similar •OH scavenging ability,
354 which might be due to their similar content of uronic acid.

355 3.4.3. $ABTS^{\bullet+}$ radical scavenging ability

356 The ABTS approach has been widely used to evaluate the total antioxidant
357 capacity of various active ingredients [48]. The $ABTS^{\bullet+}$ scavenging activity of H-SFP
358 was significantly stronger than that of H-SFP3 and H-SFP5 at concentrations of >2
359 mg/mL (p<0.05) (Fig. 7c). At 5 mg/mL, the $ABTS^{+\bullet}$ scavenging rate of H-SFP, H-SFP3
360 and H-SFP5 were 64.78 %, 39.66% and 38.85%, respectively. The IC_{50} values of HSFP,
361 H-SFP3 and H-SFP5 were found to be 3.37 ± 0.06, 10.65 ± 0.08, and 12.08 ± 0.08
362 mg/mL, respectively. In this study, the $ABTS^{+\bullet}$ scavenging activity of H-SFP was higher
363 than that of the two purified fractions, and the activity of H-SFP3 was closed to that of
364 H-SFP5, which was consistent with the results of the DPPH test. Thus, it is assumed
365 that the sulfate content and molecular weight of the polysaccharides play an important
366 role in their $ABTS^{+\bullet}$ scavenging activity.

367 3.5. Antioxidant effect of SFPs in H_2O_2-treated RAW264.7 macrophages

368 3.5.1. Effect of SFPs on cell proliferation

369 As shown in Fig. 8a, the viability of RAW 264.7 cells treated with the three
370 polysaccharides at different concentrations (100, 200 and 400 μg/mL), remained
371 above 100% after incubated for 24 h, indicating that the three polysaccharides were

372 non-toxic to RAW 264.7 cells. Particularly, H-SFP (200 μg/mL) and H-SFP3 (200
373 and 400 μg/mL) significantly promoted the cell proliferation (P<0.05). Therefore, the
374 polysaccharide concentrations of 100, 200 and 400 μg/mL were selected for further
375 study. Similarly, Jiang et al. [6] found that SFP could promote the proliferation of
376 RAW264.7 cells at a concentration of 10-1000 μg/mL.

377 3.5.2. Protective effects on H_2O_2-induced oxidative stress

378 The protective effect of SFPs on the H_2O_2-induced injured RAW 264.7 cells are
379 presented in Fig. 8b. Compared with the control group, the cell survival rate of the
380 model group fell in the range of 50-70% (53.14 ± 2.14%), indicating that the cellular
381 oxidative stress modeling was successful [24]. Compared with the model group, the cell
382 viability of SFP treatment groups was improved significantly, indicating that
383 polysaccharides had a considerable protective effect on H_2O_2-induced oxidative stress
384 of RAW264 cells. This result is similar to a recent report that phosphorylated
385 Cyclocarya paliurus polysaccharide (P-CP) has a good protective effect on
386 H_2O_2induced oxidative stress in RAW264.7 cells in a concentration-dependent manner
387 (25100 μg/mL) [1]. In the range of 100-400 μg/mL, H-SFP3 exhibited better protection
388 than H-SFP and H-SFP5 (P<0.05). At 400 μg/mL, the cell survival rate of H-SFP,
389 HSFP3 and H-SFP5 treatment groups increased by 34.30%, 54.70% and 32.33%,
390 respectively, compared with the model group. The superior protection effect of H-SFP3
391 on the celluar oxidative stress is possibly due to its relatively low molecular weight.

3.5.3. Effect on SOD activity

The intracellular antioxidant enzyme SOD can catalyze the disproportionation of superoxide anion radicals to generate H_2O_2 and oxygen, thus plays an important role in cell protection in response to oxidative stress [2]. As displayed in Fig. 8c, the SOD activity in the model cells was significantly decreased compared with the normal group ($P < 0.05$). All the three polysaccharides improved SOD activity in a dose-dependent manner. At 400 μg/mL, the SOD activity of H-SFP, H-SFP3 and H-SFP5 groups were determined to be 13.71 ± 0.78, 14.47 ± 0.96 and 13.43 ± 1.01 U/mg prot, respectively, increasing by 132.77%, 145.67% and 128.01% when compared to the model group. Among these polysaccharides, H-SFP3 exhibited the best effectiveness in the improvement of SOD activity.

3.5.4. Effect on ROS level

As presented in Fig. 8d, the fluorescence intensity of the model group was significantly higher ($P < 0.05$) than that of the normal group, indicating that cells in the model group were in a state of oxidative damage. Such increase in intracellular ROS levels was effectively alleviated by the treatment of SFPs. Among them, H-SFP3 showed the strongest activity in reducing intracellular ROS level. In the presence of HSFP3 (400 μg/mL), the ROS level in the cells was reduced by 34.3% compare with the model group, being 110.8% of that of normal cells. This result is consistent with the above SOD activity assay.

Different from the results of free radical scavenging tests, H-SFP3 showed the best antioxidant activity in the H_2O_2-treated RAW264.7 cells. It has been reported that the polysaccharides with relatively large molecular weight are difficult to span the cell membrane, consequently exerting the weak biological effects [15,49]. A previous study has found that high molecular weight of Holothurian glycosaminoglycan (HG) may have adverse effects on platelet aggregation due to its high viscosity and low cellular permeability, while the low molecular weight HG obtained by physical or chemical method can improve this situation [50]. Thus, the superior antioxidant activity of HSFP3 in the H_2O_2-treated RAW264.7 cells could be attributed to its relatively low molecular weight, which might allow it penetrate the cell membrane more readily. Indeed, many studies have found that molecular weight of polysaccharides has a significant impact on their antioxidant activity [5,29]. In general, polysaccharides with low molecular weight have higher antioxidant activity due to the exposure of more functional groups that can react adequately with free radicals [5,13,15,51]. For instance, low-molecular weight pectin polysaccharides (LMPs) showed improved bioavailability when compared to the pectin polysaccharides with high molecular weight [16]. The degraded polysaccharides have been reported to possess strong free radical scavenging and reducing power due to their lower molecular weight and more reducing and nonreducing ends [17,52].

