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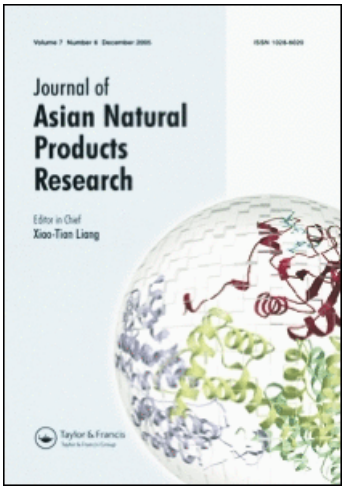

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Influence of sulfation on anti-myocardial ischemic activity of *Ophiopogon japonicus* polysaccharide

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Influence of sulfation on anti-myocardial ischemic activity of *Ophiopogon japonicus* polysaccharide

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Ophiopogon japonicus polysaccharide (FOJ-5) from *Radix ophiopogonis* has shown anti-myocardial ischemic action *in vitro* and *in vivo* in our previous studies. In order to clarify the influence of chemical modifications on the action, a series of sulfated FOJ-5 (FOJ-5-S) with different substitution degrees were prepared and the anti-myocardial ischemic action of the natural FOJ-5 and the FOJ-5-S were studied *in vitro* and *in vivo*. Langendorff isolated rat hearts and acute myocardial ischemic rats induced by isoprenaline were employed as myocardial ischemic models in our experiments. The amplitude and frequency of cardiac contraction, coronary blood flow at different time points after ischemia/reperfusion were measured *in vitro*. The ST segment shift in electrocardiogram and lactate dehydrogenase level in blood plasma were observed on the *in vivo* model. The results indicated that FOJ-5 and FOJ-5-S had the anti-myocardial ischemic action compared with non-treated vehicle groups. Furthermore, it was found that FOJ-5-S had significant action on the *in vivo* model compared with FOJ-5 ($P < 0.05$). And the obtained results from the further study also indicated that only when the degree of substitution was in a certain range, the FOJ-5-S had excellent anti-myocardial ischemic activity.

Keywords: polysaccharide; *Ophiopogon japonicus*; derivative; anti-myocardial ischemia

Abbreviations: FOJ-5, fructosan with an average molecular weight of 5 kDa from *Ophiopogon japonicus*; K–H solution, Krebs–Henseleit solution containing NaCl 118, KCl 4.74, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, $CaCl_2$ 2.5, glucose 10 (mmol/l)

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1. Introduction

Nowadays, coronary heart disease is one of the leading causes of death in the world, and myocardial ischemia is a common and dangerous symptom of this disease. The pathogenetic mechanism of myocardial ischemic damage is still not completely understood, but the role of oxygen-derived free radicals in myocardial ischemia has been established, although not completely characterized. The major cytotoxic effect of free radicals in the heart is supposed to be the peroxidation of lipid components of cellular and subcellular membranes. The loss of cellular integrity could lead to irreversible cell injury [1]. Ischemia/reperfusion damage has important implications and particular attention has been focused on the consequences of reperfusion, since the serious damage often follows. Many polysaccharides isolated from plant, animal, and microorganism have been studied in the biomedical area

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and represent an unlimited source for treating coronary heart disease because of their immunostimulating and free-radical scavenging action [2].

Radix ophiopogonis is the root of *Ophiopogon japonicus* (Thunb.) Ker-Gawl. It can nourish yin and promote the production of body fluid, moisten lung, and clear away heart-fire. Clinical researches indicated that it had the effect on myocardial ischemia in coronary artery disease [3]. It is reported that *R. ophiopogonis* contains 71% of carbohydrate [4]. In our previous research, it was found that crude polysaccharides with an average molecular weight of less than 10 kDa from *O. japonicus* had anti-myocardial ischemic activity [5]. From the crude polysaccharides, a fructosan with an average molecular weight of 5 kDa (FOJ-5) was isolated. It had a backbone chain of 1 → 2-β-D-fructan, of which 1 out of 2.8 fructose residues was substituted at O-6 by two fructosyl groups (Figure 1) [6]. Furthermore, experiments revealed that *O. japonicus* polysaccharide (FOJ-5) had the effect on anti-myocardial ischemia *in vitro* and *in vivo* [7]. Since the action of polysaccharide usually improved after being sulfated [8], in this paper a series of sulfated FOJ-5 (FOJ-5-S) with different substitution degree were prepared and their anti-myocardial ischemic activities were studied in order to find more powerful derivatives.

2. Results and discussion

2.1 Sulfation of FOJ-5

With the different reaction temperatures and initial ratios of *N*,*N*′-dicyclohexylcarbodiimide (DCC) or H_2SO_4 to FOJ-5 monomer unit, FOJ-5-Ss with different degree of

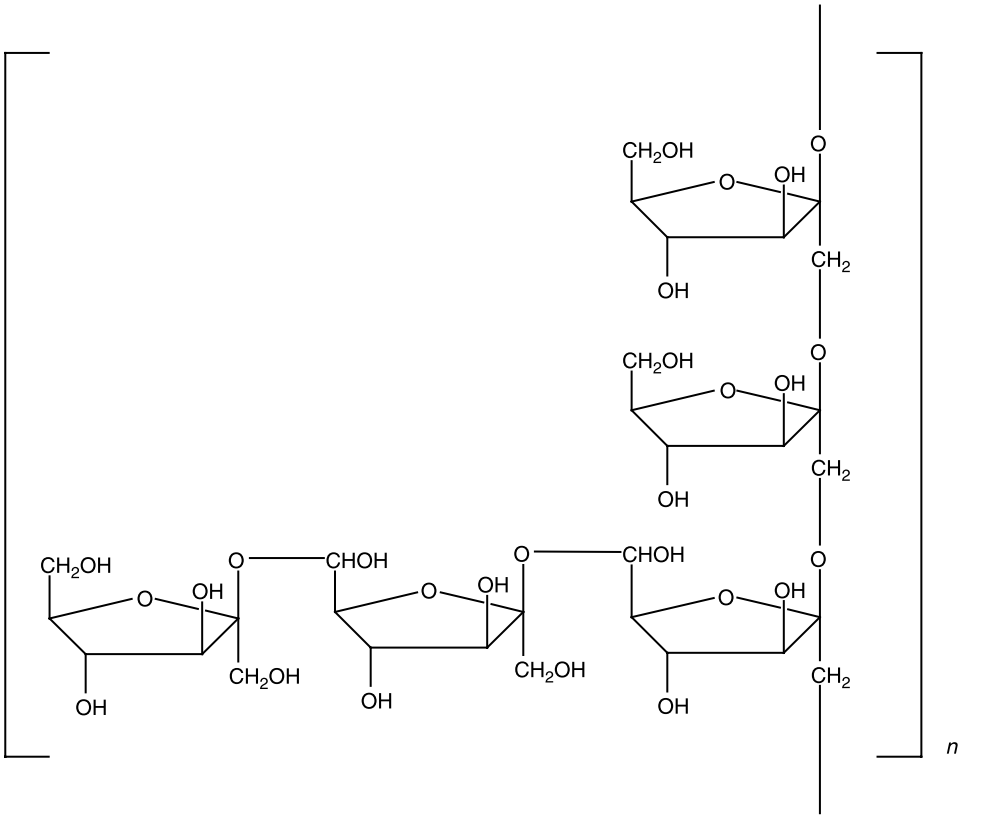

Figure 1. The possible primary structure of FOJ-5 ($n \approx 6$).

