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The structure of a sulfated galactan from *Porphyra haitanensis* and its in vivo antioxidant activity

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Abstract—The sulfated galactan fraction F1 isolated from the red seaweed, *Porphyra haitanensis*, showed typical porphyran structure. It has a linear backbone of alternating 3-linked β-D-galactosyl units and 4-linked α-L-galactosyl 6-sulfate and 3,6-anhydro-α-L-galactosyl units. The L-residues are mainly composed of α-L-galactosyl 6-sulfate units, and the 3,6-anhydrogalactosyl units are minor. Partial methylation occurred at the C-6 position of the D-galactosyl units and at the C-2 position of the 3,6-anhydro-α-L-galactosyl units. Intraperitoneal administration of F1 significantly decreased the lipid peroxidation in aging mice. F1 treatment increased the total antioxidant capacity and the activity of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) in aging mice. The results indicated that F1 had significant in vivo antioxidant activity.

Keywords: *Porphyra haitanensis*; Sulfated galactan; Chemical structure; NMR spectroscopy; In vivo antioxidant activity

1. Introduction

Sulfated polysaccharides from marine algae can have diverse physiological activities including anticoagulant, antihyperlipidemic, antiviral, and antitumor activities.[1–9] In recent years, algal polysaccharides have been demonstrated to play an important role as free-radical scavengers and antioxidants for the prevention of oxidative damage in living organisms.[10,11]

The red alga, *Porphyra*, is an important food source in many parts of the world. *Porphyra* is nutritious, having abundant protein, polysaccharide, vitamins, and minerals.[12] Besides being widely utilized as food, it has been used in traditional Chinese medicine as a drug for over 1000 years. Several investigations of the structure and function of the polysaccharides isolated from different *Porphyra* species have been undertaken.[13–17] Porphyrans, the sulfated polysaccharides comprising the hot-water soluble portion of cell wall, are the main components of *Porphyra*. Porphyran is related to agarose in that it contains disaccharide units consisting of 3-linked β-D-galactosyl residues alternating with 4-linked 3,6-anhydro-α-L-galactose, but differs in that some residues occur as the 6-sulfate.[16] In addition, the chemical components and structure of porphyrans isolated from different species show great variations.[13]

Sulfated galactans from *Porphyra* have been reported to have immunoregulatory and antitumor activities.[7,8,18,19] But most of these investigations focused on *Porphyra yezoensis*, a species widely distributed in East Asia. *Porphyra haitanensis* (*P. haitanensis*) is an important economic alga in southern China. In a preliminary study, we isolated three sulfated polysaccharide fractions (F1, F2, and F3) from *P. haitanensis* and evaluated their in vitro antioxidant activities.[20] In the present study, we have elucidated the structure of fraction F1 by nuclear magnetic resonance (NMR) spectroscopy and have investigated its in vivo antioxidant activity in aging mice.

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2. Results and discussion

2.1. Chemical features of F1

Chemical analysis indicated that the sulfate content in F1 was 17.5%, and the content of 3,6-anhydrogalactose was 12.1%, although it cannot be quantified by a GC method due to the shortage of the authentic sample (Table 1). GC analysis of the acetylated aldononitrile derivatives of F1 hydrolysate showed that galactose was the predominant component. In addition to galactose, fucose was also present in low levels. The presence of fucose should be further confirmed by mass spectroscopy in the following study. The average molecular weight of F1 was estimated to be 850 kDa by high-performance size-exclusion chromatography (HPSEC).

Infrared spectra yielded useful information on the sulfate and 3,6-anhydrogalactose content in agaroid polysaccharides. The FTIR spectra of native and alkali-treated polysaccharide F1 are shown in Figure 1. The absorption bands at 1225 and 817 cm^{-1} for the sulfate group were present in the IR spectrum of F1. The absorption peak at 817 cm^{-1} indicated that the sulfate group was attached at the C-6 position of galactose.[21] The band at 931 cm^{-1} was attributed to the absorption of 3,6-anhydrogalactose.