4. Conclusion

A novel method for the preparation of polysaccharide from *S. fusiforme* with improved yield and bioactivity, namely hydrogen peroxide/ascorbic acid-assisted extraction (HAE) was developed. The polysaccharide prepared by HAE (H-SFP) and its two purified fractions (H-SFP3 and H-SFP5) were all heteropolysaccharides, being mainly comprised of fucose, mannuronic acid, guluronic acid, galactose and xylose, together with a small amount of mannose, glucuronic acid and glucose. All the three polysaccharides exhibited considerable antioxidant activity. H-SFP was found to possess the most potent radical scavenging activity among these polysaccharides (HSFP and its two isolated fractions H-SFP3 and H-SFP5), which could be attributed to its highest sulfate content. In the oxidative stress RAW264.7 cells induced by H_2O_2, HSFP3 was found to possess the superior protection against cellular oxidative damage, and better ability to improve intracellular SOD activity and reduce ROS levels, which could be due to its relatively low molecular weight. These findings also provide scientific basis for using SFP as ingredients in health foods and pharmaceutical products. However, the antioxidant assays *in vivo* and investigation on the antioxidant mechanism are still required in the due course.

CRediT author statement

Cheng Wan: Investigation, Writing-Original draft preparation.

Hui Jiang: Methodology, Investigation.

Meng-Ting Tang: Formal analysis, Visualization.

Shaobo Zhou：Supervision.

Tao Zhou: Conceptualization, Supervision, Funding acquisition, Writing - Review & Editing.

Conflicts of Interest

The authors declare no conflict of interest.

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Tables

Table 1. Chemical composition of H-SFP, H-SFP3 and H-SFP5.

Sample	H-SFP	H-SFP3	H-SFP5
Total sugar content (%)	44.52 ± 1.98	42.86 ± 1.38	46.59 ± 1.59
Protein content (%)	1.15 ± 0.04	0.88 ± 0.01	1.34 ± 0.05
Sulfate content (%)	7.91 ± 0.10	3.14 ± 0.13	4.55 ± 0.06
Uronic acid content (%)	23.26 ± 1.16	20.45 ± 1.28	22.8 ± 0.72
Weight average molecular weight (Mw)	51.8	23.7	58.5
Number average molecular weight (Mn)	13.5	9.9	23.6
Polydispersity index (PDI) (Mw/ Mn)	3.84	2.39	2.48
Monosaccharide composition (mol%)			
Fuc	25.9	14.8	31.4
Gal	15.2	5.2	18.3
Glc	1.4	1.0	-
Xyl	7.7	7.1	19.1
Man	2.5	1.1	0.7
GulA	16.1	31.3	13.3
GlcA	2.3	1.6	1.1
ManA	29.0	37.9	16.1

Note: Fuc, Gal, Glc, Xyl, Man, GulA, GlcA and ManA represent fucose, galactose, glucose, xylose, mannose, guluronic acid, glucuronic acid and mannuronic acid

644 respectively. “-”means not detected.

645

Figure captions

Fig. 1. Screening different preparation methods of SFPs. (a) Yield of SFPs; (b) Scavenging activity of DPPH radical by different extraction methods. The different lowercase letters indicate the significant difference between different groups ($P < 0.05$). Hot water extraction (HWE), Acid-assisted extraction (ACAE), Alkali-assisted extraction (ALAE), Ultrasonic-assisted extraction (UAE), Microwave-assisted extraction (MAE) and hydrogen peroxide/ascorbic acid-assisted (HAE); SFP obtained by HWE, ACAE, ALAE, UAE, MAE and HAE denoted HW-SFP, AC-SFP, AL-SFP, U-SFP, M-SFP and HAE, respectively.

Fig. 2. The elution curve of SFP on DEAE-52 chromatography column.

Fig. 3. FT-IR spectra of H-SFP, H-SFP3 and H-SFP5.

Fig. 4. SEM imaging of H-SFP, H-SFP3 and H-SFP5.

Fig. 5. Atomic force microscope images of H-SFP, H-SFP3 and H-SFP5.

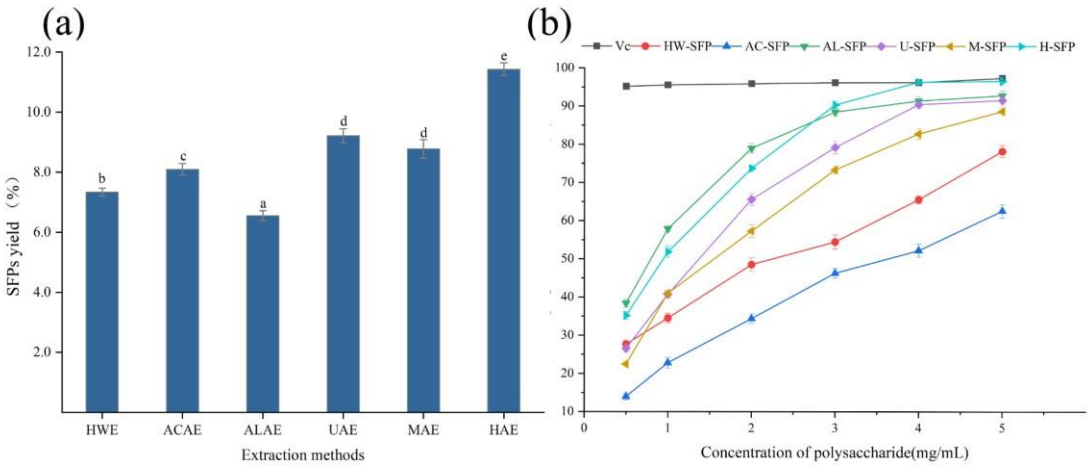
Fig. 6. Rheological properties of H-SFP, H-SFP3 and H-SFP5. (a) Apparent viscosity; (b-d) Dynamic-viscoelastic properties.

Fig. 7. Radical scavenging ability of H-SFP, H-SFP3 and H-SFP5. (A) DPPH radical; (B) Hydroxyl radical; (C) $ABTS^{\bullet+}$. The different lowercase letters indicate the significant difference between the results of different polysaccharide at the same concentration ($P < 0.05$). The different capital letters indicate the significant difference between the results of the same polysaccharide at different concentrations ($P < 0.05$).

Fig. 8. Antioxidant activity of SFPs in RAW 264.7 cell model. (a) Cytotoxicity; (b) Protective effect on H_2O_2-induced oxidative injury of RAW 264.7 cells; (c) Effect on cellular SOD activity; (d) Effect on cellular ROS level. The different lowercase letters indicate the significant difference between the results of different polysaccharide at the same concentration ($P < 0.05$). The different capital letters indicate the significant difference between the results of the same polysaccharide at different concentrations ($P < 0.05$). #$p < 0.05$ vs control; *$p < 0.05$, vs Model.