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Table 1. Sulfation of FOJ-5 with DCC–H_2SO_4 in dimethyl sulfoxide (DMSO) media.

Derivatives	[DCC]/[FOJ][a]	[H_2SO_4]/[FOJ][a]	Temp. (°C)	Time (min)	DS[b]	Molecular weight
FOJ-5-S_1	8	4	25	30	0.03	9432
FOJ-5-S_2	16	4	30	30	0.08	11,540
FOJ-5-S_3	12	8	25	30	0.10	12,111
FOJ-5-S_4	16	10	25	60	0.13	12,194

[a] The initial ratio of DCC or H_2SO_4 to FOJ-5 monomer unit.
[b] Determined by sodium rhodizonate method.

substitution (DS) were obtained. The reaction conditions and DS of the products were listed in Table 1. Preparing sulfated FOJ with chlorosulfonic acid was experimented in our preliminary study. The sulfated reactions in chlorosulfonic acid were violent. Though higher DS could be obtained, the yield was as low as 10%, and the product would change to black after several days, indicating that FOJ-5 may have degraded or deliquesced. Moreover, chlorosulfonic acid is evaporable and amyctic, so DCC was selected as derivate agent in the following study. Because of the low molecular weight of FOJ-5, the temperature of the reaction should be controlled under 40°C. With the increase in DS, the color of the product became more and more dark. The density and the hydroscopicity of the FOJ-5-S also increased.

2.2 Characterization

FT-IR spectrum of FOJ-5-S_2 showed two characteristic absorption bands at 1240 and 819.6 cm^{-1}, which originated from an asymmetrical S═O stretching vibration and a symmetrical C—O—S stretching vibration associated with a C—O—SO_3 group, respectively [9]. The result indicated that —O—SO_3 groups had been connected to FOJ-5 (Figure 2).

HPGPC figure showed that FOJ-5-S_2 was almost homogeneity. Comparing with

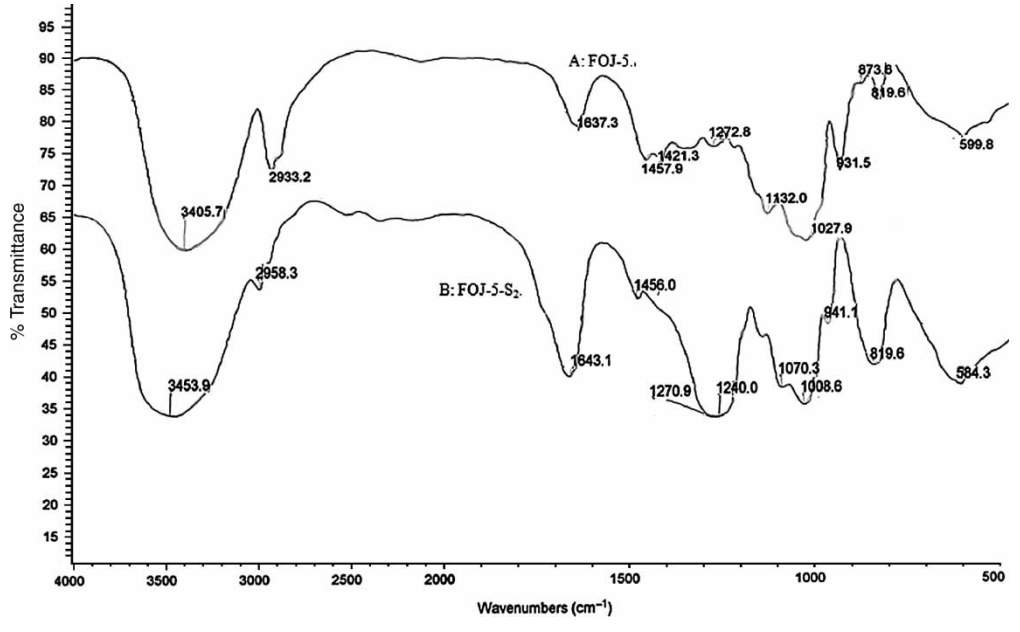

Figure 2. FT-IR spectra of FOJ-5 and FOJ-5-S (scanning wave numbers 4000–400 cm^{-1}).

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FOJ-5, its retention time decreased which indicated the increasing of the molecular weight (Figure 3). In our study, we found that the molecular weights of the FOJ-5-Ss measured by high performance gel permeation chromatography (HPGPC) were larger than that calculating from theory. The reason might be that FOJ-5-Ss have been electrified, so their retention behaviors were different from those of uncharged polysaccharides. So when the uncharged dextrans were selected as standard to measure the molecular weights of FOJ-5-Ss, there would be an error. It reminded us that we should select a standard glycan with a similar structure as the polysaccharide which we want to measure its molecular weight.

There was a good linear correlation between the values of absorbance and the amounts of Na_2SO_4 from 2.08 to 12.48 μg ($R^2 = 0.9957$) and a regression equation $Y = -0.0225X + 0.3006$ was obtained, where Y and X were absorbance and the amounts of Na_2SO_4, respectively.

According to the absorbance of the samples and the regression equation, the degrees of substitution of different FOJ-5-Ss were

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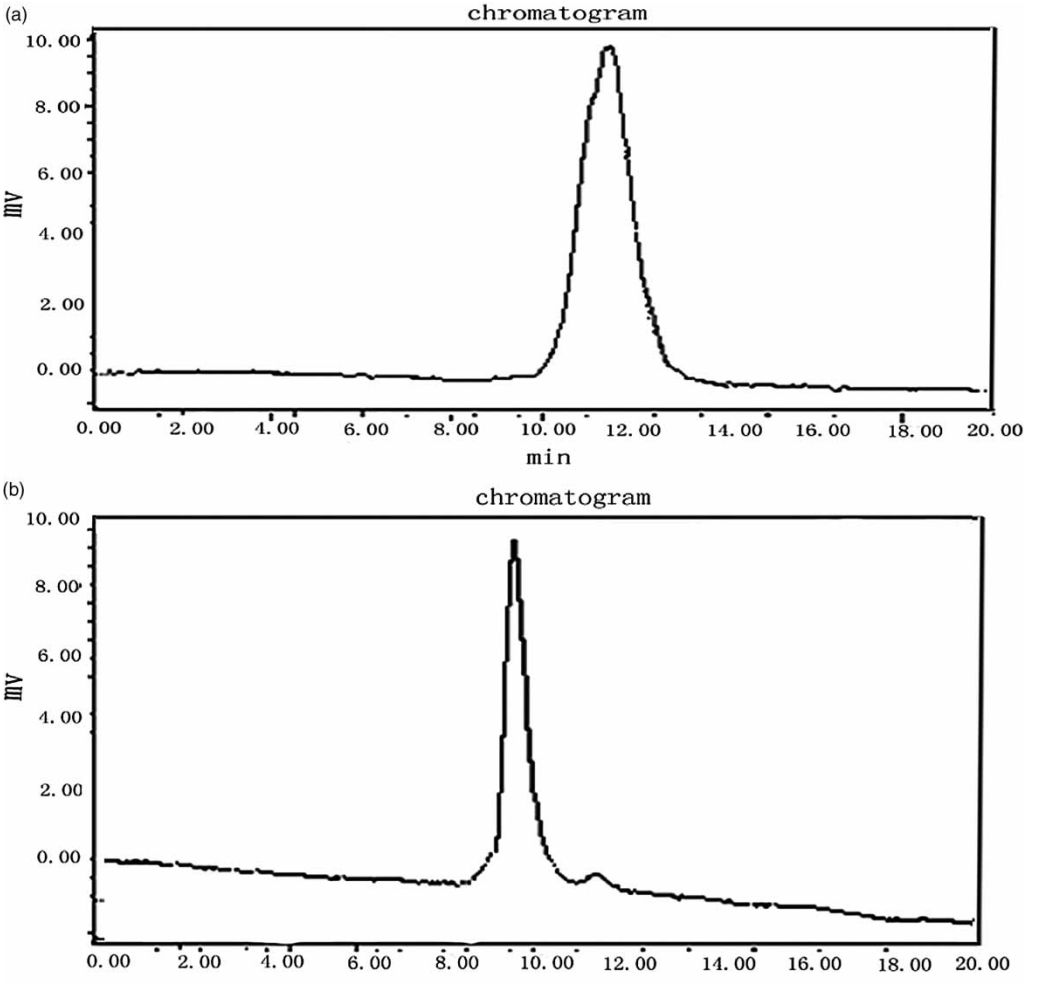

