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4-*O*-Sulfation in sea cucumber fucodians contribute to reversing dyslipidiaemia caused by HFD

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ABSTRACT

Fucodians extracted from sea cucumbers (SC-FUCs) possess linear chains with uniform repeating units. Sulfation patterns endow SC-FUCs unique structures and bioactivity. The present study investigated the anti-hyperlipidemic activity of two fucodians isolated from sea cucumbers *Pearsonothuria graeffei* (fuc-*Pg*) and *Isostichopus badionotus* (fuc-*Ib*). The results indicate fuc-*Pg* dominated with a 4-*O*-sulfation pattern shows strong activity in reducing body weight, regulating lipid disorder (TC, TG, HLD-C, and LDL-C level), improving liver function (liver weight, GOP, GPT, and TBA concentrations) and increasing adiponectin level (adiponectin concentration) caused by HFD. However, fuc-*Ib* dominated with a 2-*O*-sulfation pattern has only moderate effects. These results suggest that different sulfation pattern may contribute to the differences in the hyperlipidemic activities. Further analysis by quantitative reverse transcription-polymerase chain reaction and Western blot analysis indicate that Fuc-*Pg* can suppress the expression of CD 36, increase the level of PPARα and decrease the level of CYP7A1, thus, the transportation of fatty acids into liver tissue and lipid metabolism, while fuc-*Ib* had only limited effects. Our results indicated the sulfation pattern may contribute to anti-hyperlipidemic activity of fucodian, and fuc-*Pg* dominated with 4-O-sulfation shows better effect and could be further developed as a potential anti-hyperlipidemic food supplement.

1. Introduction

Over nutrition has been a risk factor for human beings [1], resulting in abnormalities of lipid homeostasis, is associated with hyperlipidemia, obesity, insulin resistance, type 2 diabetes mellitus, and non-alcoholic fatty liver, all risk factors for cardiovascular diseases (CVD) [2]. Recent study indicated that one of the most strongly predictive cardiovascular risk factors for myocardial infarction is dyslipidemia characterized by elevated concentrations of serum triglyceride (TG) as well as increased levels of low-density lipoprotein cholesterol (LDL-C) and decreased levels of high-density lipoprotein cholesterol (HDL-C) [3]. Therefore, any rational strategies for the prevention and treatment of atherosclerosis as well as the reduction of the incidence of related cardiovascular diseases should be closely associated with targeting hyperlipidemia by drugs and/or dietary intervention. Meanwhile, growing evidence establishes that the imbalanced gene expressions of CD36, peroxisome proliferator-activated receptors (PPARs), Acyl coenzyme A: cholesterol acyltransferase-1 (ACAT1), ATP-binding cassette transporters A1 (ABCA1), and scavenger receptor B1 (SR-B1) mediate the foam cell formation by impairing the balance of cholesterol influx and efflux [4]. Among these proteins, some were reported to be strongly associated with the regulation of dyslipidemia. For instance, CD36 functions as a scavenger receptor that can transfer fatty acids from serum into cellular tissues and responsible for increasing hepatic fatty acid uptake [5]. Statins are modern lipid-modifying therapy in patients with hypercholesterolemia, mixed hyperlipidemia and a history of cardiovascular disease [6]. However, excessive intake of statins brings about side effects such as myopathy. Since dietary intervention has been one of the promising strategies for prevention and treatment of metabolic diseases,

Abbreviations: SC-FUC, sea cucumber fucoidan; fuc-*Ib*, fucoidan from *Isostichopus badionotus*; fuc-*Pg*, fucoidan from *Pearsonothuria graeffei*; HFD, high-fat diet; TC, total cholesterol; TG, triglyceride; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; GOT, glutamic-oxaloacetic transaminase; GPT, glutamic pyruvic transaminase; TBA, total bile acids; MW, molecular weight; SDF, soluble dietary fiber.

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it is important to explore food-grade ingredients to prevent and treat lipid disorder and improve the health of body.

Fucoidans, are a type of polysaccharide with a backbone of sulfated L-fucose units isolated from marine algae and marine invertebrates, which possess various activities for health [7]. Administration of fucoidan from *Laminaria japonica* decreased serum TC, TG, and LDL-C concentrations, while increasing HDL-C levels, in a high-fat emulsion-fed hyperlipidemic rat model [8]. Fucoidan from *Cladosiphon okamuranus* also reduced the isoproterenol-induced increases in concentrations of TC, TG, and LDL-C but increased that of HDL-C in a rat model of myocardial infarction [9]. These benefits of fucoidan may be mediated by the inhibition of lipid accumulation [10]. However, it remains unclear whether and how the structure of fucoidans affects hypolipidemic activities. This is because the fucoidans from marine algae are typically hetero-polysaccharides with complex chemical compositions and side chains [11–13].

Fucoidans from marine invertebrates usually contain linear structures with uniform repeating units, which makes it possible to investigate their structure–function relationship. Fucoidans containing uniform repeating tetrasaccharides units with α-1, 3-glycosdic linkage have been isolated from sea cucumbers (SC-FUC) (Fig. 1) [14–18]. These fuoidans differ in their sulfation pattern (i.e., 2-*O*-, 4-*O*-and 2, 4-*O*- sulfo group substitution) among different species. Although there have been reports showed that SC-FUC showed anticoagulant and antithrombotic activities [19], anti-hyperglycemic effects [20], neural protection [21] and anti-inflammation [22], little research has focused on the structure-activity relationship of SC-FUCs because of a lack of understanding of the structure of the fucoidan used in previous studies.