674 **Fig. 1**

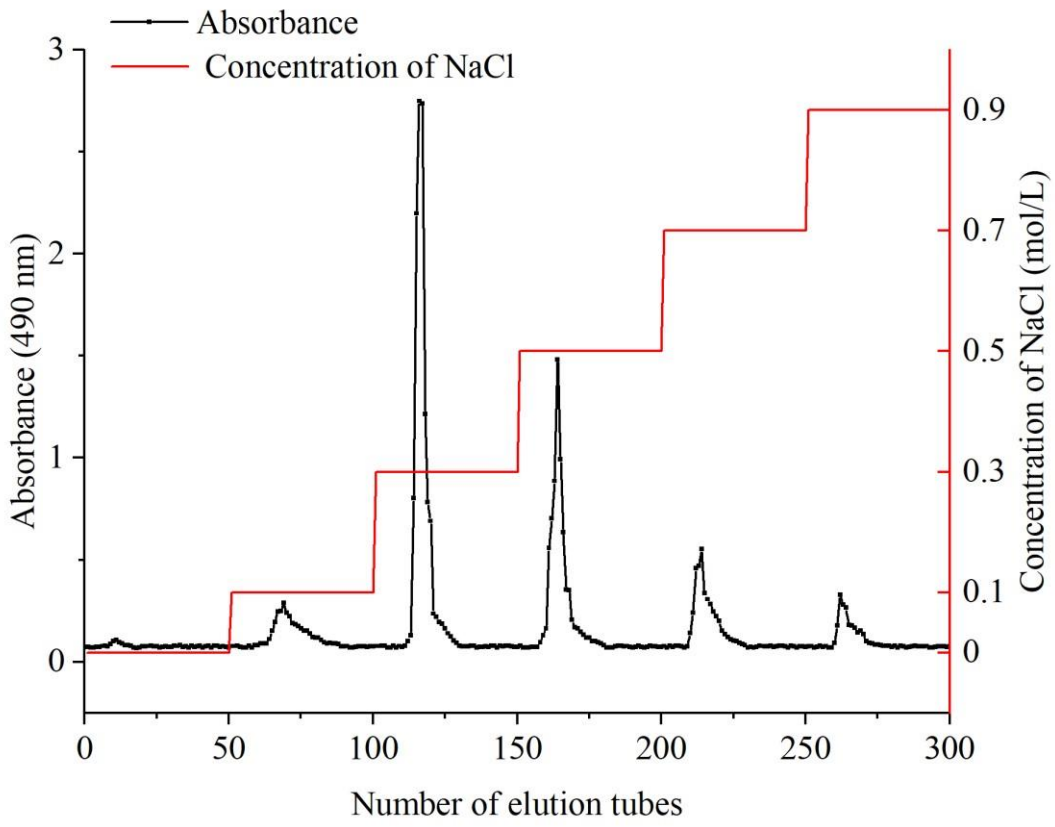
675



676

677 **Fig. 2**

678



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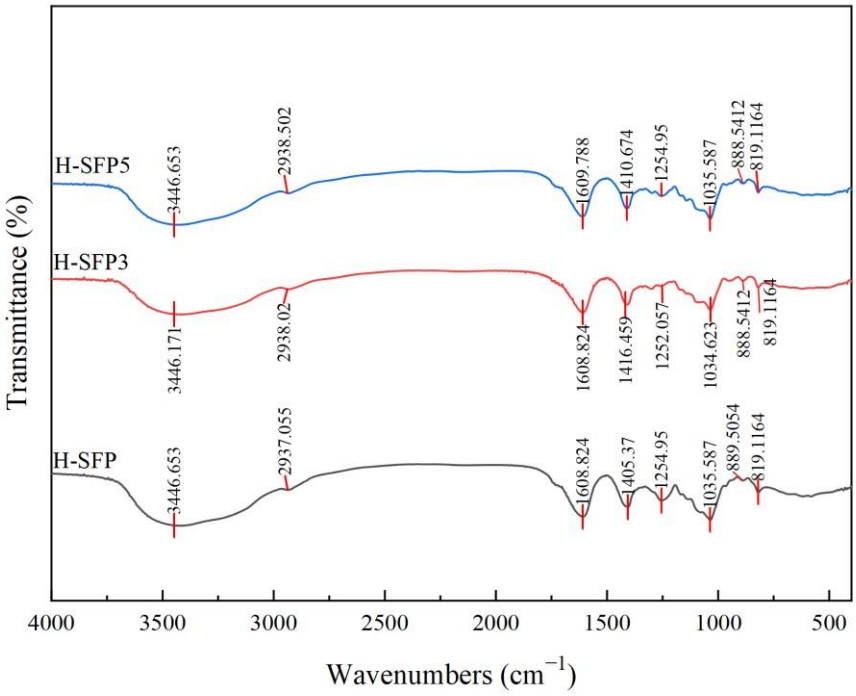
682 **Fig. 3**