Figure 3. HPGPC of (a) FOJ-5 and (b) FOJ-5-S_2.

calculated. Results showed that substitution degrees of FOJ-5-S obtained under the reaction conditions were low (Table 1).

The picture of atom force microscope (AFM) showed that FOJ-5 appeared to be spherical with a diameter of 19–29 nm and had good dispersivity at a concentration of 0.1 μg/ml but aggregated at a concentration of 10 μg/ml (Figure 4). Since there is no long branched chain in the structure,

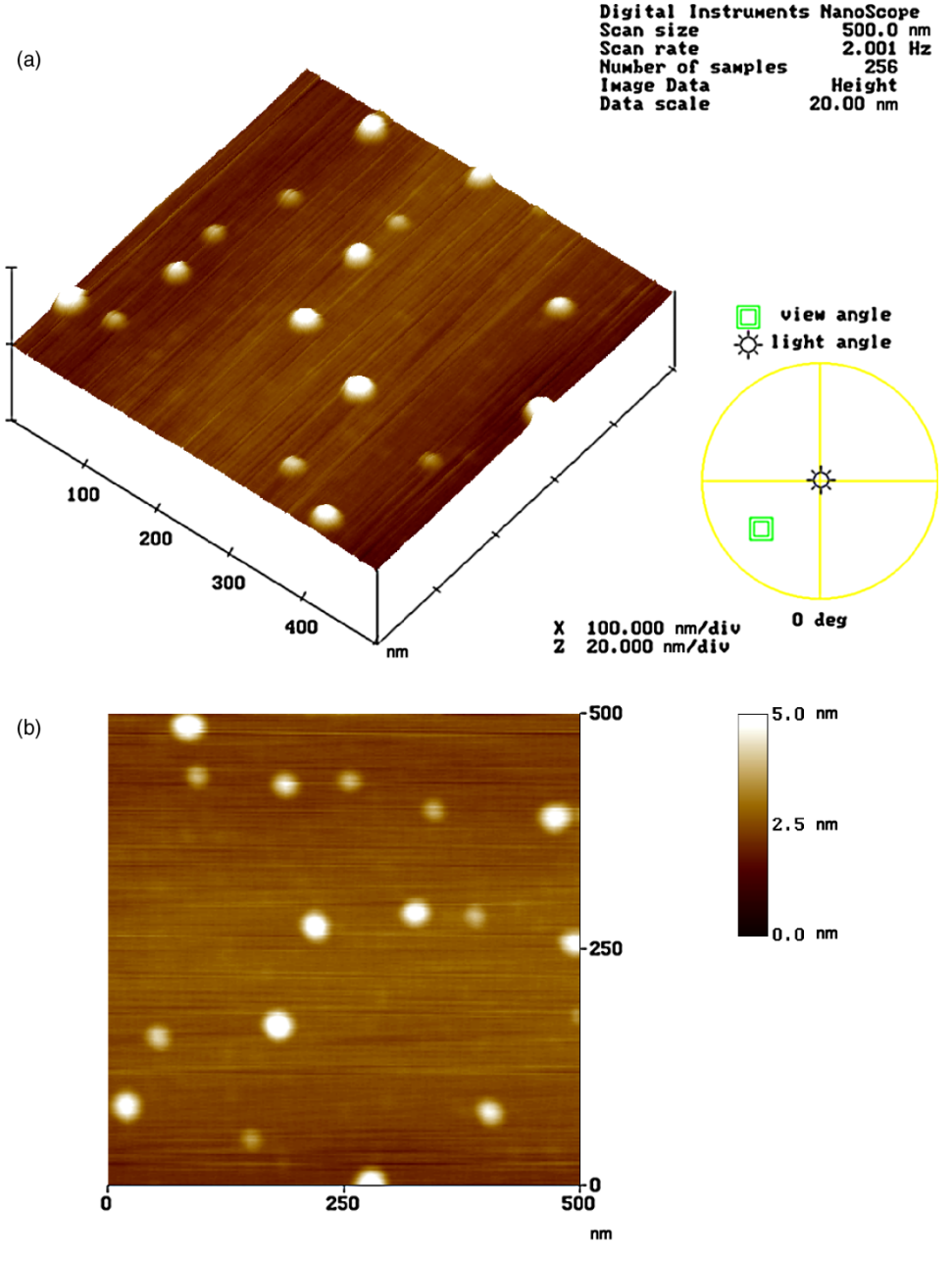

Figure 4. (a) Three-dimensional and (b) planar graphs of AFM picture of FOJ-5 solution (0.1 μg/ml) in 500 nm scale.

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it was deduced that the molecular conformation of FOJ-5 might be curl based on the above results. The surface topography of FOJ-5-S_2 was also spherical with a larger diameter of 59–67 nm which is three times as that of FOJ-5 (Figure 5). Since the DS was not high, the size of the FOJ-5-S_2 is not likely to increase much theoretically. Therefore, the