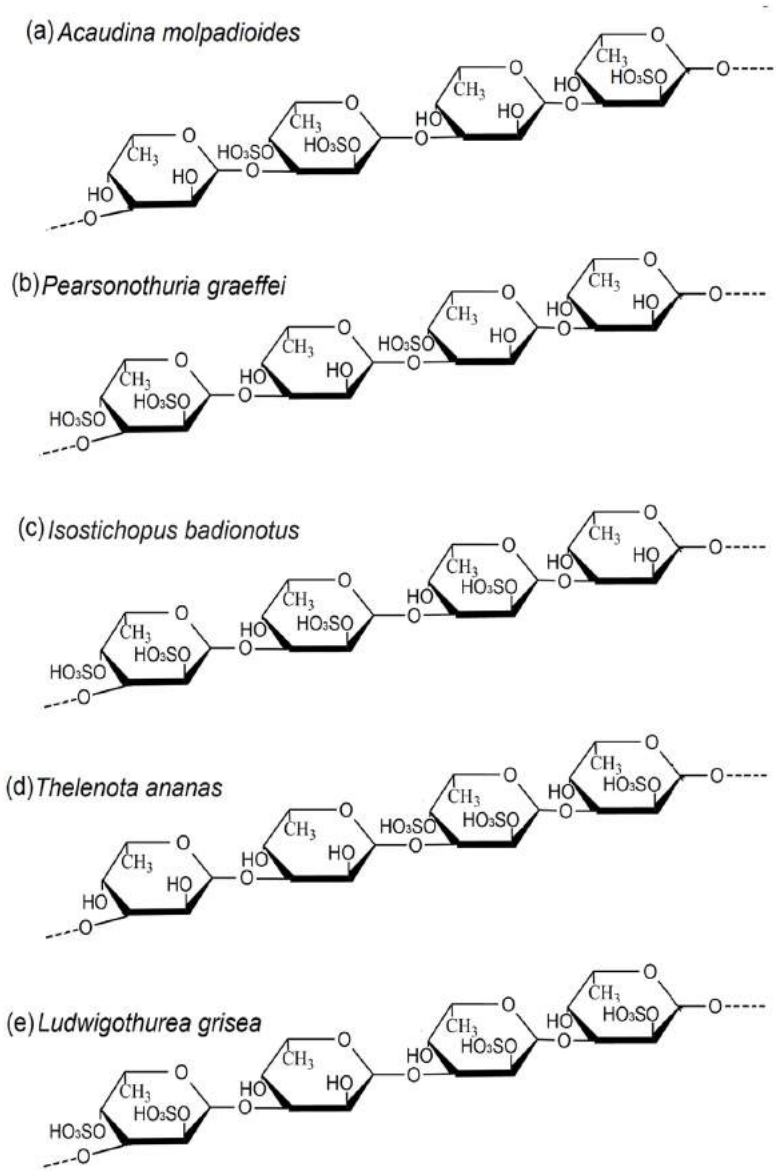

Fig. 1. Chemical structures of the well-repeated tetrasaccharide units of the fucodians from sea cucumber. The structures are the following: (a)*Acaudina molpadioides*, [Fuc α1 → 3Fuc (2S; 4S) α1 → 3Fucα1 → 3Fuc (4S)α] $_n$; (b) *Pearsonothuria graeffei*, [Fuc (2S; 4S) α1 → 3Fucα1 → 3Fuc (4S) α1 → 3Fucα]$_n$; (c) *Isostichopus badionotus*, [Fuc (2S; 4S) α1 → 3Fucα(2S)1 → 3Fuc (2S) α1 → 3Fucα]$_n$; (d) *Thelenota ananas*, [Fuc α1 → 3Fucα1 → 3Fuc (2S; 4S) α1 → 3Fuc(2S)α]$_n$; (e) *Ludwigothurea grisea*, [Fuc (2S; 4S) α1 → 3Fucα1 → 3Fuc (2S) α1 → 3Fuc(2S)α]$_n$;.

In our former work, two fucoidans with regular structures, named as fuc-*Ib*[15] and fuc-*Pg* [14] were characterized by a combination of ES–CID–MS/MS and 2D NMR. Fuc-*Ib* having mainly 2, 4-disulfation pattern showed anticoagulant, anti-antithrombotic [15] and alleviated hepatic inflammation and insulin resistance [22]. In addition, a unique repeating structure was recently established for fuc-*Pg* and there have only been limited studies to explore its activities as a functional food ingredient or for pharmaceutical use. In the present study, fuc-*Pg* and fuc-*Ib*, the two fucodians with uniform repeating tetrasaccharides structures but having quite different sulfation patterns were used to explore anti-hyperlipidemic activity of SC-FUC. The relationship between structures and anti-hyperlipidemia activity was investigated using SD rats fed on HFD from lipid profile, liver function, adiponectin levels. The expression level of the target protein CD36, PPARα and CYP7A1 were investigated to better understand the effects of the fucoidan on the lipid metabolism. As far as could be ascertained from the literature, this is the first study exploring functional activity of fuc-*Pg* and comparing the functional activity of two SC-FUCs.

2. Materials and methods

2.1. Materials

Two species of sea cucumbers, *Pearsonothuria graeffei* (from Indo-Pacific) and *Isostichopus badionotus* (from Western Atlantic Ocean) were purchased from a local market in Qingdao, Shandong, China.

2.2. Preparation of sea cucumber fucodians

The fuc-*Pg* and fuc-*Ib* used in this paper were obtained from the same resource, prepared based on a previously described method [14,15], confirmed by high performance liquid chromatography (HPLC) and ^{1}H NMR. Briefly, the dry sea cucumber body wall (ca. 100 g) was minced and homogenized. The homogenate was digested with papain at 60 °C for 10 h in a solution containing 5 mM EDTA and 5 mM cysteine. Polysaccharide in the clear supernatant fractions was precipitated with 160 ml of 10% cetylpyridinium chloride solution. After incubation at room temperature for 24 h, the mixture was centrifuged (2000 × *g* for 15 min). The precipitated sulfated polysaccharide was dissolved with 1000 ml of 3 M NaCl: ethanol (100:15, v/v) solution and then 600 ml of 95% ethanol were added to precipitate chondroitin sulfate. After centrifugation (2000 × *g* 15 min) and removal of the precipitate, another 900 ml of ethanol was added to the supernatant to a final concentration of 60%. The precipitate formed was collected by centrifugation (2000 × *g*, 15 min) and dissolved in water before dialysis against water for 24 h. The retained solution was lyophilized and crude fucoidans was obtained.

The crude fucodians solution was fractionated by anion exchange chromatography on a Q-Sepharose Fast Flow column (4.6 × 20 cm) with elution by a linear gradient of 0–3.0 M NaCl in1000 min at a flow rate of 2 ml/min. Carbohydrate fractions were collected every 6 min with a test tube. Polysaccharide content was determined by the improved phenol-sulfuric acid method at 490 nm. The purified polysaccharide was collected, dialyzed, and lyophilized.

2.3. Animals and experimetal design

Fifty-six Sprague–Dawley rats, male, weighting from 180 to 220 g (4 weeks old), were purchased fromthe Animal Lab Center of Zhejiang Chinese Medical University (Certificate No. SCXK131(Hu) 2007-2005, China). The animals were housed in stainless steel cages at room temperature (25 ± 2 °C) and 12 h light cycle. The animals were fed with a commercial mice chow for 7 days to acclimatize to animal facilities. Then, animals were weighed and randomly divided into five groups of 8 rats. Group (1) was normal control while group (2) served as hyperlipidemic control group (3) had the standard drug(simvastatin, 5 mg/kg) treated animals that served as positive control. Groups (4), (5) received fuc-*Pg*,fuc-*Ib* in doses of 40 mg/kg. After the period of acclimation ended, group (1) continued tobe provided with the common commercial mice chow and others were fed with a HFD for 28 days. At the same time, groups (3)–(5) were given different doses of simvastatin, fuc-*Pg* and fuc-*Ib* by oral administration for 28 days by gavage. Simvastatin, fuc-*Pg* and fuc-*Ib* were dissolved in 0.9% saline. The rats were allowed free access to food and water during the experimental period. The composition of HFD was 1% cholesterol, 10% lard, 10% yolk powder, and 79% commercial chow. The weight gains of rats were measured once per week.

2.4. Plasma biochemistry analysis

At the end of the experimental period (28 days), the rats were starved for 24 h, weighed and anesthetized. Blood samples were collected from the eyeballs for following analysis: Levels of serum lipids including total cholesterol (TC), TG, HDL-C and LDL-C levels were measured enzymatically by assay kits (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) as the manufacturer's instructions.

The concentrations of glutamate oxaloacetate (GOT) and GPT were measured using commercial kits (Sigma Chemical Co., MO, USA) based on the former method [23]. TBA was measured by a direct spectrophotometry method according to the former report [24] using a commercial kit (Nanjing Jiancheng Bioengineering Institute, Jiangsu,China).

The adiponectin content was measured using commercial ELISA kits (R&D Systems, USA).