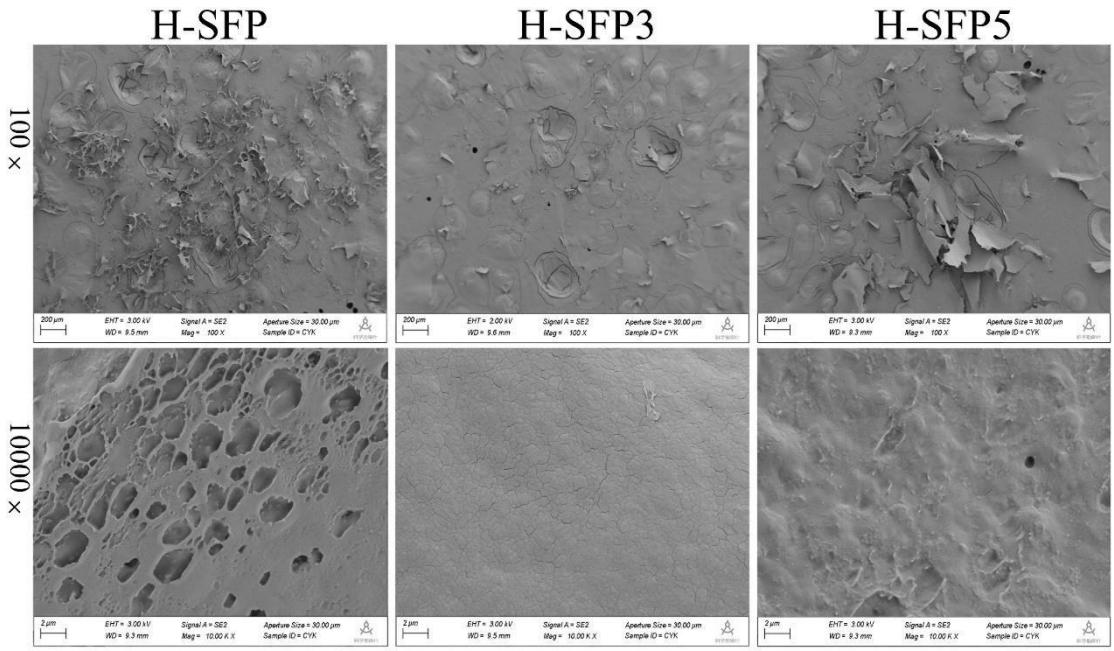
683



684 685

Fig. 4

686



687

688 **Fig. 5.**

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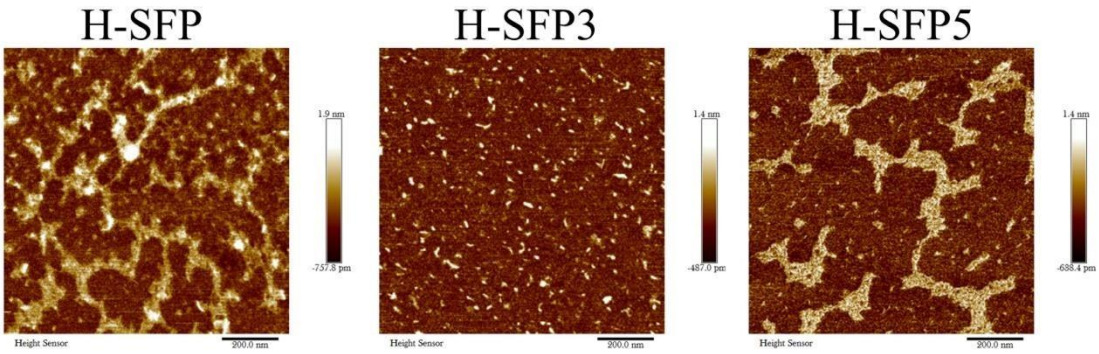

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692 **Fig. 6.**

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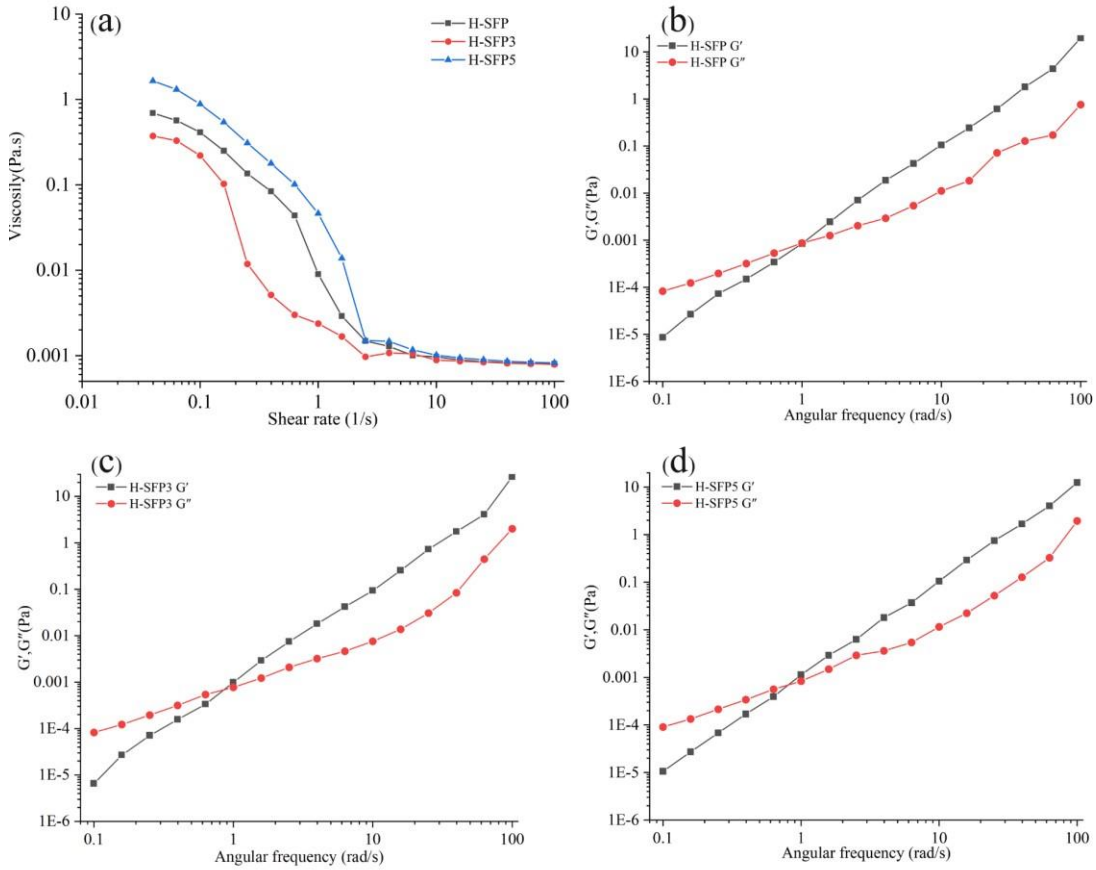