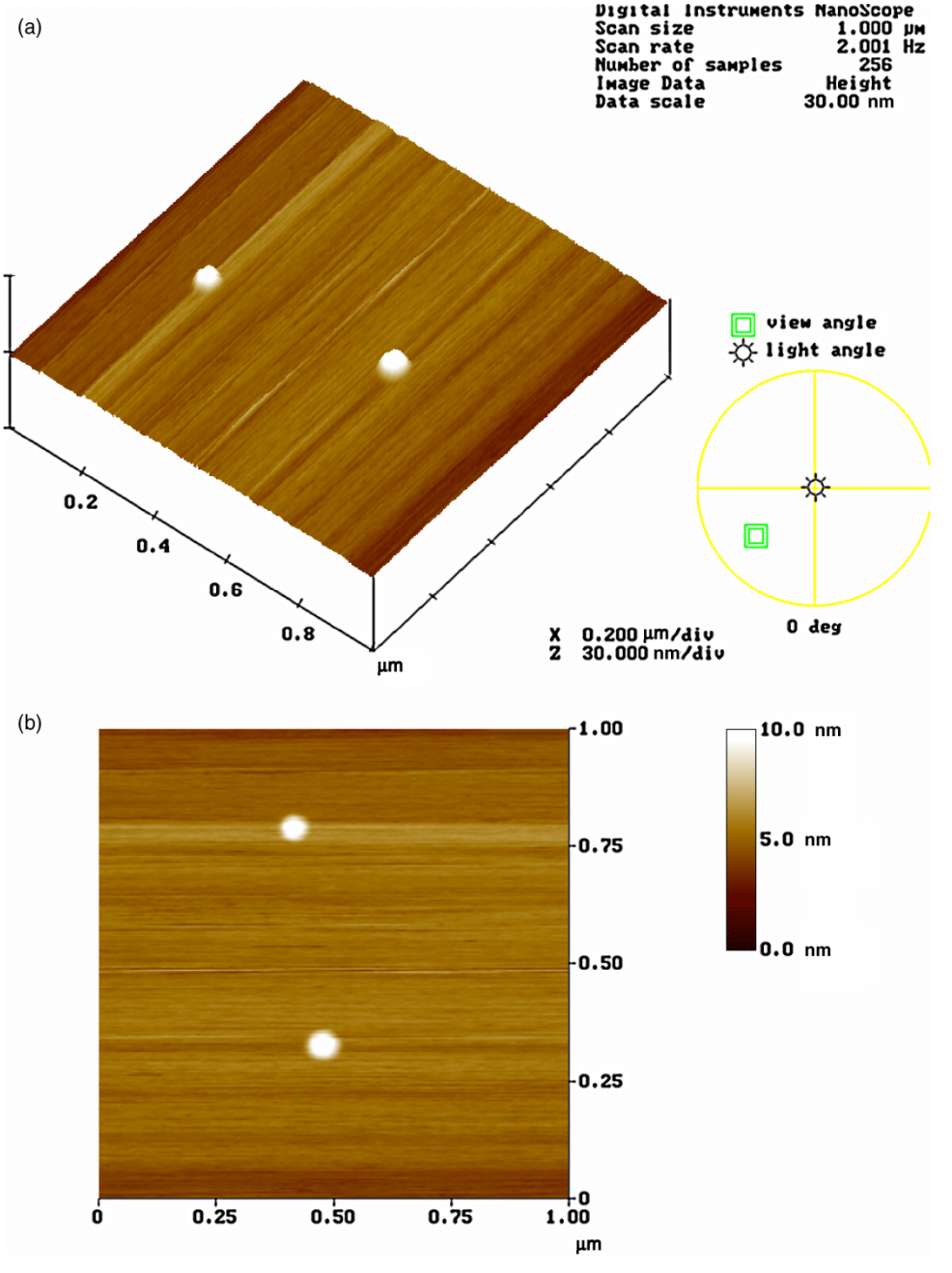

Figure 5. (a) Three-dimensional and (b) planar graphs of AFM picture of sulfated FOJ-5-S_2 solution (0.1 µg/ml) in 1 µm scale.

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FOJ-5-S_2 might have been electrified after being substituted by HSO_3 group.

Structures of polysaccharides are very complex with heterogeneity. It is very difficult to reveal their microscopic structures. Moreover, chemical modification often leads to the change in the structures of polysaccharides, which results in much more difficulty in researches. With the development of analytical techniques such as AFM and 3D NMR, researches on the advanced structures of polysaccharides will make a great progress.

2.3 Effects on isolated myocardial ischemia–reperfusion model

The *in vitro* anti-myocardial ischemic activities of FOJ-5 and its sulfated derivatives were tested on Langendorff model. The value of all the groups after perfusion with Krebs–Henseleit (K–H) for 15 min was considered as 100%. In the control group, the heart contraction (42.3 ± 5.7) and coronary flux (57.6 ± 3.3) were decreased and the heart rate (115.1 ± 4.2) was increased significantly ($P < 0.01$) when compared with the value before ischemia (101.1 ± 3.1, 95.1 ± 7.7, and 99.6 ± 0.9), respectively. Results showed that 1–100 μg/ml of fructose diphosphate (FDP), FOJ-5, and FOJ-5-S_2 could restore heart contraction, resume coronary blood flux, and restrain the increasing of heart rate caused by ischemia–reperfusion of isolated rat myocardium.

FDP (1–100 μg/ml) could significantly restore heart contraction at 15 min after reperfusion (69.4 ± 6.4, 72.7 ± 1.5, 75.7 ± 9.5, $P < 0.01$) when compared with the vehicle (42.3 ± 5.7).

Comparing with the vehicle (42.3 ± 5.7), FOJ-5 (1–100 μg/ml) could also significantly restore heart contraction at 15 min after reperfusion (64.3 ± 14.9, 59.7 ± 3.3, 60.9 ± 6.2, $P < 0.01$). And FOJ-5 (100 μg/ml) could restore heart contraction quickly; the value at 8 min (70.6 ± 3.0, $P < 0.01$) was significantly different from the control (43.3 ± 16.4).

FOJ-5-S_2 (1 μg/ml) could significantly restore heart contraction in a shorter time than all the other groups as reperfusion going. The value at 5 min after reperfusion (89.1 ± 12.4, $P < 0.01$) was significantly different from the control (44.3 ± 19.4). However, FOJ-5-S_2 (10–100 μg/ml) have no significant difference from the control. It indicated that the effect of FOJ-5-S_2 might be better in a lower dosage than in a higher dosage (Figure 6).

FDP (1–100 μg/ml) could significantly restrain the increasing of heart rate at 15 min after reperfusion (100.0 ± 0.0, 97.6 ± 4.3, 98.5 ± 10.9, $P < 0.01$) when compared with the control (115.1 ± 4.2).

Comparing with the control (115.1 ± 4.2), FOJ-5 (1–100 μg/ml) could significantly restrain the increasing of heart rate at 15 min (101.1 ± 1.7, 100.9 ± 6.2, 102.0 ± 3.6, $P < 0.01$). After reperfusion for 12 min, FOJ-5 (100 μg/ml) (103.6 ± 6.4, $P < 0.01$) have significant difference from the control (115.0 ± 5.0).

FOJ-5-S_2 (1–100 μg/ml) could significantly restrain the increasing of heart rate at 15 min (99.7 ± 6.9, 93.3 ± 9.0, 100.2 ± 8.8, $P < 0.01$) when compared with the control (115.1 ± 4.2; Figure 7).

The heart contraction and heart rate of all the sample groups have no significant difference between before and after ischemia–reperfusion for 15 min, but in the control group the difference was significant ($P < 0.01$).

FDP (1–100 μg/ml) could significantly resume coronary blood flux at 15 min after reperfusion (89.1 ± 10.3, 99.8 ± 10.4, 102.0 ± 8.6, $P < 0.01$) when compared with the control group (57.6 ± 3.3).

Comparing with the control (57.6 ± 3.3), FOJ-5 (1–100 μg/ml) could significantly resume coronary blood flux at 15 min (79.8 ± 9.8, 92.7 ± 10.2, 92.0 ± 6.7, $P < 0.01$). More intriguingly, the value of coronary blood flux in FOJ-5 (1–100 μg/ml) groups significantly increased at the beginning of reperfusion after 30 min ischemic (182.8 ± 34.6, 191.3 ± 20.9, 179.8 ± 35.1, $P < 0.01$) when compared with the control (122.2 ± 15.8).