2.5. Determination of liver weight

At the end of the experimental period (28 days), the rats were starved for 24 h and anesthetized. Their livers were quickly removed and weighed.

2.6. Real-time quantitative PCR

Total RNA was isolated using a total tissue TRIzol® Plus RNA Purification Kit (Invitrogen, America). Equal amounts of total RNA were used to synthesize cDNA with the Quant II fast RT kit (Tools, Taiwan). Quantitative real-time reverse-transcription PCR (qRT–PCR) was performed in triplicate using SYBR Green, 384-well plates and the CFX384 Touch Real-Time PCR System (Bio-Rad, USA). Each well was loaded with a total of 20 μl containing 1 μl of cDNA, 1 μl of target primers, and 8 μl of SDW and 10 μl of Power SYBR® Green Master Mix. Hot-start PCR was performed for 40 cycles, with each cycle consisting of denaturation for 15 s at 94 °C, annealing for 30 s at 60 °C and elongation for 30 s at 72 °C. The primers of CD36, forward: 5′GGCGATGAGAAAGCAGAAATG3′, Reverse: 5′CACTACTCCAACACCAA-GTAAGA 3′. The housekeeping gene β-actin was used as a control. PCR products were quantitated using the software iCycler iQ5 (Bio-Rad, USA). The mRNA relative expression levels were expressed as the ratio of signal intensity for the target genes to that of β-actin.

2.7. Western blot

Liver tissue (100 mg) was homogenized in a commercial Pro-Prep Protein Extraction Solution (Intron Biotechnology, South

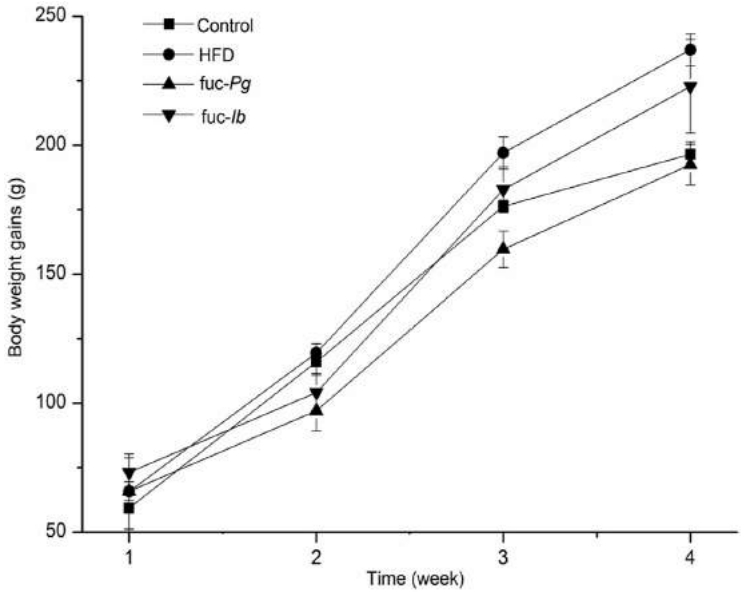

Fig. 2. The body weight gains of normal group, HFD group, fuc-*Pg* group and fuc-*Ib* group per week compared with initial weight. Body weight was measured every day. Data are expressed as mean ± SD (n = 8).

Korea). All proteins were denatured at 100 °C for 10 min and stored at −80 °C for Western blot analysis. Total protein lysates were fractionated on a 10% sodium dodecyl sulfate–polyacrylamide gel and electro-blotted onto polyvinylidene difluoride membranes (Immobilon TM-P; Millipore, USA). Membranes were blocked with 5% non-fat milk for 1 h at room temperature in TBST buffer (Tris 10 mM, NaCl 150 mM, pH 7.6, 0.1% Tween 20) and probed with primary antibodies (Santa Cruz, America) overnight at 4 °C. Membranes were then incubated with horseradish peroxidase-conjugated secondary antibody, exposed by X-ray film for 10 min and the density of bands were analyzed by BandScan5.0.

2.8. Statistical analysis

All of the numeric results are the mean ± SD. Repeated measures ANOVA was used to evaluate any changes in food utilization among groups. Other comparisons among the groups were performed with one-way ANOVA followed by an LSD or Dunnett's T3 post-hoc test. SPSS 22 was used for all analysis. Differences were defined as statistically significant for values of $P < 0.05$.

3. Results

3.1. Effects of SC-FUCs on reducing weight gains caused by HFD

After four weeks, all the five groups of rats gained weight more than two-fold compared with their initial weight. Compared with the normal group, HFD group had a 20.6% weight gain (Table 1), indicating SD rats became obese by excessive fat intake. Simvastatin, fuc-*Pg*, and fuc-*Ib* groups all reduced body weight gains than that of HFD groups, and the fuc-*Pg* gains less weight than normal control group, despite no significant difference between two groups ($P > 0.05$). The weekly changes in body weight also indicated two types of fucoidans exhibited different ability to control the body weight (Fig. 2), and fuc-*Pg* group gained less body weight than fuc-*Ib*. Fuc-*Ib* was also gained less weight than HFD Group, but the effect was limited ($P > 0.05$ compared with HFD group). The results indicated that fuc-*Pg* exhibited an excellent ability to control the weight gains caused by HFD.

3.2. Effects of SC-FUCs on the regulating hyperlipidemia caused by HFD

Hyperlipidemia can be characterized as raised blood cholesterol and triglycerides levels. It is often associated with raised levels of LDL, a type of lipoprotein that transports cholesterol and triglycerides from the liver to peripheral tissues. HDL, another type of lipoprotein, enables cholesterol and triglycerides to be transported within the blood stream [25]. Elevated LDL-C levels are a severe risk factor for atherosclerosis and the role of HDL-C is controversial [6]. The results indicated that HFD disturbed the plasma lipid level of SD rats, elevated TC, TG, and LDL-C content, reduced HDL-C content compared with the control group (as shown in Table 2). The hyperlipidemia caused by HFD was alleviated in different extent treated with simvastatin, fuc-*Pg*, and fuc-*Ib*.

For the TC content, fuc-*Pg* group significantly lowered the TC level by 25.6% ($P < 0.05$, compared with the HFD rats), which was no significant difference ($P < 0.05$) compared to normal group. The

Table 1
Effects of simvastatin, fuc-*Pg*, and fuc-*Ib* on body weight in HFD-fed rats.

Groups	initial weight(g)	1st week(g)	2nd week(g)	3rd week(g)	final weight(g)	gain weight(g)
Normal	183.7 ± 4.0	239.7 ± 17.0	299.1 ± 14.7	355.5 ± 9.0	380.7 ± 8.8	196.5 ± 4.8*
Hyperlipidemia	178.2 ± 9.5	245.7 ± 17.6	300.8 ± 14.5	372.7 ± 14.1	414.3 ± 6.1	237.0 ± 7.0
simvastatin	188.0 ± 8.6	262.0 ± 13.0	297.0 ± 23.3	357.6 ± 27.4	388.5 ± 34.8	201.3 ± 19.9*
fuc-*Pg*	177.7 ± 9.2	243.4 ± 13.3	274.5 ± 18.9	337.9 ± 19.0	370.4 ± 20.3	193.8 ± 10.5*
fuc-*Ib*	180.4 ± 13.2	257.3 ± 20.1	283.1 ± 17.8	359.5 ± 24.7	398.8 ± 36.7	222.8 ± 18.2

* $P < 0.05$: compared with HFD group. The data are given as mean ± SD (n = 8). Body weight was measured per week.