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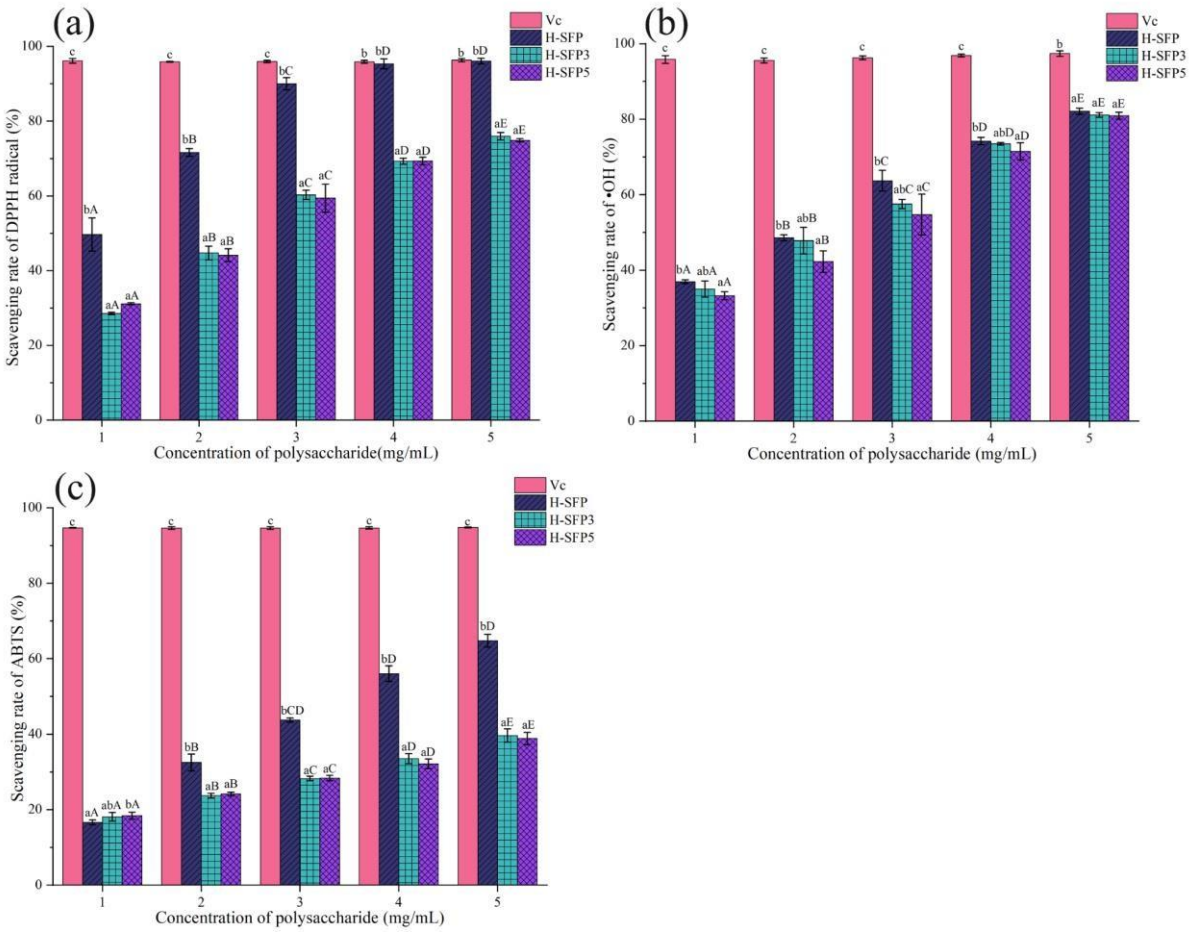
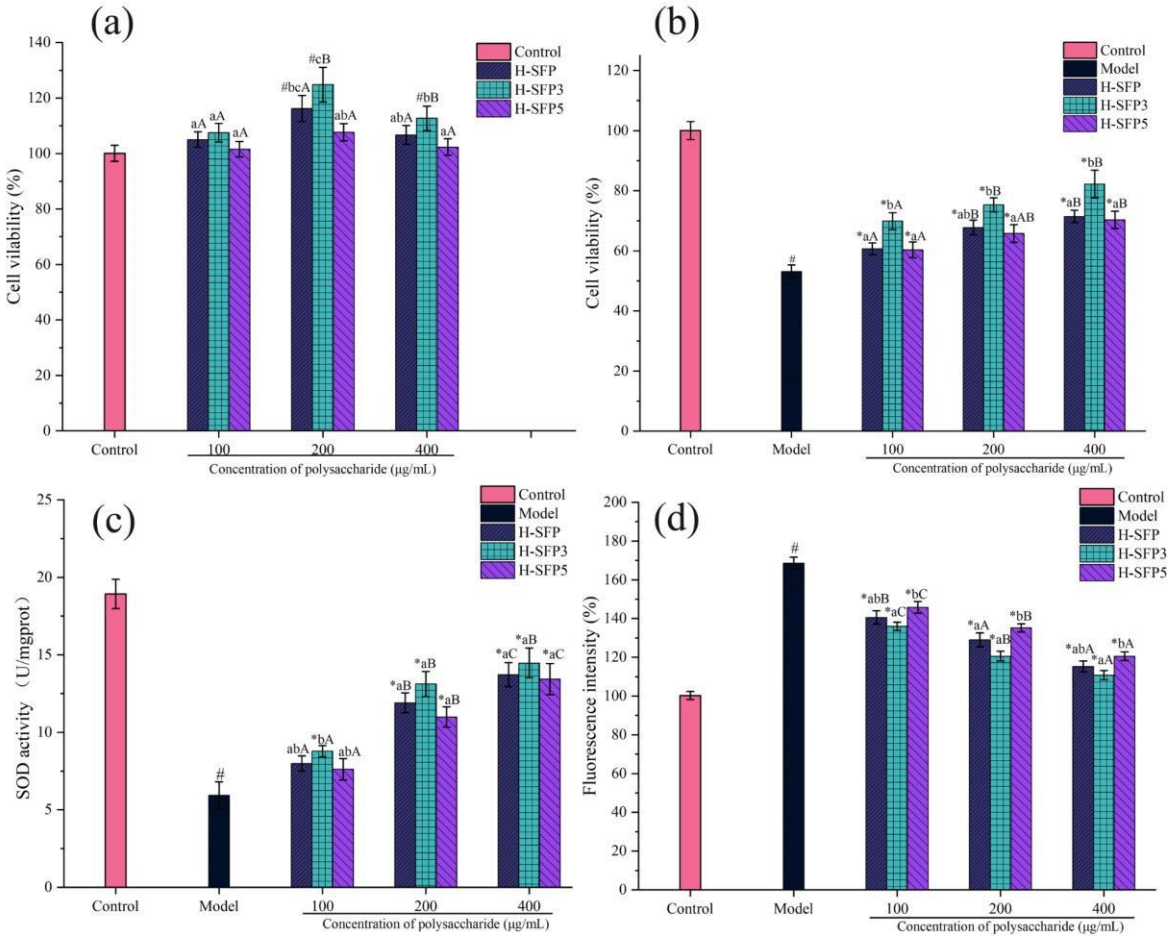
Fig. 7.