NMR spectroscopy gave valuable structural information on the red algal galactans. In the ^1H NMR spectrum of F1, the anomeric resonances at 5.15–5.28 ppm originated from (1→4)-linked α-L-galactopyranose units.[22,23] The signal at 5.28 ppm was attributed to the anomeric proton of the agarose precursor, 4-*O*-linked 6-sulfated α-L-galactose units. The signal at 5.15 ppm was due to the anomeric proton of 3,6-anhydro-α-L-galactose units. In the ^1H NMR spectrum of F1, the ratio of the intensity of signals at 5.28 to the intensity of the signal at 5.15 is 2.5:1. The high-intensity signal at 5.28 and the low intensity signal at 5.15 ppm indicated that the precursor, 4-*O*-linked 6-sulfated α-L-galactose units was predominant, while 3,6-anhydro-α-L-galactose units were present in low amounts. The signals at 3.50 and 3.40 ppm in the ^1H NMR spectrum were attributed to the methyl group of 3,6-anhydro-2-*O*-methyl-α-L-galactose and 6-*O*-methyl-D-galactose, respectively.[24,25] F1 was partially methylated as evidenced by their peak areas.

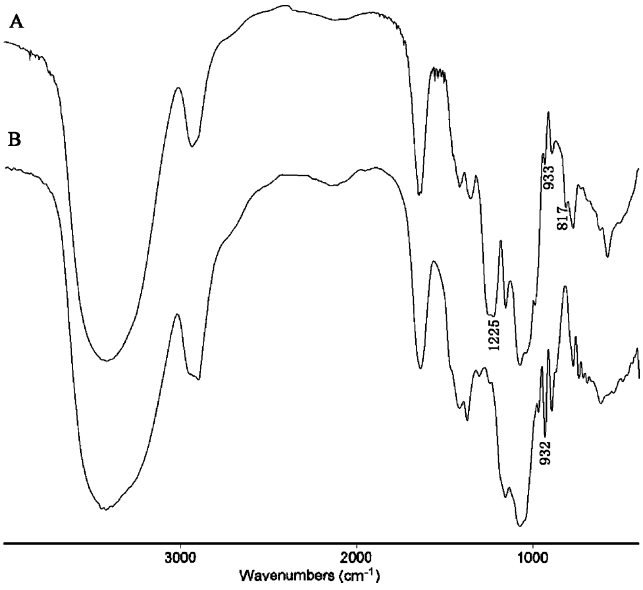

Figure 1. FTIR spectra of native (A) and alkali-treated (B) sulfated polysaccharide fraction F1 from *P. haitanensis*. The band at 817 cm^{-1} was due to the C-6 sulfate of galactose; the band at 933 cm^{-1} was attributed to the absorption of 3,6-anhydrogalactose.

^{13}C NMR spectra for the native and alkali-treated polymer F1 were obtained and are shown in Figure 2. The signal assignments were made on the basis of comparison with spectra of model compounds, agarose, and other related polysaccharides.[16,26–30] The resonances observed for F1 are given in Table 2, together with assignments from the literature for relevant structural elements. This spectrum could be best interpreted as consisting of signals of high intensity and of low intensity, together with additional signals due to 6-*O*-methyl-β-D-galactose (G6M) and 3,6-anhydro-2-*O*-methyl-α-L-galactose (A2M). The high-intensity signals correspond well to those reported for the (G–L6S) units, the repeating units of the agarose biological precursor, while the low-intensity signals correspond well with those reported for (G–A) units.[16,26,27] The methylation of F1 was indicated by the characteristic signals due to 3,6-anhydro-2-*O*-methyl-α-L-galactose (82.5, 78.5, and 59.1 ppm) and 6-*O*-methyl-β-D-galactose (73.6, 71.8, and 59.1 ppm).[26]

Treatment of agaroids containing L-galactosyl 6-sulfate residues (precursor units) with alkali results in an

Table 1. Chemical analysis of polysaccharide fraction F1 from *P. haitanensis*

Total sugar[a]	Sulfate[a]	3,6-AG[a,b]	N[a,c]	Monosaccharide composition[d] (mol %)	
				Galactose	Fucose
60.4	17.4	12.4	0.27	92	8

[a] Percentage of the dry weight of F1.
[b] 3,6-Anhydro-α-L-galactose.
[c] The nitrogen content was determined by elemental analysis.
[d] The monosaccharide composition was detected by GC analysis.