Table 2
Effects of simvastatin, fuc-*Pg*, and fuc-*Ib* on the serum lipids of HFD-fed rats.

Groups	TC(mmol/L)	TG(mmol/L)	HDL-C(mmol/L)	LDL-C(mmol/L)
Normal	3.24 ± 0.07*	2.56 ± 0.16*	1.45 ± 0.21*	0.74 ± 0.04*
Hyperlipidemia	4.41 ± 0.15	4.22 ± 0.61	0.93 ± 0.06	0.91 ± 0.09
simvastatin	4.07± 0.57	3.55 ± 0.40*	1.13 ± 0.11*	0.60 ± 0.04*
fuc-*Pg*	3.28 ± 0.34*	2.86 ± 0.35*	1.17 ± 0.25*	0.73 ± 0.10*
fuc-*Ib*	4.36 ± 0.39	3.25 ± 0.21*	0.89 ± 0.11	0.91 ± 0.13

*$P < 0.05$: compared with HFD group. The data are given as mean ± SD (n = 8).

simvastatin groups could only reduce 7.7% ($P > 0.05$) than the HFD group, whereas fuc-*Ib* had almost no effect on lowering the content of TC.

The concentration of HDL-C at the serum of HFD rats decreased by 35.9% compared with the normal control. Treatments with simvastatin and fuc-*Pg* alleviated HDL-C level decreasing caused by HFD, which were improved 21.5% and 25.8% respectively compared with HFD group. However, fuc-*Ib* had no effect on the HDL-C level ($P > 0.05$ *vs*. HFD group). LDL-C level increased by 23.0% for SD rats fed on HFD, compared with the normal group ($P < 0.05$). Simvastatin and fuc-*Pg* administration alleviated the LDL-C improving, lowering the content by 34.1% and 19.8% respectively ($P < 0.05$ *vs*. HFD group). Besides, fuc-*Ib* remained no effect on the LDL-C level as HDL-C level ($P > 0.05$ *vs*. HFD group).

As for TG levels, simvastatin, fuc-*Pg*, and fuc-*Ib* administration significantly decreased TG by 15.9%, 32.2%, and 23.0% respectively, as compared with the HFD group ($P < 0.05$). Both two fucoidans had impact on lowering TG level and fuc-*Pg* was better. It was noteworthy that there was no significant difference between fuc-*Pg* group and the normal group ($P > 0.05$).

Our results showed both fucoidans could low the TG level caused by HFD. Fuc-*Pg* had better effect on lowing the TG level than fuc-*Ib*. As for TC, HDL-C, and LDL-C, fuc-*Pg* also exhibited the ability to alleviated serum lipid disorder caused by HFD. However, fuc-*Ib* had no effect on regulating abnormal cholesterol level.

3.3. Effects of the two SC-FUCs on protecting liver from HFD

Liver plays a key role in lipid metabolism, which is the hub of fatty acid synthesis and lipid circulation [26]. Excess lipid intake will cause the damage of liver or even induce fatty liver diseases, which is a major contributor to cardiovascular and overall obesity-related morbidity and mortality [2]. The final liver weight, the content of GOP, GPT, and TBA were detected in this work to evaluate the function of liver.

As shown in the Fig. 3a, liver weight of HFD group was significantly increased by 34.85% compared with the liver weight of the normal group ($P < 0.05$). As for sample groups, oral administration of simvastatin, fuc-*Pg*, fuc-*Ib* decreased the liver weight of rats fed on HFD by 12.4%, 19.5% and 10%, respectively ($P < 0.05$ *vs*. HFD). Both two types of fucoidans inhibited SD rats from liver weight gain

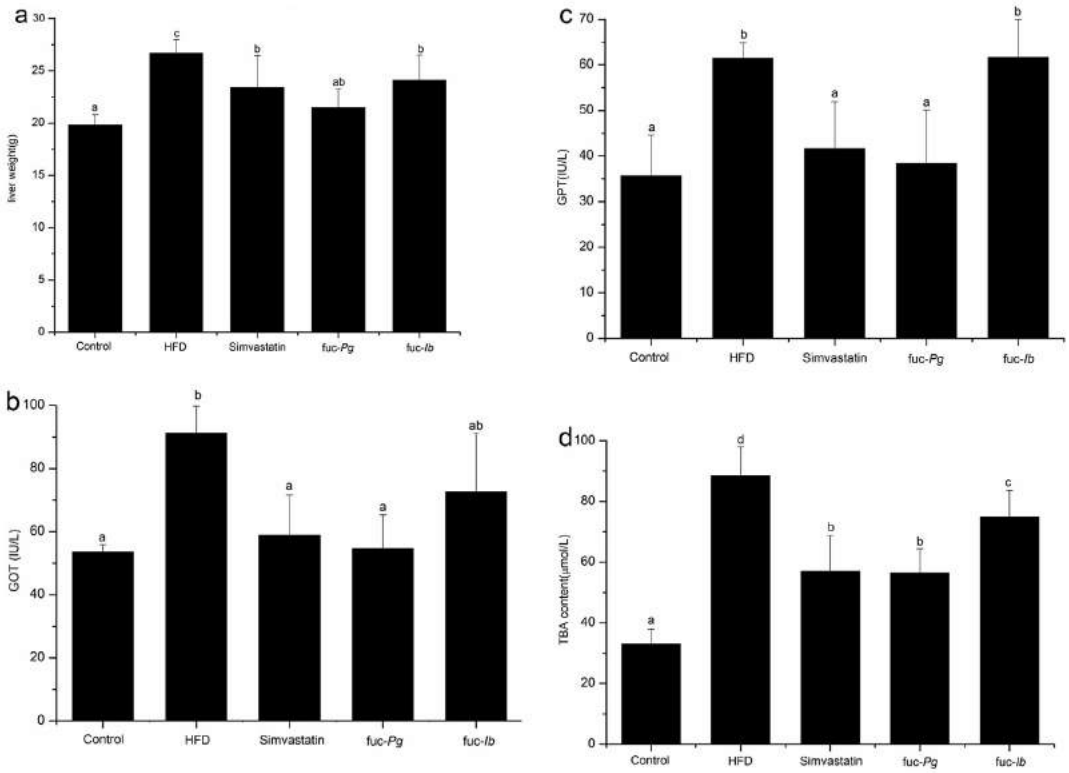

Fig. 3. Effects of simvastatin, fuc-*Pg* and fuc-*Ib* on liver weight (**a**); Effect of, simvastatin, fuc-*Pg* and fuc-*Ib* on levels of GOP **(b)**, GPT**(c)**, and TBA **(d)** in hyperlipidemia rats fed on HFD. Data are expressed as mean ± SD (n = 8). Multiple comparisons were done using one way ANOVA analysis followed by Dunnett's T3 post-hoc test. a, b, c, d: $P < 0.05$, compared between five groups.