(a)
Scavenging rate of DPPH radical (%)
Concentration of polysaccharide(mg/mL)
Vc
H-SFP
H-SFP3
H-SFP5
(b)
Scavenging rate of •OH (%)
Concentration of polysaccharide (mg/mL)
(c)
Scavenging rate of ABTS (%)
Concentration of polysaccharide (mg/mL)

707

708 **Fig. 8.**



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Supporting Information

Purification, physico-chemical properties and antioxidant activity of polysaccharides from *Sargassum fusiforme* by hydrogen peroxide/ascorbic acid-assisted extraction

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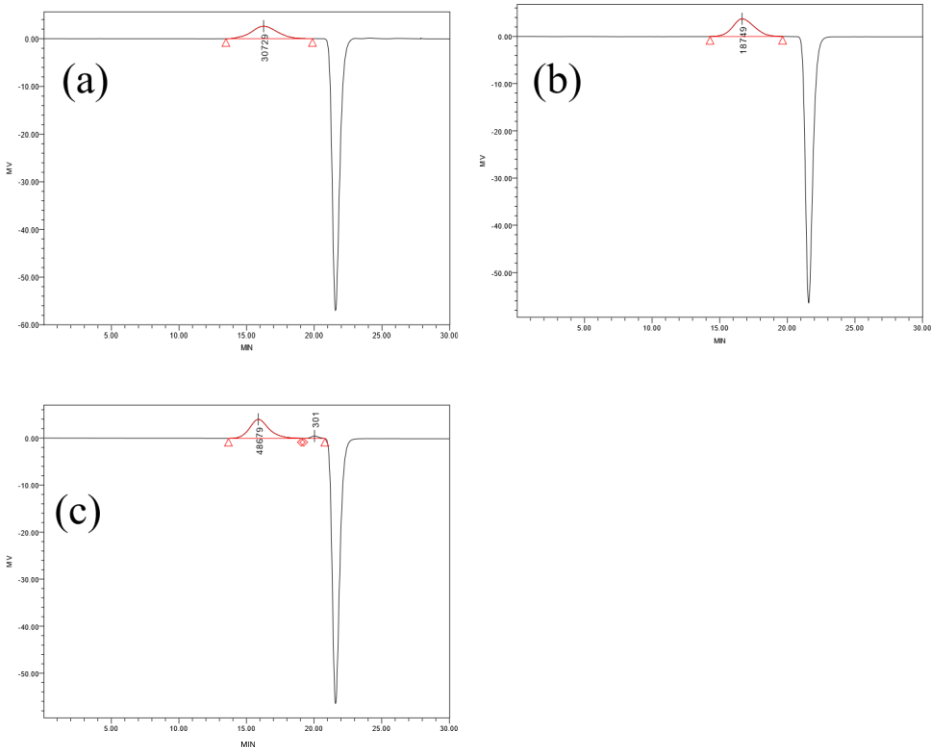

Fig. S1. HPGFC chromatograms of H-SFP (a), H-SFP3 (b) and H-SFP5 (c).

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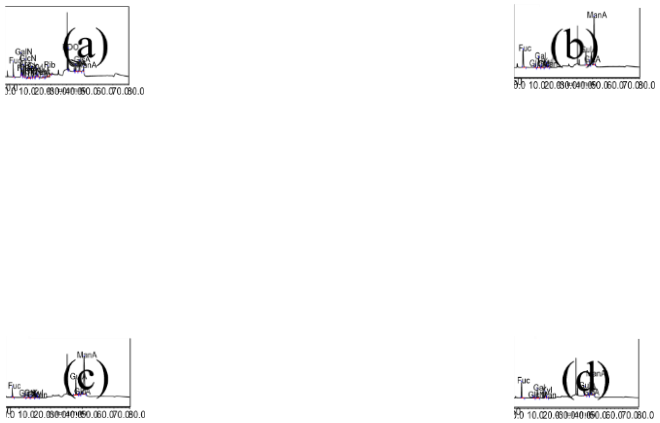

Fig. S2. HPAEC-PAD chromatograms of standards (a), H-SFP (b), H-SFP3 (c) and H-SFP5(d).

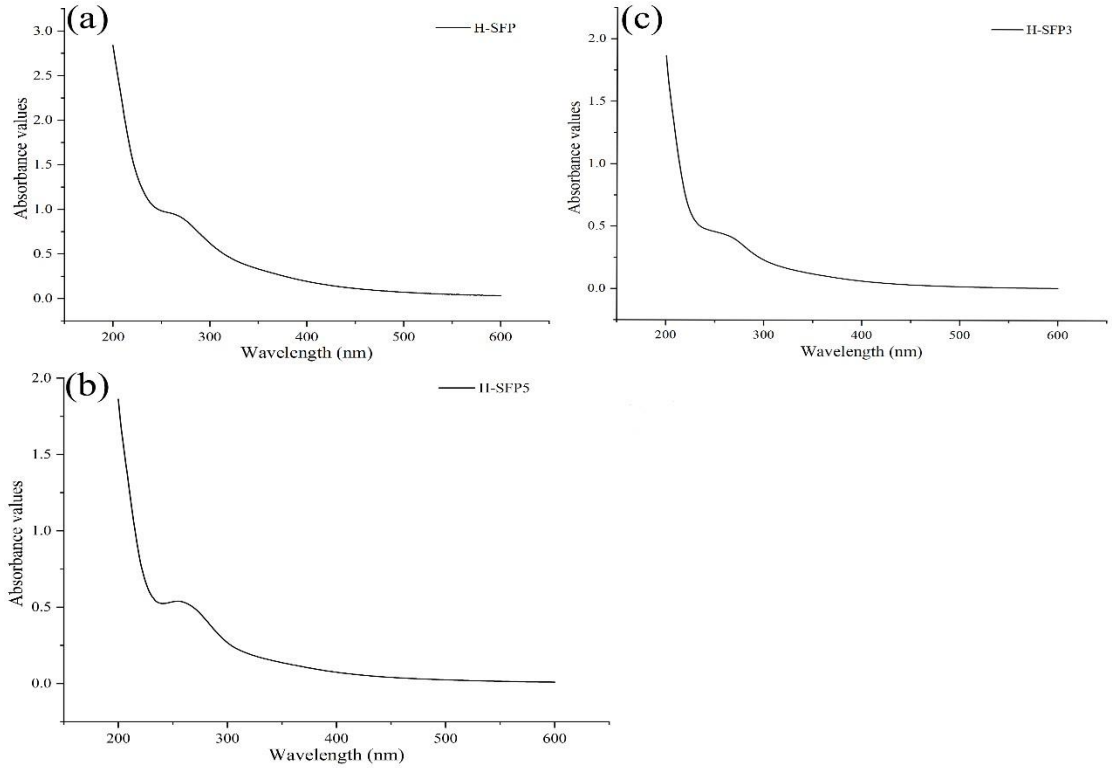

Fig. S3. UV-Vis spectra of SFPs. (a) H-SFP; (b) H-SFP3; (c) H-SFP5.