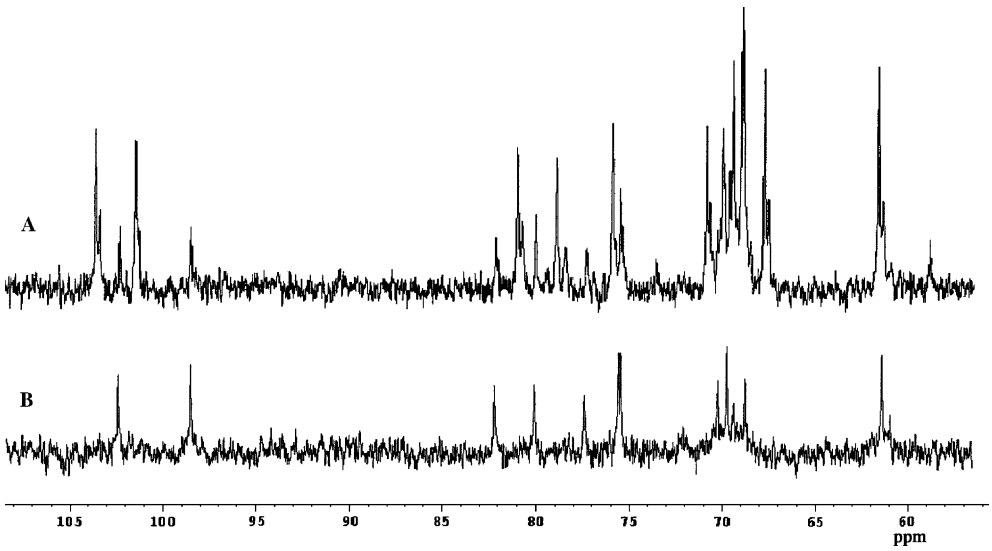

Figure 2. ^{1}H-decoupled ^{13}C NMR spectra of native (A) and alkali-treated (B) polysaccharide fraction F1 from *P. haitanensis*.

Table 2. Chemical shift assignments for ^{13}C NMR spectra of native polysaccharide fraction F1 from *P. haitanensis*

Residue unit		^{13}C chemical shift (ppm)						
		C-1	C-2	C-3	C-4	C-5	C-6	Me
Lahaye et al.[26]								
$(G–A)_n$	G	102.4	70.2	82.2	68.8	75.3	61.4	
	A	98.3	69.9	80.1	77.4	75.7	69.4	
$(G–L6S)_n$	G	103.7	69.8	81.2	69.1	75.9	61.8	
	L6S	101.3	69.3	71.1	79.1	70.3	67.9	
$(G–A2M)_n$	G	102.7	70.2	82.7	68.8	75.3	61.4	
	A2M	98.7	78.9	78.5	77.6	75.7	69.5	59.2
$(G6M–A)_n$	G6M	102.3	70.1	82.1	69.0	73.5	71.7	59.1
	A	98.2	69.9	80.1	77.3	75.6	69.4	
Observed spectra								
(G–A) unit	G	102.4	70.1	82.2	69.1	75.4	61.5	
	A	98.5	70.1	80.1	77.3	75.5	69.6	
(G–L6S) unit	G	103.6	69.7	81.0	69.1	75.9	61.7	
	L6S	101.5	69.1	71.0	78.9	70.8	67.9	
(G6M–A) unit	G6M	102.4	70.1	82.1	69.0	73.6	71.8	59.1
	A	98.5	69.9	80.1	77.3	75.6	69.4	
(G–A2M) unit	G	102.4	70.1	82.5	69.1	75.4	61.5	
	A2M	98.5	78.5	78.5	77.3	75.5	69.6	59.1