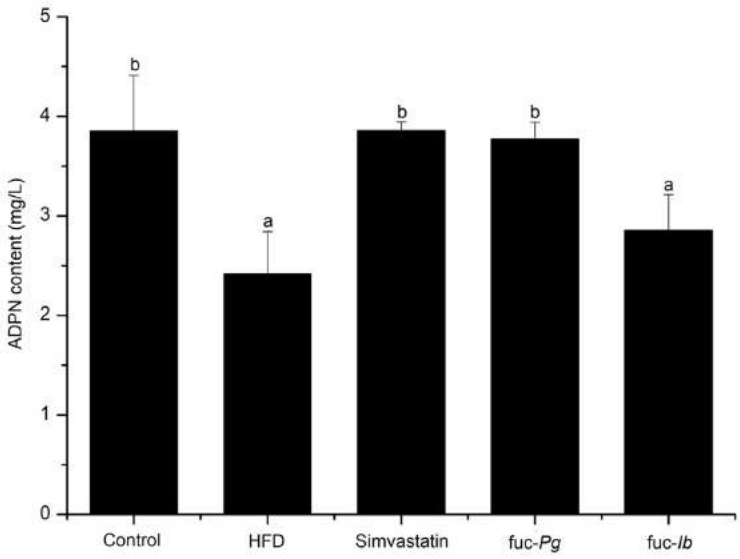

Fig. 4. Effects of simvastatin, fuc-*Pg* and fuc-*Ib* on adiponectin level in hyperlipidemia rats fed on HFD. Data are expressed as mean ± SD (n = 8). Multiple comparisons were done using one way ANOVA analysis followed by Dunnett's T3 post-hoc test. a, b, c, d: $P < 0.05$, compared between four groups.

caused by HFD. Oral administration of fuc-*Pg* decreased the weight significantly compared with the HFD group ($P < 0.01$). However, fuc-*Ib* showed less potent effects compared with fuc-*Pg* ($P > 0.01$, fuc-*Ib* *vs*. HFD).

Blood GOP and GPT levels are the most frequently reliable biomarkers of liver injury. GOP and GPT are primarily localized to liver. When there is damage to hepatocytes, GOP and GPT are released to the extracellular space and ultimately enter into circulation [27]. As shown in the Fig. 3b and c, administration of simvastatin and fuc-*Pg* made the level of GOT and GPT significantly decrease compared with the level of HFD group ($P < 0.05$), which showed no significant difference compared to the normal group ($P > 0.05$). However, the fuc-*Ib* showed no significant effect on ameliorating the abnormal content of GOT and GPT ($P > 0.05$ *vs*. the normal group).

Bile acids (BAs) play a number of roles in lipid metabolism. They are synthesized by a multistep enzymatic conversion of cholesterol in the liver, and then are delivered to the lumen of the small intestine acting as emulsifiers of dietary lipids, cholesterol, and fat-soluble vitamins [28]. Efficient reabsorption of BAs in the terminal ileum results in the accumulation of a certain mass of BAs within the body, referred to as the BA pool, which cycles between intestine and liver in the enterohepatic circulation [29]. When the liver is damaged, BAs are released into body circulation and leading a relatively high level [30]. According to our result (Fig. 3d), HFD increased the TBA level in the serum compared to the normal group. Simvastatin, fuc-*Pg* and fuc-*Ib* ameliorated the high content of TBA to 57.04 ± 11.78, 56.48 ± 7.96 and 74.85 ± 8.80 μmol/L, respectively ($p < 0.05$, *vs*. HFD). Although fuc-*Pg* group showed a significant difference ($p < 0.05$) in TBA level when compared with the normal group, the effect of fuc-*Pg* was still greater than fuc-*Ib*.

According to the results of final liver weight, GOP, GPT, and TBA levels, a conclusion was drew that fuc-*Pg* had a good ability to protect liver from HFD damage, while fuc-*Ib* had a limited effect on that.

3.4. Effects of SC-FUCs on the adiponectin level

Adiponectin is a hormone secreted by adipocytes that regulates energy homeostasis, glucose and lipid metabolism [31]. For the increase of the total body fat mass, the adipocytokines should be secreted more by adipose tissue in obesity. However, it is found that the levels of adiponectin in obesity were lower than those in non-obese subjects [32]. High adiponectin concentrations in the plasma are needed to perform normal physiological actions in the cardiovascular system. Adiponectin increases tissue fat oxidation, resulting in reduced circulating fatty acid levels and reduced intracellular triglyceride contents [33].

The function of fat tissues was evaluated by the adiponectin level in the serum (Fig. 4). HFD feeding made the adiponectin level lower than that of normal control group. The adiponectin levels of normal group, simvastatin and fuc-*Pg* were 3.85 ± 0.56 mg/L, 3.85 ± 0.09 mg/L and 3.77 ± 0.17 mg/L, respectively. There was no significant difference between three groups ($P > 0.05$). However, the fuc-*Ib* almost had no effect on ameliorating the adiponectin decreasing, 2.85 ± 0.36 mg/L, which was similar to the adiponectin level of HFD group 2.65 ± 0.55 mg/L ($P > 0.05$).

3.5. Effects of SC-FUCs on the related protein expression

Further molecular mechanism of the two fucodians, expression of some protein related lipid metabolism were investigated. As shown in the Fig. 5a, the HFD significantly increased the expression of CD36 in liver. Since CD36 acts as a receptor on cell surface, we further confirmed the mRNA level of CD36 was also increased by HFD, which is consistent with the high concentration of this protein concentration (Fig. 5b). Fuc-*Pg* reversed the increased CD36 caused by HFD, fuc-*Ib* showed no significant difference compared with HFD group.

The HFD increased the level of PPARα and decreased the level of CYP7A1, and fuc-*Pg* reversed these changes while fuc-*Ib* had no significant effects on both proteins (Fig. 5c). PPARα is a member of the nuclear receptor family of ligand-activated transcription factors [34]. It plays an important role in the regulation of genes involved in lipid metabolism and transport. CYP7A1 is a cytochrome P450 enzyme that converts cholesterol to 7a-hydroxycholesterol [35].