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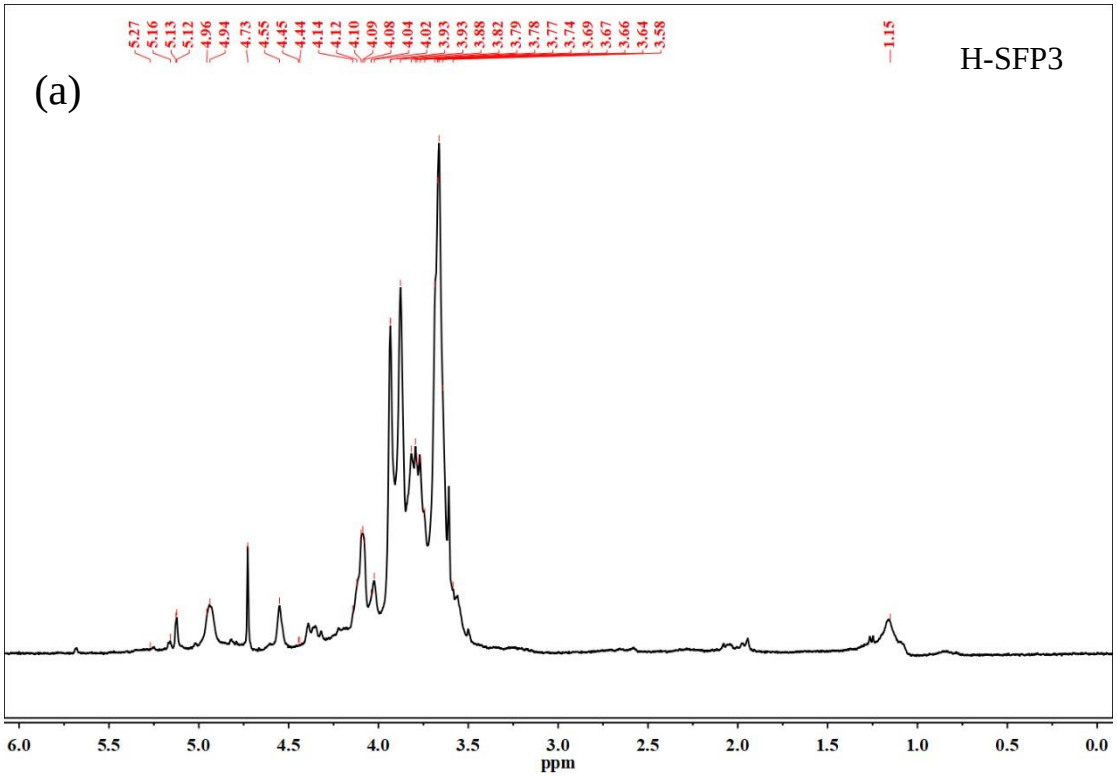

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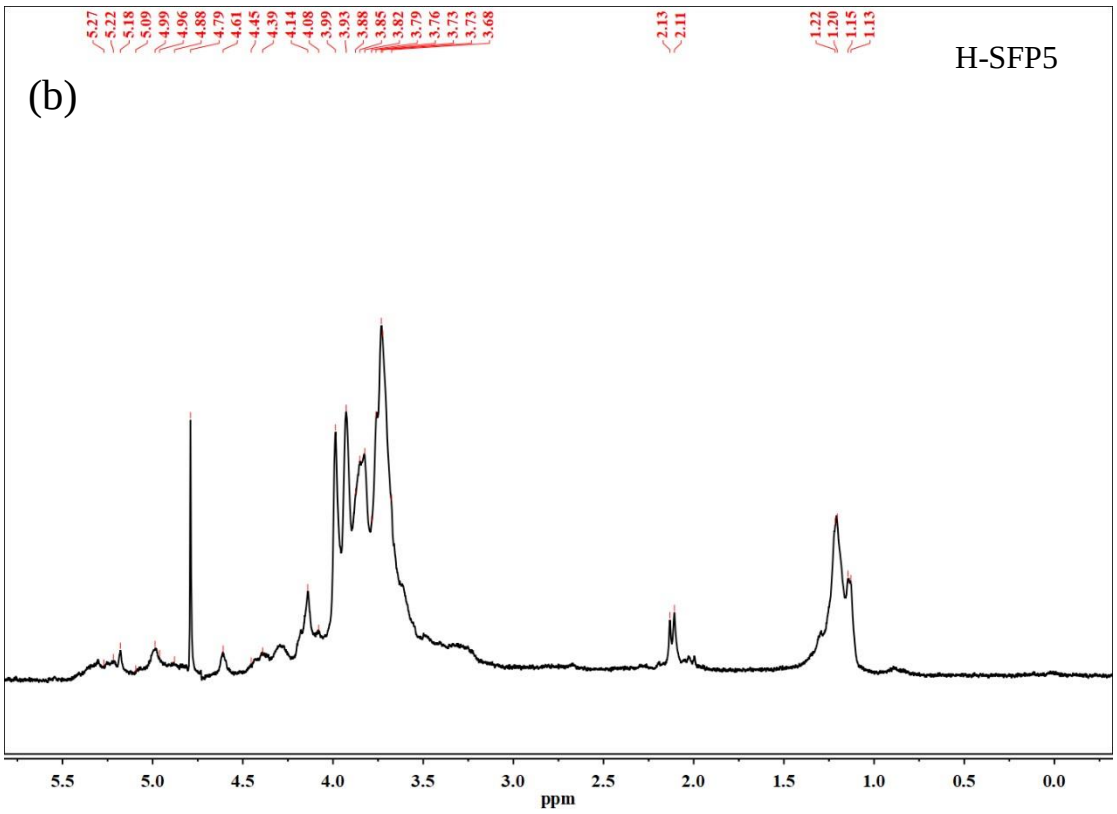

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768 **Fig. S4.** ^{1}H NMR spectra of H-SFP3 (a) and H-SFP5 (b)