G: (1→3)-linked β-D-galactose; A: (1→4)-linked 3,6-anhydro-α-L-galactose; L6S: (1→4)-linked α-L-galactose 6-sulfate; G6M: (1→3)-linked 6-*O*-methyl-β-D-galactose; A2M: (1→4)-linked 2-*O*-methyl-3,6-anhydro-α-L-galactose.

intramolecular displacement of the sulfate group and formation of 3,6-anhydro-L-galactosyl residues. To confirm the presence of such precursor units in F1, a sample was treated with alkali, and its infrared and NMR spectra were recorded. After alkali modification, the bands at 1225 and 817 cm^{-1} for the sulfate group disappeared, while the intensity of the absorption band for 3,6-anhydrogalactose at 933 cm^{-1} increased (Fig. 1). This confirmed that the band at 817 cm^{-1} was due to a 6-sulfate on the L-galactose unit, which can be converted to 3,6-anhydrogalactose after alkali treatment.[16]

In the ^{1}H NMR spectrum of the alkali-treated polysaccharide, the ratio of the intensity of signals at 5.28 to the intensity of signal at 5.15 changed from 2.5:1 to 1:2.9, which means most of the 6-sulfated α-L-galactose units were transformed into 3,6-anhydro-α-L-galactose units. This modification was also confirmed by the ^{13}C NMR spectrum. The signals corresponding to the G–L6S units disappeared, and only the signals corresponding to the G–A units were observed in the ^{13}C NMR spectrum (shown in Fig. 2). As the intensities of all the signals were much weaker in the alkali-treated sample than in the native one, the low-intensity signal for the *O*-methyl group at 59.1 ppm could not be clearly distinguished in the ^{13}C NMR spectrum of the alkali-treated sample.

It can be concluded from the above analysis that the polysaccharide fraction F1 from *P. haitanensis* has a typical porphyran structure, with the linear backbone of alternating 3-linked β-D-galactosyl units and 4-linked α-L-galactosyl 6-sulfate and 3,6-anhydro-α-L-galactosyl units. The L-residues are mainly composed of α-L-galactosyl 6-sulfate units, and the 3,6-anhydrogalactosyl units are minor. Partial methylation occurred at the C-6 position of the D-galactosyl units and at the C-2 position of the 3,6-anhydro-α-L-galactosyl units.

2.2. In vivo antioxidant activity of F1 in aging mice

Malondialdehyde (MDA) is a main marker of endogenous lipid peroxidation. In this study, MDA levels in all the organs significantly increased with aging (shown in Fig. 3). The increase of MDA production indicates that peroxidative damage increases with the aging process.[31,32] F1 treatment at low dose (50 mg kg^{-1}) significantly decreased the MDA levels in the liver and heart, had no influence on the MDA level in the serum, and slightly increased the MDA production in the brain. F1 treatment at the doses of 100 and 200 mg kg^{-1} significantly inhibited the formation of MDA in all tested organs. In general, F1 at high dose exhibited similar or stronger effects than vitamin C at a dose of 200 mg kg^{-1}. The data suggest that the administration of F1 resulted in an effective inhibition of the lipid peroxidation in aging mice.

Superoxide dismutase (SOD) is an intracellular antioxidant enzyme that protects against oxidative processes initiated by the superoxide anion. As shown in Figure 4, there is no significant difference on SOD activities between the aging and young mice. Treating aging mice with F1 significantly and dose dependently increased the SOD activities in tested organs.

The data on glutathione peroxidase (GSH-Px) activity are shown in Figure 5. Significant decreases in the activity of GSH-Px were observed in the brain and liver in aging mice. The activity of GSH-Px in the heart was also slightly decreased in aging mice. Administration of F1 increased the GSH-Px activity significantly. The maximal activity of GSH-Px in liver and brain was achieved by the administration of F1 at a dose of 50 mg kg^{-1}, F1 treatment at a dose of 100 mg kg^{-1} maximally increased the GSH-Px activity in the heart. It is likely that the effect of F1 on the activity of GSH-Px has an optimal dose.