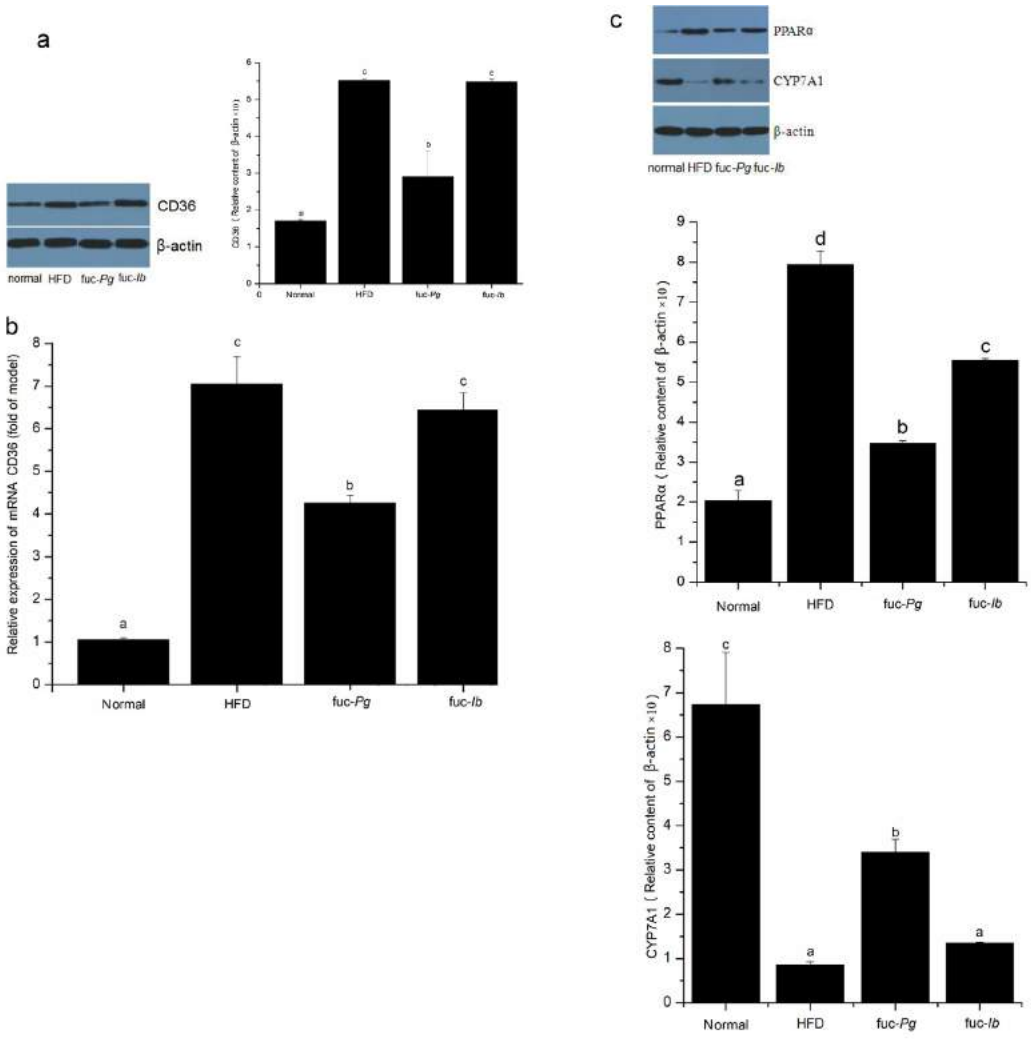

Fig. 5. Effects of fuc-*Pg* and fuc-*Ib* on CD36 protein expression in the liver of rats fed on HFD using by Western blot **(a)**. Effects of fuc-*Pg* and fuc-*Ib* on CD36 mRNA expression in the liver of rats fed on HFD using qRT-PCR, and the results were normalized by β-actin **(b)**. Effects of fuc-*Pg* and fuc-*Ib* on PPARα and CYP7A1 protein expression in the liver of rats fed on HFD using by Western blot**(c).** Data are expressed as mean ± SD (n = 4). Multiple comparisons were done using one way ANOVA analysis followed by Dunnett's T3 post-hoc test. a, b, c, d: $P < 0.05$, compared between four groups.

4. Discussion

In our former studies, the structures of fuc-*Pg* and Fuc-*Ib* were identified by a combination of NMR and ES-MS–MS analysis of the oligosaccharides fragments obtained by mild acid hydrolysis with no obvious loss of sulfate groups, which were different from other reported fucoidans with regular structure composition (Fig. 1). The delicate structures of fuc-*Pg* and fuc-*Ib* were [Fuc (2S; 4S) α1 → 3Fucα1 → 3Fuc (4S) α1 → 3Fucα]$_n$ and [Fuc (2S; 4S) α1 → 3Fucα (2S) 1 → 3Fuc (2S) α1 → 3Fucα]$_n$, respectively [14,15]. Both of the two fucodian are composed by fucose tetrasaccharide repeat units, with an initial 2, 4-di-*O*-sulfated fucose. Fuc-*Pg* contains other 4-*O*-sulfated fucose units whereas fuc-*Ib* has two 2-*O*-sulfated fucoses in the middle of the chain. Thus, total 3 sulfate groups were presented in fuc-*Pg*, lower than 4 sulfation groups found in fuc-*Ib* (Table 3). Also, the molecular weight (MW) is another difference that fuc-*Pg* were only slightly lower than of fuc-*Ib* (320 kDa *vs.* 460 kDa). Thus, the main difference in the structure of two fucoidans is their unique sulfation patterns.

In the present study, SD rats fed on HFD developed into hyperlipidemic rats and had an abnormal lipid profile compared with the normal group fed on a regular chow. In addition, excess fat intake induced damages to the liver of the HFD group. The low adiponection concentration of HFD indicated abnormal fat tissue accumulation. Oral administration of fuc-*Pg* alleviated the lipid disorder caused by HFD, and it even showed the similar weight gains, lipid profile, and adiponection level as normal group. However, fuc-*Ib* only had limited effect on TG level, liver weight, and TBA level, and slightly decreased the weight gain. The different effects may

Table 3
Chemical composition of fuc-*Ib* and fuc-*Pg*.

Sample	MW(kDa)	Molar ratio		Sulphate patterns
		Fuc	Sulfate	
fuc-*Pg*	320	1.0	0.8	2,4-di-*O*-S, 4-*O*-S
fuc-*Ib*	460	1.0	1.0	2,4-di-*O*-S, 2-*O*-S, 2-*O*-S

"S", sulfate group.

contribute to the different sulfation patterns of the two fucodians. The additional 4-*O*-mono-sulfated pattern facilitates fuc-*Pg* for anti-hyperlipidemic activity, rather than the simply sulfate content. It has been reported a 2, 4-*O*-disulfated fucose unitis important for anticoagulant activity [15,36]. Pereira, Melo, and Mourao [37] found the occurrence of 2, 4-di-*O*-sulfated units and single 4-*O*-sulfated fucose units are amplifying motifs for anticoagulation, while 2-*O*-sulfated fucose residues have a deleterious effect on anticoagulant activity. In our study, a similar founding was found that an additonal 4-*O*-sulfated fucose may contribute to high anti-hyperlipidemic activity.

Typically, functional polysaccharides exhibit anti-hyperlipidemic effect similar to the soluble dietary fiber (SDF), which are considered as indigestible food ingredients [38]. The SDFs prevent bile salt (BS) re-absorption, thus lowering the fat absorption and insulin stimulation of hepatic cholesterol synthesis, or modulating the composition of the gut microbiota [39]. The interpretation of polysaccharides as dietary fiber or prebiotic means polysaccharides prevents lipid absorption and modulates lipid metabolism in the digestive tract. In our study, the higher liver weight and TBA level of simvastatin and fuc-*Pg* group means the livers of SD rats were still injured by HFD, or rather injured by hyperlipidemia in the circulation. Simvastatin reportly inhibit sthe activity of HMG-CoA reductase, thus alleviating hyperlipidemia [40]. As for fuc-*Pg*, low dose and powerful impact on alleviating dyslipidemia indicated the underlying mechanism cannot simply be explained by its behavior as a dietary fiber adjusting dyslipidemia. We further investigated several important key proteins about lipid metabolism in liver to find out the possible mechanism. Since liver seems to be the most vulnerable organs, HFDs induce steatosis, even when no changes in insulin signaling or weight are found [41]. Fuc-*Pg* reduced expression of both mRNA CD36 and CD36. CD36 functions as a scavenger receptor that can transfer fatty acids from serum into cellular tissues. Elevated CD36 in liver was proved to be responsible for increasing hepatic fatty acid uptake, and the expression of CD36 in liver was aberrant when rats were exposed to a HFD for 5 weeks [5]. Increased expression of hepatic CD36 protein in response to diet-induced obesity is thought to be sufficient to exacerbate hepatic triglyceride storage and secretion [5]. Here, in our study, the expression of hepatic CD36 was decreased by administrating fuc-*Pg*, and reduced the influx of fatty acids into hepatocyte, thus, protecting liver from excessive fatty acids. Fatty acids, as the main endogenous ligands of PPARα, can activate PPARα to promote hepatic fatty acid oxidation [34]. However, some studies have been reported showing that the level of hepatic PPARα increased in response to HFD feeding [42,43]. Meanwhile, the higher level of PPARα in response to HFD was associated with producing ROS during β-oxidation of fatty acids, which can cause hepatic oxidative stress [43]. Fuc-*Pg* reversed the increased expression of PPARα, which might contributed to less exposure to fatty acids. In additon, fuc-*Pg* can reverse the decrement of CYP7A1, thus promoting the conversion of cholesterol into bile acids through several pathways [35]. PPARα indirectly represses CYP7A1 by reducing HNF4α binding to the DR-1 response element in the CYP7A1 promoter [44,45]. Therefore, the anti-hyperlipidemic activity of fuc-*Pg* can be preliminarily attributed to reducing the expression of CD36. Then, the lower expression of CD36 reduced the hepatic uptake of fatty acids and thus protecting liver from further damages caused by over influx of fatty acids. However, fuc-*Ib* exerted almost no effects on the expression of CD36. This difference may be attributed to the different anti-hyperlipidemic activities of fucoidans.