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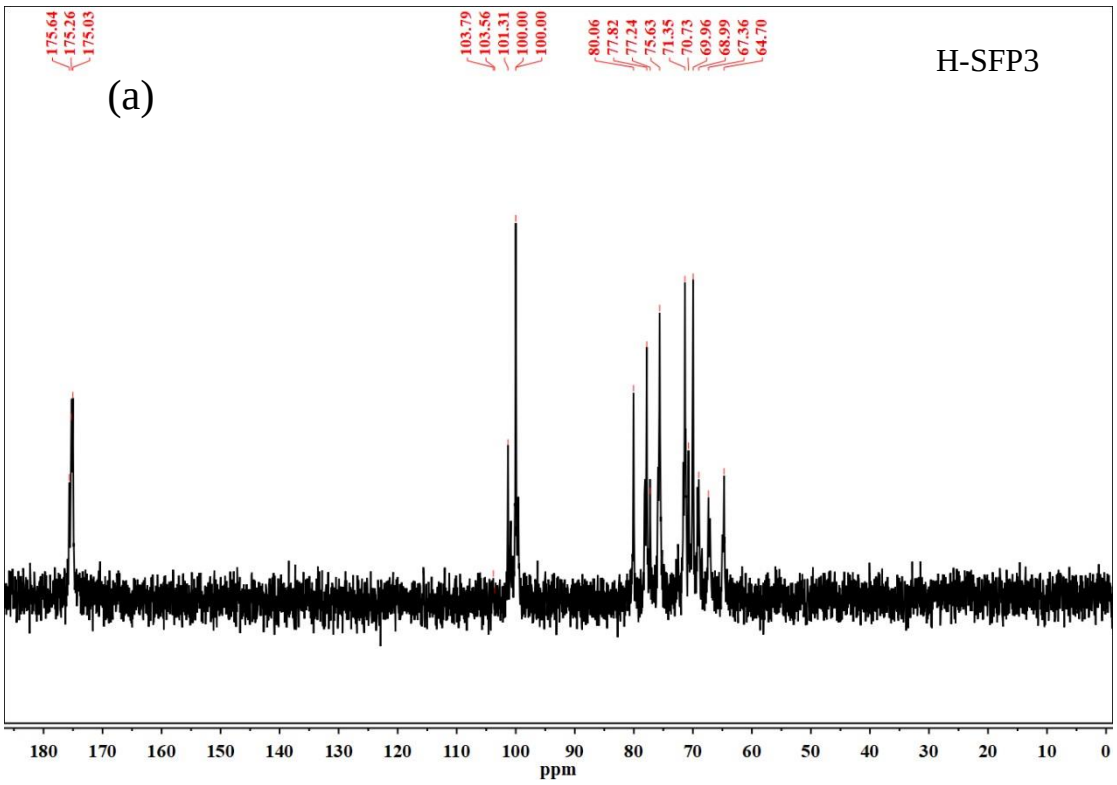

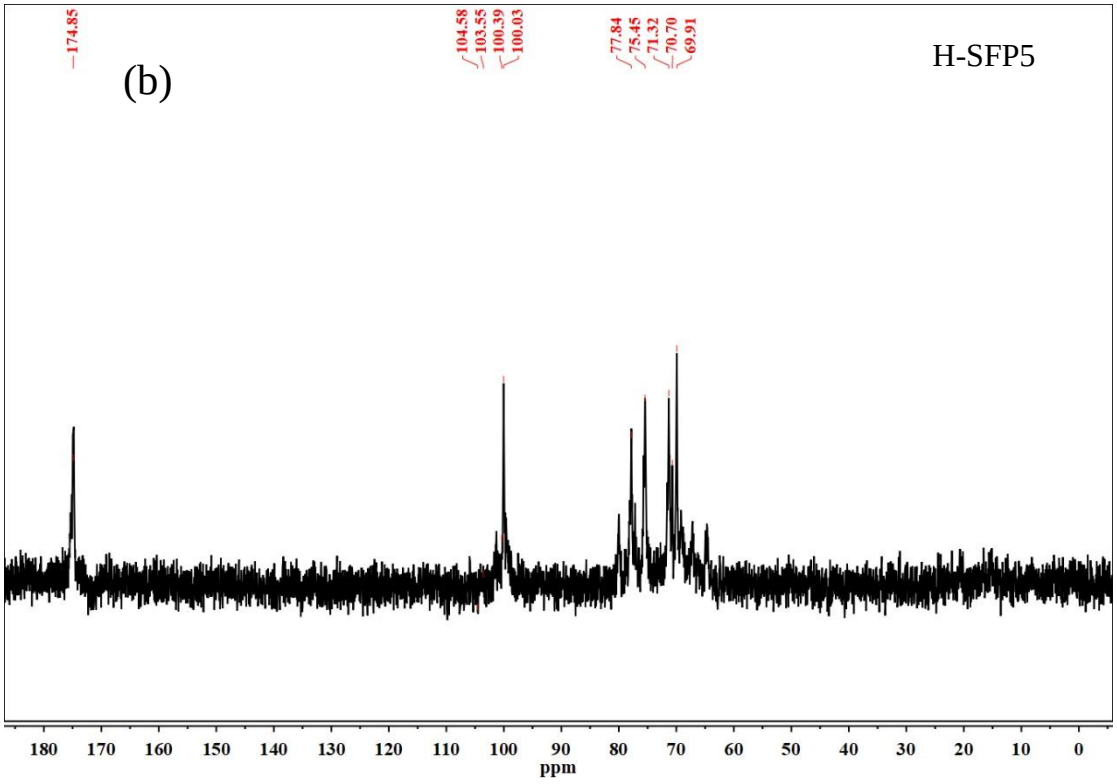

Fig. S5. ^{13}C NMR spectra of H-SFP3 (a) and H-SFP5 (b).

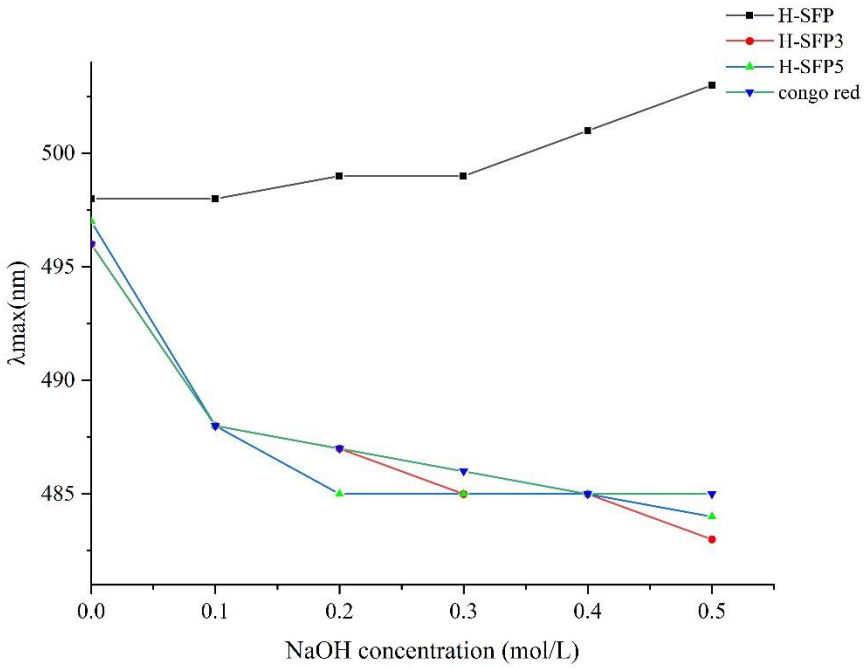

Fig. S6. The maximum absorption wavelength of SFP-Congo red complexes in different alkali concentration.