Total antioxidant capacity (TAOC) reflects the capacity of the non-enzymatic antioxidant defense system. As shown in Figure 6, total antioxidant capacity decreased markedly with age in all the organs. F2 administration greatly elevated the total antioxidant capacity in all organs. F1 treatment at a dose of 100 mg kg^{-1} had the maximal increase in total antioxidant capacity in all tested organs.

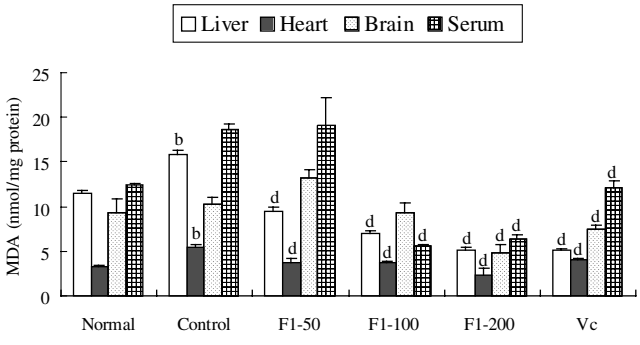

Figure 3. Effect of F1 on the MDA levels in aging mice. Normal: young mice group; Control: aging mice group; F1-50, F1-100, and F1-200 refer to the three dosage F1 treatment groups, respectively; Vc: the positive control (vitamin C) group. (a) $P < 0.05$ (vs Normal group); (b) $P < 0.01$ (vs Normal group); (c) $P < 0.05$ (vs Control group); (d) $P < 0.01$ (vs Control group).

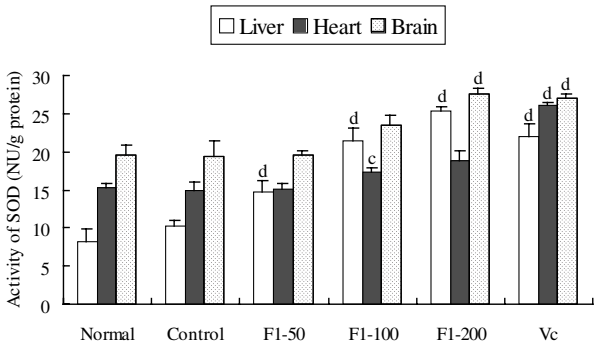

Figure 4. Effect of F1 on the SOD activity in different organs in aging mice. Normal: young mice group; Control: aging mice group; F1-50, F1-100, and F1-200 refer to the three dosage F1 treatment groups, respectively; Vc: the positive control (vitamin C) group. (a) $P < 0.05$ (vs Normal group); (b) $P < 0.01$ (vs Normal group); (c) $P < 0.05$ (vs Control group); (d) $P < 0.01$ (vs Control group).

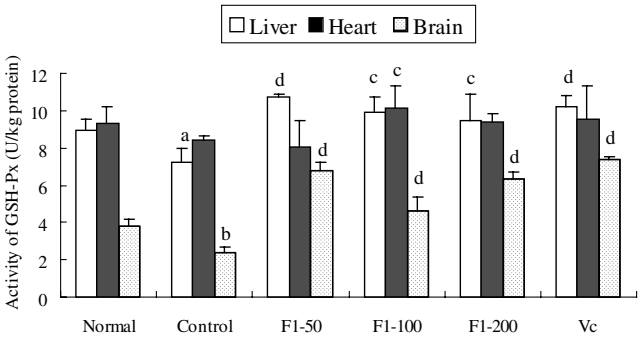

Figure 5. Effect of F1 on the activity of GSH-Px in different organs in aging mice. Normal: young mice group; Control: aging mice group; F1-50, F1-100, and F1-200 refer to the three dosage F1 treatment groups, respectively; Vc: the positive control (vitamin C) group. (a) $P < 0.05$ (vs Normal group); (b) $P < 0.01$ (vs Normal group); (c) $P < 0.05$ (vs Control group); (d) $P < 0.01$ (vs Control group).