In addition, our results suggested that the oral administration of fuc-*Pg* might stimulate the secretion of adiponectin by adipocyte, which can promote the lipid increases tissue fat oxidation, resulting in reduced circulating intracellular triglyceride contents, thus protecting liver from excess fat. Fucoidans are reported to inhibit lipid accumulation by stimulating lipolysis [46] and inhibit adipogenesis through the mitogen-activated protein kinase pathway in 3T3-L1 preadipocytes [47]. These publications indicate fucodians can act on adipose tissue to intervene lipid metabolism. Besides, polysaccharides including fucoidans are argued for absorption for the huge MW. For past years, more and more evidences have been found to prove the possible absorption of polysaccharides [48], also including fucoidans [49]. It is possible for fuc-*Pg* to be absorbed at some extent and exhibit its anti-hyperlipidemic activity on particular tissues.

5. Conclusion

In conclusion, both fuc-*Pg* and fuc-*Ib* are functional food components that can adjust dyslipidiaemia caused by abnormal diet. However, fuc-*Pg* has a more powerful impact on protecting rats from HFD. The different hypolipidemic behaviors of the two fucodians are related to their unique sulfation patterns. The occurrence of 2, 4-di-*O*-sulfated units and single 4-*O*-sulfated fucose units may benefit the anti-hyperlipidemic activity of liner fucoidan. Our results indicated that the fuc-*Pg* with 4-*O*-sulfation group may have better anti-hyperlipidemic activity, which can be preliminarily assigning to preventing transfer of fatty acids by lowering the expression of CD36, thus reducing PPARα and decreasing the level of CYP7A1.

Conflict of interest

The authors have declared no conflicts of interest.

Author contributions

Shiguo Chen, Shan Li, Yaqin Hu, Xingqian Ye, and Tian Ding were responsible for the concept and design of the studies. Shan Li and Junhui Li prepared polysaccharides. Zijian Zhi and Jian Ge were responsible for feeding rats. Indices in serum, RT-PCR and western blot were performed by Shan Li. Shiguo Chen, Shan Li, and Linhardt Robert were responsible for drafting of the manuscript. All authors read and approved the final iteration of the paper.

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References

[1] L. Cordain, S.B. Eaton, A. Sebastian, N. Mann, et al., Origins and evolution of the Western diet: health implications for the 21st century, Am. J. Clin. Nutr. 81 (2005) 341–354.
[2] K.S. Jain, M.K. Kathiravan, R.S. Somani, C.J. Shishoo, The biology and chemistry of hyperlipidemia, Bioorg. Med. Chem. 15 (2007) 4674–4699.
[3] K. Kotani, M.C. Serban, P. Penson, G. Lippi, M. Banach, Evidence-based assessment of lipoprotein(a) as a risk biomarker for cardiovascular diseases—some answers and still many questions, Crit. Rev. Clin. Lab. Sci. 53 (6) (2016) 370–378.
[4] X.H. Yu, Y.C. Fu, D.W. Zhang, K. Yin, C.K. Tang, Foam cells in atherosclerosis, Clin. Chim. Acta 424 (2013) 245–252.
[5] D.P.Y. Koonen, R.L. Jacobs, M. Febbraio, M.E. Young, et al., Increased hepatic CD36 expression contributes to dyslipidemia associated with diet-induced obesity, Diabetes 56 (2007) 2863–2871.
[6] S. Fischer, U. Schatz, U. Julius, Practical recommendations for the management of hyperlipidemia, Atheroscler. Suppl. 18 (2015) 194–198.
[7] J.H. Fitton, D.N. Stringer, S.S. Karpiniec, Therapies from fucoidan: an update, Mar. Drugs 13 (2015) 5920–5946.
[8] L. Huang, K. Wen, X. Gao, Y. Liu, Hypolipidemic effect of fucoidan from *Laminaria japonica* in hyperlipidemic rats, Pharm. Biol. 48 (2010) 422–426.