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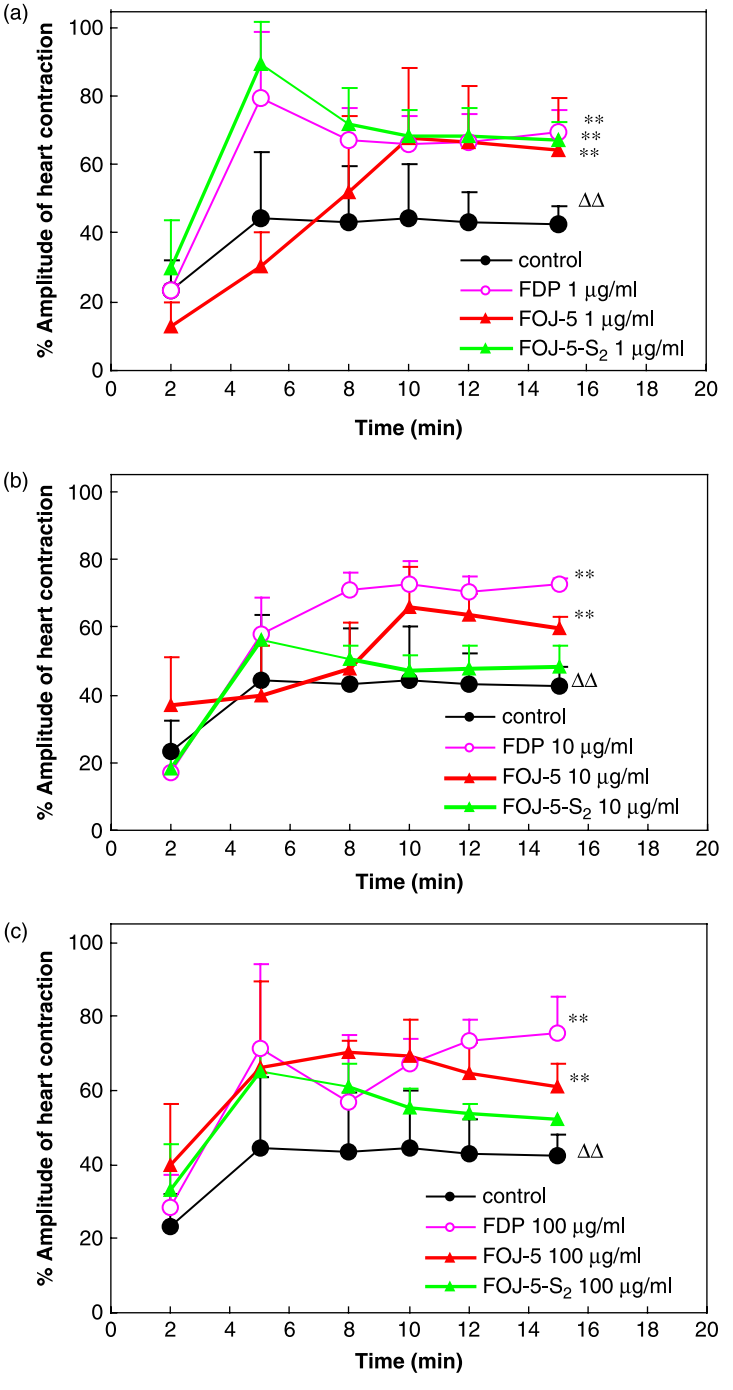

Figure 6. The action of FOJ-5 and FOJ-5-S_2 on the heart contraction of isolated myocardium of Guinea pigs (the value after perfusion with K–H for 15 min was considered as 100%). When compared with model, pretreatment with samples (in concentration of (a) 1, (b) 10, and (c) 100 μg/ml, respectively) significantly increased the amplitude of heart contraction. Data are mean ± SD ($n = 6$). $*P < 0.05$, $**P < 0.01$ versus control; $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$ versus before ischemia.

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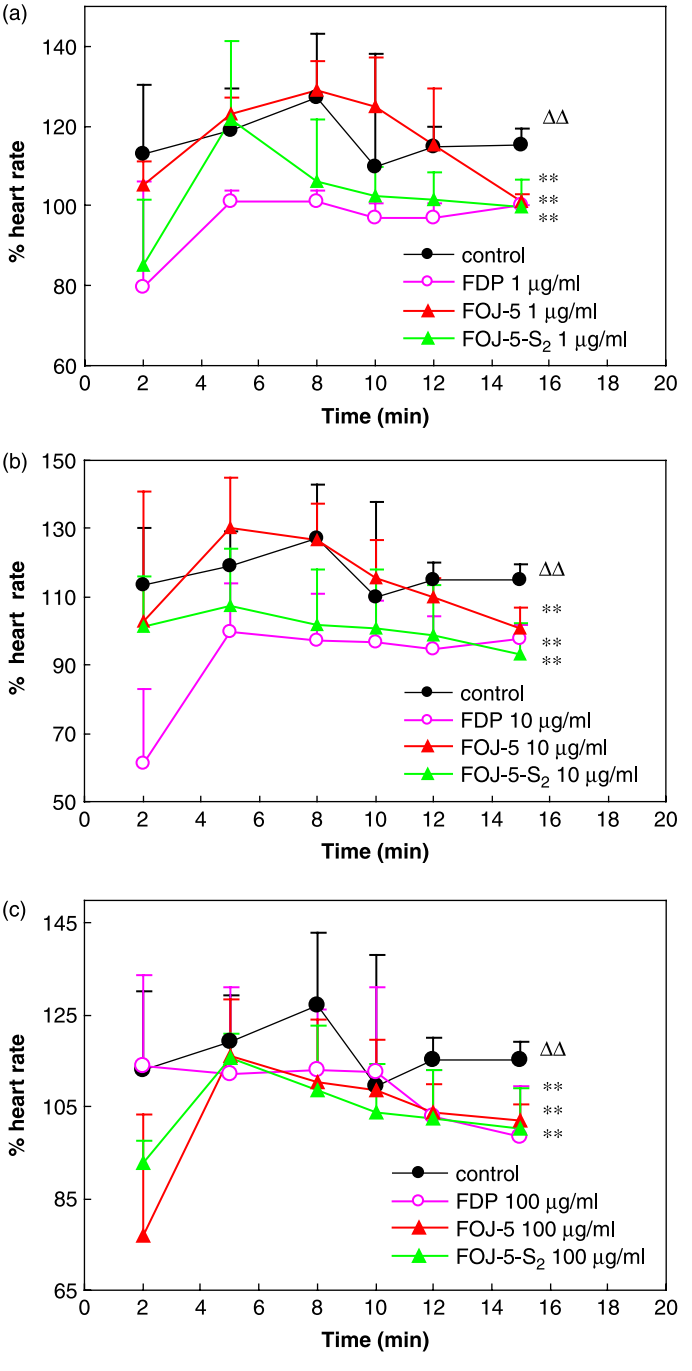

Figure 7. The action of FOJ-5 and FOJ-5-S_2 on the heart rate of isolated myocardium of Guinea pigs (the value after perfusion with K–H for 15 min was considered as 100%). When compared with the model, pretreatment with samples (in concentration of (a) 1, (b) 10, and (c) 100 μg/ml, respectively) significantly decreased the heart rate. Data are mean ± SD ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$ versus control, $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$ versus before ischemia.

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FOJ-5-S_2 (1–100 μg/ml) could significantly resume coronary blood flux at 10 min (98.7 ± 12.6, 90.2 ± 6.8, 93.0 ± 1.6, $P < 0.01$) and 15 min (88.8 ± 4.2, 82.8 ± 8.1, 84.4 ± 2.2, $P < 0.01$) after reperfusion when compared with the control (57.6 ± 3.3; Figure 8).

The above results indicated that FOJ-5 and FOJ-5-S_2 could reduce the injuries caused by ischemia and reperfusion.