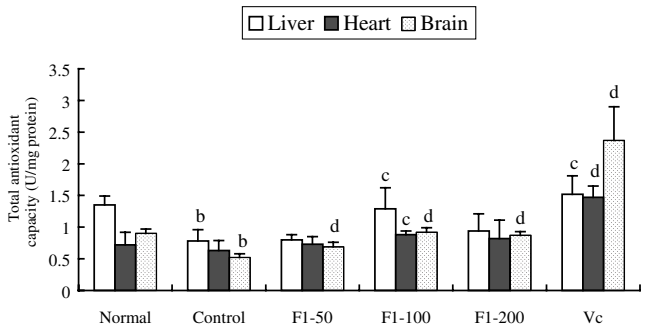

Figure 6. Effect of F1 on total antioxidant capacity in different organs in aging mice. Normal: young mice group; Control: aging mice group; F1-50, F1-100, and F1-200 refer to the three dosage F1 treatment groups, respectively; Vc: the positive control (vitamin C) group. (a) $P < 0.05$ (vs Normal group); (b) $P < 0.01$ (vs Normal group); (c) $P < 0.05$ (vs Control group); (d) $P < 0.01$ (vs Control group).

A vast amount of evidence has implicated that aging is associated with a decrease in antioxidant status, and that age-dependent increases in lipid peroxidation are a consequence of diminished antioxidant protection.[33] Antioxidant enzymes are considered to be a primary defense that prevents biological macromolecules from oxidative damage. SOD and GSH-Px are two important enzymatic antioxidant defense mechanisms. Sulfated polysaccharide fraction F1 was successful in inhibiting lipid peroxidation as observed in the reduction of MDA production in aging mice. The result was consistent with the in vitro inhibitory effect of F1 on the liver microsomal lipid peroxidation observed in our previous study. F1 also induced the activities of SOD and GSH-Px and increased the total antioxidant capacity in aging mice. The enhanced activity of SOD and GSH-Px and increased total antioxidant capacity in the aging animals can be very effective in scavenging the various types of oxygen free radicals and their products. So the inhibitory effect of F1 on lipid peroxidation might be, at least in part, attributed to its influence on the antioxidant enzymes and nonenzymatic system. In our previous study, F1 showed significant scavenging activity on the superoxide anion and hydroxyl free radical. The direct oxygen free-radical scavenging activity of F1 might also contribute to its in vivo antioxidant activity.

Evidence implicates oxygen-derived free radicals as important causative agents of aging, and the free-radical theory of aging suggests that damage produced by the interaction of such free radicals with cellular macromolecules results in cellular senescence and aging.[34–36] The inhibitory effect of sulfated polysaccharide fraction F1 on lipid peroxidation in aging animals suggests that F1 might be an effective agent to reduce the risk of lipid peroxidation in the aging process.

3. Experimental

3.1. Materials and chemicals

P. haitanensis T. J. Chang and B. F. Zheng, cultured near the coast of Lianjiang County, Fujian Province, China, was collected in January 2001. Polysaccharide fraction F1 was isolated and fractionated from *P. haitanensis* as described previously.[20] Briefly, F1 was fractionated from the hot-water extract by ion-exchange cellulose column chromatography.

Assay kits for protein, MDA, SOD, GSH-Px, and TAOC were obtained from Nanjing Institute of Jiancheng Bioengineering. Vitamin C injection was provided by Tianjin Amino Acid Co. Ltd.