[9] P. Thomes, M. Rajendran, B. Pasanban, R. Rengasamy, Cardioprotective activity of Cladosiphon okamuranus fucoidan against isoproterenol induced myocardial infarction in rats, Phytomedicine 18 (2010) 52–57.
[10] M.J. Kim, U.J. Chang, J.S. Lee, Inhibitory effects of fucoidan in 3T3-L1 adipocyte differentiation, Mar. Biotechnol. 11 (2009) 557–562.
[11] M.T. Ale, A.S. Meyer, Fucoidans from brown seaweeds: an update on structures, extraction techniques and use of enzymes as tools for structural elucidation, RSC Adv. 3 (2013) 8131–8141.
[12] M.I. Bilan, A.A. Grachev, A.S. Shashkov, N.E. Nifantiev, A.I. Usov, Structure of a fucoidan from the brown seaweed Fucus serratus L, Carbohydr. Res. 341 (2006) 238–245.
[13] J. Wang, Q.B. Zhang, Z.S. Zhang, H. Zhang, X.Z. Niu, Structural studies on a novel fucogalactan sulfate extracted from the brown seaweed *Laminaria japonica*, Int. J. Biol. Macromol. 47 (2010) 126–131.
[14] Y.Q. Hu, S. Li, J.H. Li, X.Q. Ye, et al., Identification of a highly sulfated fucoidan from sea cucumber *Pearsonothuria graeffei* with well-repeated tetrasaccharides units, Carbohydr. Polym. 134 (2015) 808–816.
[15] S.G. Chen, Y.Q. Hu, X.Q. Ye, G.Y. Li, et al., Sequence determination and anticoagulant and antithrombotic activities of a novel sulfated fucan isolated from the sea cucumber *Isostichopus badionotus*, BBA Gen. Subj. 2012 (1820) 989–1000.
[16] B. Mulloy, A.C. Ribeiro, A.P. Alves, R.P. Vieira, P.A.S. Mourao, Sulfated fucans from echinoderms have a regular tetrasaccharide repeating unit defined by specific patterns of sulfation at the O-2 and O-4 positions, J. Biol. Chem. 269 (1994) 22113–22123.
[17] L. Yu, L. Ge, C.H. Xue, Y.G. Chang, et al., Structural study of fucoidan from sea cucumber Acaudina molpadioides: a fucoidan containing novel tetrafucose repeating unit, Food Chem. 142 (2014) 197–200.
[18] L. Yu, C.H. Xue, Y.G. Chang, X.Q. Xu, et al., Structure elucidation of fucoidan composed of a novel tetrafucose repeating unit from sea cucumber *Thelenota ananas*, Food Chem. 146 (2014) 113–119.
[19] S. Chen, Y. Hu, X. Ye, G. Li, et al., Sequence determination and anticoagulant and antithrombotic activities of a novel sulfated fucan isolated from the sea cucumber *Isostichopus badionotus*, Biochim. Biophys. Acta 2012 (1820) 989–1000.
[20] Y.M. Wang, J.F. Wang, Y.L. Zhao, S.W. Hu, et al., Fucoidan from sea cucumber *Cucumaria frondosa* exhibits anti-hyperglycemic effects in insulin resistant mice via activating the PI3K/PKB pathway and GLUT4, J. Biosci. Bioeng. 121 (2016) 36–42.
[21] Y.J. Zhang, S.L. Song, D. Song, H. Liang, et al., Proliferative effects on neural stem/progenitor cells of a sulfated polysaccharide purified from the sea cucumber *Stichopus japonicus*, J. Biosci. Bioeng. 109 (2010) 67–72.
[22] J.H. Wang, S.W. Hu, W. Jiang, W.D. Song, et al., Fucoidan from sea cucumber may improve hepatic inflammatory response and insulin resistance in mice, Int. Immunopharmacol. 31 (2016) 15–23.
[23] S. Reitman, S. Frankel, A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases, Am. J. Clin. Pathol. 28 (1957) 56–63.
[24] F. Mashige, N. Tanaka, A. Maki, S. Kamei, M. Yamanaka, Direct spectrophotometry of total bile-acids in serum, Clin. Chem. 27 (1981) 1352–1356.
[25] N.A. Smart, B.J. Marshall, M. Daley, E. Boulos, et al., Low-fat diets for acquired hypercholesterolaemia, Cochr. Database Syst. Rev. (2) (2011), CD007957, http://dx.doi.org/10.1002/14651858.CD007957.pub2.
[26] P. Tessari, A. Coracina, A. Cosma, A. Tiengo, Hepatic lipid metabolism and non-alcoholic fatty liver disease, Nutr. Metab. Cardiovasc. 19 (2009) 291–302.
[27] J. Ozer, M. Ratner, M. Shaw, W. Bailey, S. Schomaker, The current state of serum biomarkers of hepatotoxicity, Toxicology 245 (2008) 194–205.
[28] D.W. Russell, The enzymes, regulation, and genetics of bile acid synthesis, Annu. Rev. Biochem. 72 (2003) 137–174.
[29] P. Lefebvre, B. Cariou, F. Lien, F. Kuipers, B. Staels, Role of bile acids and bile acid receptors in metabolic regulation, Physiol. Rev. 89 (2009) 147–191.
[30] M.B. Thompson, Bile acids in the assessment of hepatocellular function, Toxicol. Pathol. 24 (1996) 62–71.
[31] T. Kadowaki, T. Yamauchi, Adiponectin and adiponectin receptors, Endocr. Rev. 26 (2005) 439–451.
[32] Y. Arita, S. Kihara, N. Ouchi, M. Takahashi, et al., Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity, Biochem. Biophys. Res. Commun. 257 (1999) 79–83.
[33] J.J. Diez, P. Iglesias, The role of the novel adipocyte-derived hormone adiponectin in human disease, Eur. J. Endocrinol. 148 (2003) 293–300.
[34] R.M. Evans, G.D. Barish, Y.X. Wang, PPARs and the complex journey to obesity, Nat. Med. 10 (2004) 355–361.
[35] T.Q.D. Vallim, E.J. Tarling, P.A. Edwards, Pleiotropic roles of bile acids in metabolism, Cell Metab. 17 (2013) 657–669.
[36] R.J.C. Fonseca, G.R.C. Santos, P.A.S. Mourao, Effects of polysaccharides enriched in 2,4-disulfated fucose units on coagulation, thrombosis and bleeding Practical and conceptual implications, Thromb. Haemost. 102 (2009) 829–836.
[37] M.S. Pereira, F.R. Melo, P.A.S. Mourao, Is there a correlation between structure and anticoagulant action of sulfated galactans and sulfated fucans? Glycobiology 12 (2002) 573–580.
[38] P. Gunness, M.J. Gidley, Mechanisms underlying the cholesterol-lowering properties of soluble dietary fibre polysaccharides, Food Funct. 1 (2010) 149–155.
[39] C. Chassard, C. Lacroix, Carbohydrates and the human gut microbiota, Curr. Opin. Clin. Nutr. 16 (2013) 453–460.
[40] T.R. Pedersen, J.A. Tobert, Simvastatin: a review, Expert Opin. Pharmacol. 5 (2004) 2583–2596.
[41] P.A. Kakimoto, A.J. Kowaltowski, Effects of high fat diets on rodent liver bioenergetics and oxidative imbalance, Redox Biol. 8 (2016) 216–225.
[42] J. Ciapaite, N.M. van den Broek, H.T. Brinke, K. Nicolay, et al., Differential effects of short- and long-term high-fat diet feeding on hepatic fatty acid metabolism in rats, BBA Mol. Cell Biol. Lipids 2011 (1811) 441–451.
[43] N. Matsuzawa-Nagata, T. Takamura, H. Ando, S. Nakamura, et al., Increased oxidative stress precedes the onset of high-fat diet-induced insulin resistance and obesity, Metabolism 57 (2008) 1071–1077.
[44] G. Zollner, M. Trauner, Nuclear receptors as therapeutic targets in cholestatic liver diseases, Br. J. Pharmacol. 156 (2009) 7–27.
[45] M. Marrapodi, J.Y.L. Chiang, Peroxisome proliferator-activated receptor alpha (PPAR alpha) and agonist inhibit cholesterol 7 alpha-hydroxylase gene (CYP7A1) transcription, J. Lipid Res. 41 (2000) 514–520.
[46] M.K. Park, U. Jung, C. Roh, Fucoidan from marine brown algae inhibits lipid accumulation, Mar. Drugs 9 (2011) 1359–1367.
[47] K.J. Kim, O.W. Lee, B.Y. Lee, Fucoidan, a sulfated polysaccharide, inhibits adipogenesis through the mitogen-activated protein kinase pathway in 3T3-L1 preadipocytes, Life Sci. 86 (2010) 791–797.
[48] X.Q. Zha, L. Xue, H.L. Zhang, M.N. Asghar, et al., Molecular mechanism of a new Laminaria japonica polysaccharide on the suppression of macrophage foam cell formation via regulating cellular lipid metabolism and suppressing cellular inflammation, Mol. Nutr. Food Res. 59 (2015) 2008–2021.
[49] T. Nagamine, K. Nakazato, S. Tomioka, M. Iha, K. Nakajima, Intestinal absorption of fucoidan extracted from the brown seaweed, cladosiphon okamuranus, Mar. Drugs 13 (2015) 48–64.