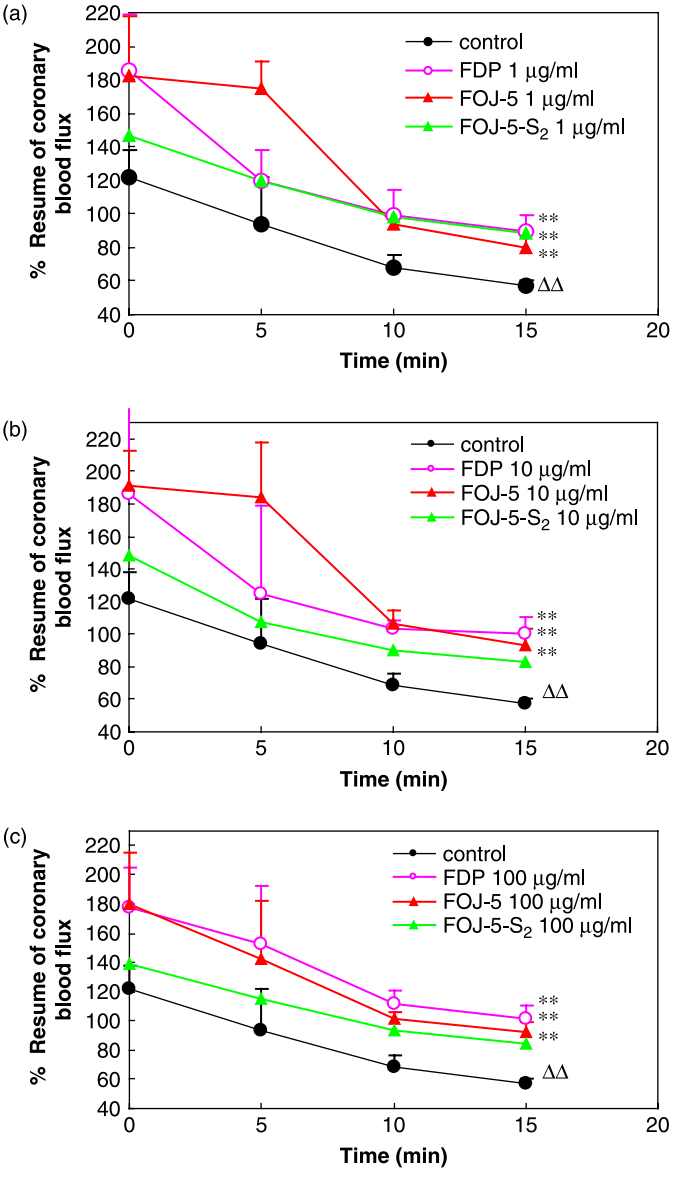

Figure 8. The action of FOJ-5 and FOJ-5-S_2 on the coronary blood flux of isolated myocardium of Guinea pigs (the value after perfusion with K–H for 15 min was considered as 100%). When compared with the model, pretreatment with samples (in concentration of (a) 1, (b) 10, and (c) 100 μg/ml, respectively) significantly resume coronary blood flux. Data are mean ± SD ($n = 6$). $*P < 0.05$, $**P < 0.01$ versus control, $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$ versus before ischemia.

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Among all the groups, the actions in 1 μg/ml of FOJ-5-S_2 and 100 μg/ml of FOJ-5 groups were distinguished.

2.4 Influences on acute myocardial ischemia

Since lactate dehydrogenase (LDH) always exists in endochylema, the release of LDH might indicate the damage of cell membrane. It showed that the value of LDH (3979 ± 720) and *J* point shift (0.71 ± 0.26) of electrocardiograms (ECG) in the model group was significantly increased when compared with the vehicle (2785 ± 209, 0.23 ± 0.10, $P < 0.01$).

About 20 mg/kg of FOJ-5-S_2 could significantly reduce the release of LDH (2897 ± 348, $P < 0.01$) and *J* point shift of ECG (0.45 ± 0.19, $P < 0.05$) compared with the model (3979 ± 720, 0.71 ± 0.26). The effects of 40 mg/kg of FOJ-5 and FOJ-5-S_2 on reducing the release of LDH (3250 ± 551 and 3148 ± 433) were also significant ($P < 0.05$). More intriguingly, 20 mg/kg of FOJ-5-S_2 had more significant effect on the release of LDH (2897 ± 348) than the same amount of FOJ-5 (3407 ± 566, $P < 0.05$). Moreover, 20 mg/kg of FOJ-5-S_2 decreased *J* point shift of ECG significantly (0.45 ± 0.19, $P < 0.05$), but either 20 mg/kg or 40 mg/kg FOJ-5 had no significant effect (0.58 ± 0.27, 0.58 ± 0.21) when compared with the model (0.71 ± 0.26). These results indicated that the action of FOJ-5 was enhanced after being sulfated (Figures 9 and 10).

Fructan can stabilize the lipid structure of cell membrane under dry and freezing conditions in plants, but the mechanism is unclear yet [10]. Did the results obtained in our experiment indicate that FOJ-5 and its sulfate can protect cell membrane of cardiomyocyte? In order to answer the question, researches on the protection of FOJ-5 and FOJ-5-S on cell damage in cultured cardiac cells were carried out. The results demonstrated that FOJ-5 and FOJ-5-S could protect cardiomyocyte from the damage induced by hypoxia/reoxygenation. The mechanism might be related to the protection of cells and inhibition of their apoptosis.

2.5 Anti-myocardial ischemic activity of FOJ-5-S with different DS

Following the finding that FOJ-5-S had the significant anti-myocardial ischemic effect, the activities of FOJ-5-S with different DS were studied. In the experiment, the dosages of FOJ-5-S were selected according to the previous results. Results showed that the value of LDH (3675 ± 596) and *J* point shift (1.29 ± 0.38) of ECG in the model group was significantly increased when compared with the vehicle (2944 ± 521, $P < 0.05$; 0.69 ± 0.15, $P < 0.01$).

It was found that 20 mg/kg of FOJ-5-S_1 (DS = 0.03) and FOJ-5-S_2 (DS = 0.08) could significantly reduce the release of LDH (2716 ± 453, 2765 ± 335, $P < 0.01$) and *J* point shift of ECG (0.83 ± 0.20, 0.75 ± 0.31, $P < 0.01$) compared with the model (3675 ± 596, 1.29 ± 0.38). In addition, the actions of 10 mg/kg of FOJ-5-S_1–FOJ-5-S_3 and 20 mg/kg of FOJ-5-S_3 were also significant ($P < 0.05$). However, the action of 20 mg/kg of FOJ-5 was not significant. These results indicated that sulfation could enhance the anti-myocardial ischemic activity of FOJ-5. Moreover, the DS of FOJ-5-S had considerable influence on its activity. FOJ-5-S had anti-myocardial ischemic activity only when the DS was between 0.03 and 0.1 and further increasing of DS to 0.13 led to the disappearance of activity, indicating that there was an optimal DS range for FOJ-5-S (Figures 11 and 12). Furthermore, detailed works on finding out the optimal DS and dose should be done in the later researches.

From all the results, it can be deduced that FOJ-5 and its sulfated derivatives could reduce the injury of myocardial ischemia, and the mechanism might be related to increasing coronary blood flux and the protection of cell membrane. Moreover, the degree of sulfation was important to its activity.