3.2. Alkaline modification of polysaccharide F1

Polysaccharide fraction F1 was treated with $NaBH_4$–NaOH according to Gretz and McCandless.[16]

3.3. Analytical methods

3.3.1. General methods. Total sugar content was analyzed by the phenol–sulfuric acid method[37] using D-galactose as standard. Sulfate content was determined according to the method of Kawai.[38] 3,6-Anhydrogalactose content was determined as described previously.[39]

3.3.2. Acid-stable sugar analysis. The polysaccharide sample was hydrolyzed with 2 M trifluoroacetic acid for 4 h at 100 °C. The acid was removed under reduced pressure by several evaporations with distilled water, then the hydrolysates were converted to acetylated aldononitrile derivatives according to conventional procedures and analyzed by GLC (HP5890) using an AC-20 capillary column (30 m × 0.32 mm i.d.). As references, the following neutral sugars were converted to their acetylated aldononitrile derivatives and analyzed: rhamnose, fucose, arabinose, xylose, mannose, galactose, and glucose.

3.3.3. Molecular weight. The molecular weights of the samples were determined by HPSEC on a PL Aquagel 32 mixed column, eluted with 0.7% Na_2SO_4 solution at a flow rate of 0.5 mL min^{-1} at 35 °C. Elution was monitored by a refractive index detector (Perkin–Elmer). Column calibration was performed with standard dextrans (molecular weight: 7.5, 10, 21.1, 41.1, 84.4, 133.6, and 2000 kDa, respectively, provided by National Institute for the Control of Pharmaceutical and Biological Product, China).

3.3.4. Spectroscopic methods. Fourier-transformed infrared spectra were recorded from the polysaccharide

powder in KBr pellets on a Nicolet Avatar 360 FTIR spectrophotometer. For NMR spectroscopic analysis, the lyophilized sample was dissolved in D_2O, and ^1H and ^{13}C nuclear magnetic resonance spectra were acquired on a Bruker DRX-500 NMR spectrometer at 25 °C. ^1H chemical shifts were measured relative to external DSS, and ^1H-decoupled ^{13}C NMR chemical shifts were measured in ppm relative to internal dimethyl sulfoxide at 39.6 ppm.

3.4. In vivo antioxidant activity in aging mice

Aging Kunming mice (20 months old, 35–45 g) and young Kunming mice (3 months old, 18–22 g) were provided by Henan Province Laboratory Animal Center, China. The animals were maintained on a 12-h dark/light cycle at about 22 °C and allowed free access to standard laboratory pellet diet (Henan Province Laboratory Animal Center, China) and water during the experiments.

Aging Kunming mice were randomly divided into five groups, each consisting of 10 animals. One group was the aging control; polysaccharide fraction F1 (50, 100, 200 mg kg^{-1} body weight) and vitamin C (200 mg kg^{-1}, as positive control) were administered by intraperitoneal injection to the other four groups, respectively, once daily for 20 consecutive days. Young Kunming mice were used as the normal control. Aging control and normal control were treated with the same volume of physiological saline. Twenty-four hours after the last drug administration, the animals were killed by decapitation. Blood samples were collected and processed to obtain serum. Three organs (liver, heart, and brain) were excised from the animal, and tissue homogenates were processed for biochemical analysis.

3.5. Biochemical analysis

Serum samples were obtained by centrifuging the whole blood at 1000*g* at 4 °C for 10 min, and the serum MDA levels were measured. For biochemical assays, the organ homogenates (including liver, heart, and brain) were prepared in 0.1 g mL^{-1} wet weight of ice-cold isotonic physiological saline. Samples were centrifuged, and the supernatants were then subjected to the measurement of SOD, GSH-Px, MDA, and TAOC levels by spectrophotometric methods. SOD activity was analyzed by monitoring the inhibition of the reduction of nitro blue tetrazolium by the sample at 560 nm;[40] GSH-Px activity was detected with 5,5′-dithiobis-*p*-nitrobenzoic acid;[41] MDA levels were analyzed with 2-thiobarbituric acid;[42] the TAOC was measured by the method of ferric reducing/antioxidant power assay.[43]

3.6. Statistical analysis

The data were presented as means ± SEM and evaluated by one-way analysis of variance followed by the Student's *t*-test to detect intergroup differences. Differences were considered to be statistically significant if $P < 0.05$.

Acknowledgements

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