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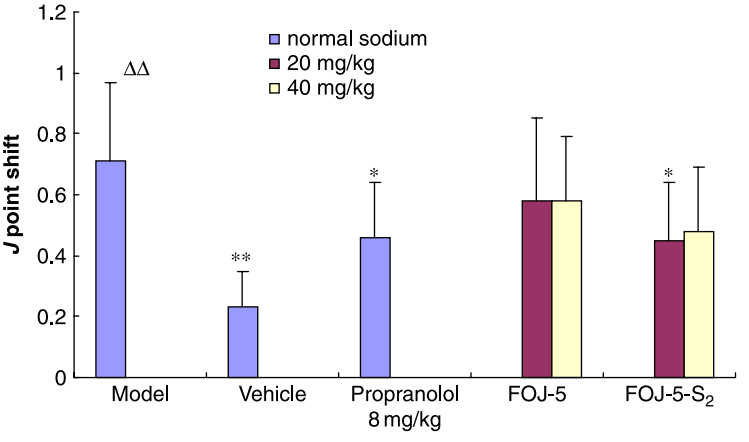

Figure 9. The effects of FOJ-5 and FOJ-5-S_2 on the *J* point shift (0.1 mV) of rats after isoprenaline (Iso) s.c. When compared with the model, pretreatment with samples, respectively (20, 40 mg/kg, p.o. for 3 days, once a day), significantly decreased the *J* point shift. Data are mean ± SD ($n = 8$). $*P < 0.05$, $**P < 0.01$ versus model; $^{\Delta\Delta}P < 0.01$ versus vehicle.

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3. Experimental

3.1 Materials

3.1.1 Animals

All animal experiments were conducted by the Pharmacological Laboratory of Shanghai Institute of Pharmaceutical Industry. Male Guinea pigs weighing ~200 g were purchased from Experimental Animal Center of Songjiang Chedun (Shanghai, China). Animal certification number was SCXK(Hu)2002-0013. Male Sprague–Dawley rats weighing 190–210 g were supplied by Experimental Animal Center of Silaike Co. Ltd (Shanghai, China). Animal certification number was SCXK(Hu)2003-0003. All the rats were maintained under standard laboratory conditions at 25 ± 2°C, relative humidity 50–70%, and a natural light. The animals were provided with standard diet and water *ad libitum.*

3.1.2 Reagents

O. japonicus from Cixi City, Zhejiang Province was supplied by Shanghai Decoction Pieces Factory. The isolation process and

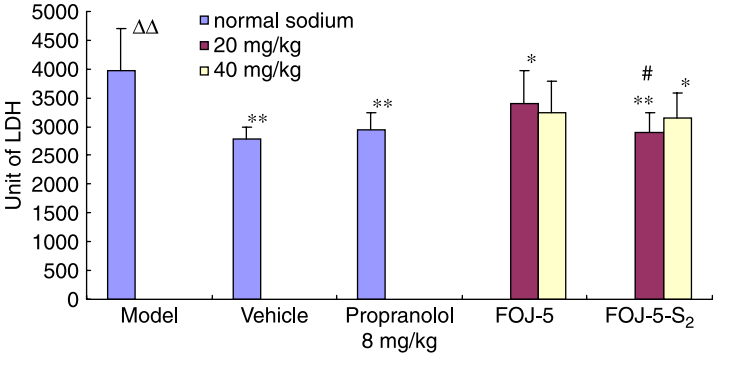

Figure 10. Effects of FOJ-5 and FOJ-5-S_2 on the release of LDH of rats after Iso s.c. When compared with the model, pretreatment with samples, respectively (20, 40 mg/kg, p.o. for 3 days, once a day), significantly decreased the release of LDH. Data are mean ± SD ($n = 8$). $*P < 0.05$, $**P < 0.01$ versus model; $^{\Delta\Delta}P < 0.01$ versus vehicle; $^{\#}P < 0.05$ versus 20 mg/kg FOJ-5 group.

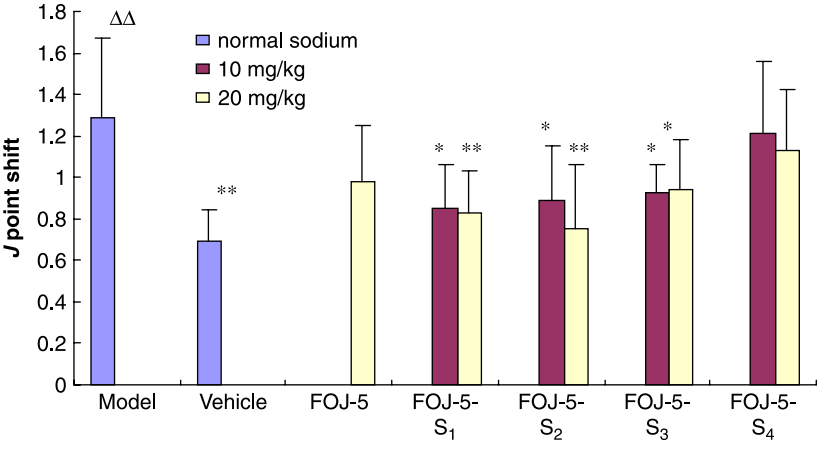

Figure 11. Effects of the DSF of FOJ-5-S on the activity of the *J* point shift (0.1 mV) of rats after Iso s.c. When compared with the model, pretreatment with samples, respectively (10, 20 mg/kg, p.o. for 3 days, once a day), significantly decreased the *J* point shift. Data are mean ± SD ($n = 8$). $*P < 0.05$, $**P < 0.01$ versus model; $^{\Delta\Delta}P < 0.01$ versus vehicle.

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the average molecular weight of FOJ-5 were the same as those described in the literature [6]. Positive control sample – FDP powder for injection was purchased from Xinya Pharmaceutical Co. Ltd (Shanghai, China). LDH assay kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Standard dextran with molecular weight of 5900, 5000, and 738 kDa, sucrose (342 kDa), and glucose (180 kDa) were purchased from Pharmacia Co. (Piscataway, NJ, USA). All other chemicals were reagents of analytical grade obtained from standard commercial sources.

3.1.3 *Apparatus*

515 HPLC, Waters 2410 RI detector (Waters, Milford, MA, USA); Perkin-Elmer Infrared Analyzer (Perkin-Elmer, Boston, MA, USA);

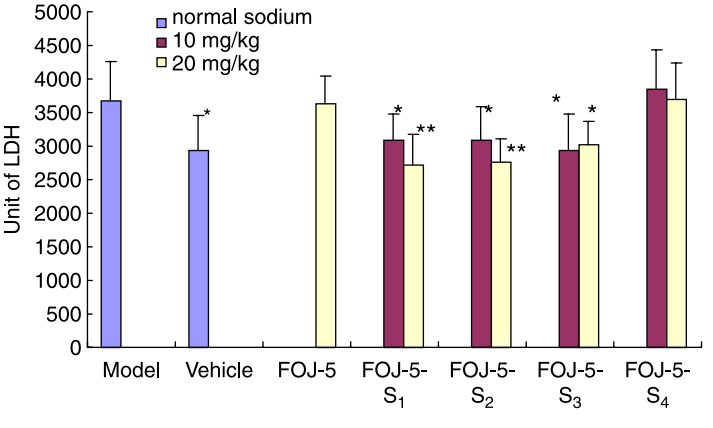

Figure 12. Effects of the DSF of FOJ-5-S on the activity of LDH of rats after Iso s.c. When compared with the model, pretreatment with samples, respectively (10, 20 mg/kg, p.o. for 3 days, once a day), significantly decreased the release of LDH. Data are mean ± SD ($n = 8$). $*P < 0.05$, $**P < 0.01$ versus model; $^{\Delta}P < 0.05$ versus vehicle.

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Büchi 461 rotation evaporator (Büchi, Flawil, Switzerland, Sweden); 722 spectrophotometer (Shanghai, China); Nanoscope IIIa (Digital Instruments, Santa Barbara, CA, USA); chromatographic column Shodex Sugar KS-G and Shodex Sugar KS-802 (Shoxwa, Tokyo, Japan); Langendorff perfusion system (powerlab/8sp, Castle Hill, Australia); LMS-2B physiological grapher (Chengdu, China); ECG 6511 electrocardiograph (NIHON-KOHDEN, Tokyo, Japan).

3.2 Methods

3.2.1 Sulfation of FOJ-5

Sulfated FOJ-5 was prepared as follows [11]: FOJ-5 (1.0 g) was suspended and stirred to dissolve in 100 ml of DMSO. Then 150 ml of DMF containing DCC (15.3 g) was added. The mixture was stirred and cooled to 0°C rapidly. After that, 100 ml of DMF containing H_2SO_4 (9.7 g) was added slowly. After being stirred at 30°C for 30 min, the mixture was poured into ice in a beaker, neutralized with 1 M NaOH solution, filtered, and then dialyzed for 48 h with tap water and 48 h with distilled water in dialysis tubing. The dialysate was concentrated under reduced pressure at 40°C. After being dried by lyophilization, the FOJ-5-S_2 was collected.

3.2.2 Characterization

FT-IR spectra were recorded with KBr tablets on a Perkin-Elmer IR spectrophotometer with the scanning wave number ranging from 4000 to 400 cm^{-1}.

The determination of DS of FOJ-5-S is based on the reaction between sodium rhodizonate and barium, which forms a colored complex [12]. Sodium rhodizonate solution was prepared as follows: 5 mg sodium rhodizonate was dissolved in 20 ml water with 100 mg vitamin C was added to promote dissolution. After that, all of them were dissolved, ethanol was added to make up the volume to 100 ml. A kind of bright yellow solution was obtained, which can be used after 30 min. Barium chloride ($BaCl_2$) buffer, which contained 2 M acetic acid 10 ml, 0.005 M $BaCl_2$ 2 ml, and 0.02 M $NaHCO_3$ 8 ml, was made up the volume to 100 ml by ethanol. Standard stock solutions of Na_2SO_4 contained 208 μg/ml natrii sulfas exsiccatus. Aspirate precisely standard solution of Na_2SO_4 proper amount to obtain solution containing 0, 2.08, 4.16, 6.24, 8.32, 12.48, and 16.64 μg of Na_2SO_4, respectively, then make up the volume to 0.5 ml with water. After that, 2 ml alcohol was added (if there is precipitation, centrifuge to clear is needed), then 0.1 ml $BaCl_2$ buffer and 1.5 ml sodium rhodizonate were added. The solution was mixed sufficiently and standby in dark at room temperature for 10 min; then the absorbance was determined at 520 nm. The regression equations were calculated in the form of $Y = AX + B$, where Y and X were absorbance and the amount of Na_2SO_4, respectively. All the FOJ-5-S samples were dissolved in water to 200 μg/ml in concentration and the measurement method was the same as what was described above. The amount of SO_4^{2-} and the DS of FOJ-5-S were calculated according to the following formula:

$$S\% = (SO_4^{2-}\% \times 32)/96$$

$$DS = (1.62 \times S\%)/(32 - 1.02 \times S\%)$$

The molecular weight and the purity of samples were measured by HPGPC. GPC system incorporated with a Waters® HPLC. The column was Shodex sugar KS-802. The mobile phase was 0.1 M NaAc at a flow rate of 1.0 ml/min. The temperature of the column was maintained at 30°C. The eluent was monitored with RI 2410 refractive index detector. The samples and standard dextrans dissolved in 0.1 M NaAc to 2.0 mg/ml were filtered with a 0.22 μm filter (Anpu Technology Co., Hunan, China), and then kept in sealed glass bottles before injection into the GPC column. The retention times of all the samples and standard dextrans were recorded.

The molecular morphology FOJ-5 and FOJ-5-S were observed directly by AFM. FOJ-5 and FOJ-5-S_2 were dissolved in

distilled water and diluted to 0.1 μg/ml, and 5 μl of the two samples were deposited on freshly cleaved mica surface, respectively. After open-air drying, they were imaged with AFM in air at room temperature in tapping model with the scan frequency of 2 Hz.

3.2.3 Isolated myocardial ischemia–reperfusion action on Langendorff model

FOJ-5 and its FOJ-5-S were dissolved in K–H solution to 1, 10, and 100 μg/ml as different dosage perfusion solution for isolated rat heart. FDP was dissolved in K–H solution to 1, 10, and 100 μg/ml for positive control perfusion solution. Control group was provided with K–H solution in the experiment.

Male Guinea pigs with a weight of about 200 g were knocked down by pound at occiput. A thoracotomy was performed and the hearts were rapidly isolated and immersed in ice-cold K–H solution. The aorta was cannulated, and the hearts were perfused in a retrograde fashion, at a pressure of 7.0–7.5 kPa and the flow rate of 7–10 ml/min with K–H solution which was continuously bubbled with 95% oxygen and 5% carbon dioxide and the temperature was maintained at 37 ± 0.5°C [13]. When the independent rhythm was restored, the heart was continuously perfused with K–H solution for 15 min to achieve a stable condition. After that, the amplitude, frequency of cardiac contraction, and coronary blood flow were measured as the basic values 100%. After that, ischemia reperfusion injury group continuously perfused with K–H solution for 10 min, but the other groups were perfused with K–H solution containing samples with concentration of 1, 10, and 100 μg/ml, respectively, for 10 min. The amplitude and frequency of heart contraction and coronary blood flux of all the groups were measured.

After that, all isolated hearts were subjected to 30 min of ischemia followed by 15 min of reperfusion with the same solution as before. The amplitude and frequency of heart contraction of all the groups were measured after perfusion with different samples solution for 2, 5, 8, 10, 12, and 15 min. Coronary blood flux of all the groups was measured after reperfusion with different samples solution for 0, 5, 10, and 15 min.

3.2.4 The protection of FOJ-5-S_2 in vivo *(acute myocardial ischemic action)*

FOJ-5 and FOJ-5-S_2 were dissolved in normal sodium to 2 and 4 mg/ml, respectively. Propranolol was dissolved in normal sodium to 8 mg/ml as positive control. Blank control and model control were set up in the study. The experiment was carried out as follows [14]: FOJ-5 and FOJ-5-S_2 were given to rats by i.g. administration at a dose of 20 and 40 mg/kg body weight, respectively, once a day for 3 days. On the second and third days, groups with the exception of blank control were given Iso at a dose of 8 mg/kg body weight by subcutaneous injection at 1 h after the sample medicines were given. The blank control rats were given normal saline with the same method. The absolute values of the difference of electrocardiographic *J* points before the medication and 30 min after the second administration of isoprenaline were recorded. Then the rats were given anesthesia with chloral hydrate and the blood sample in abdominal aorta was taken with heparin as anticoagulant. The plasma was separated and LDH in plasma was determined spectrophotometrically using LDH assay kits.

3.2.5 The protection effect of FOJ-5-S with different DS in vivo

FOJ-5 was dissolved in normal sodium to 2 mg/ml and all the FOJ-5-Ss were dissolved in normal sodium to 1 or 2 mg/ml. All the samples were given to rats by i.g. administration at a dose of 10 and 20 mg/kg body weight, respectively, once a day for 3 days. The following administration and measurement are the same as what was described above. The blank control and model control were set up in the study.

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3.3 Statistical analysis

Quantitative data are expressed as mean ± SD of n observations. Data were analyzed by independent-samples t-test. In all cases, differences were considered statistically significant if $P < 0.05$.

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