Gracilaria lemaneiformis polysaccharide with significant hypolipidemic activity, preparation method thereof and use therefor

Technical field

The invention belongs to the field of deep processing of *Gracilaria lemaneiformis*, and in particular relates to a *Gracilaria lemaneiformis* polysaccharide with significant hypolipidemic activity, a preparation method thereof and use therefor.

Background

Gracilaria lemaneiformis is a plant belonging to Rhodophyta and Gracaialri, which is mainly produced in Guangdong, Hainan, Shandong, etc. in China. It is rich in dietary fiber, vitamins and trace elements as well as active components like polysaccharides, phycobiliprotein and unsaturated fatty acids with low fat content. *Gracilaria lemaneiformis* is a traditional food with long history in eastern China. Traditional Chinese doctors believe that *Gracilaria lemaneiformis* has the effect of "facilitating defecation and releasing heats in the galls". Modern medical researchers believe that it may help digestion, relieve the symptom of dyspepsia, clean the intestines and stomach, stop bleeding and lower blood pressure.

In the early days, the production yield of *Gracilaria lemaneiformis* was so low that domestic studies on it were mainly focused on planting and processing, aiming to increase its yield and reproduction rate. In recent years, the focus of research on *Gracilaria lemaneiformis* has gradually shifted from production to the extraction of active substances and the study of physiological functions. There have been reports of its antioxidant, hypoglycemic, immune and anti-tumor activities. For example, Yisheng Zhu disclosed a method for increasing antioxidant components of *Gracilaria lemaneiformis* extract (patent application No. 201010188414.3); Guangdong Pharmaceutical University disclosed a *Gracilaria lemaneiformis* extract and preparation method thereof and use therefor (patent application No. 201611024913.2). The extract may scavenge more than 90% of DPPH, hydroxyl and superoxide ion free radicals. The Third Institute of Oceanography of the State Oceanic Administration has disclosed a *Gracilaria lemaneiformis* agar oligosaccharide and preparation method thereof, which can be used in antioxidants, anti-ultraviolet health products and cosmetics (patent application No. 201210347105.5); South China University of Technology disclosed a polysaccharide extract of *Gracilaria lemaneiformis* vulgaris and preparation method thereof and use therefor (patent application No. 201410252784.7). The obtained polysaccharide may well inhibit the activity of dipeptidyl peptidase IV and may be used in the development of hypoglycemic drugs or health care products; Shanghai Fisheries University disclosed a preparation method of *Gracilaria lemaneiformis* vulgaris polysaccharide, the obtained polysaccharide may promote the proliferation of mouse lymphocytes (patent application No. 200710044610.1); Shanghai Jiaotong University disclosed a use of *Gracilaria lemaneiformis* vulgaris polysaccharide in the preparation of inhibitors that induce the expression of FADD and GADD genes in cells, which shows application prospects in the preparation of anti-tumor drugs or anti-cancer health products (patent application No. 201610082951.7).

Summary of the invention

In order to improve the deep processing technology of *Gracilaria lemaneiformis* and broaden its application, a primary object of the present invention is to provide a *Gracilaria lemaneiformis* polysaccharide.

Another object of the present invention is to provide a method for preparing the *Gracilaria lemaneiformis* polysaccharide.

A further object of the present invention is to provide a use of the *Gracilaria lemaneiformis* polysaccharide.

The objects of the present invention are achieved through the following technical solutions.

A method for preparing *Gracilaria lemaneiformis* polysaccharide with significant hypolipidemic activity, comprising the following steps:

(1) washing, air-drying, crushing and sieving *Gracilaria lemaneiformis* to obtain *Gracilaria lemaneiformis* powder;

(2) adding an organic acid solution to the *Gracilaria lemaneiformis* powder; after pre-homogenization, extracting the *Gracilaria lemaneiformis* powder by dynamic high pressure microfluidization, neutralizing with a base and filtering to obtain a filter residue and an extract solution;

(3) performing ultrafiltration of the extract solution in step (2), discarding a permeate, and collecting a retentate;

(4) separating the retentate with an ion exchange resin, and concentrating a component with the highest hypolipidemic activity under vacuum to obtain a concentrated solution;

(5) mixing the concentrated solution with ethanol, standing and filtering to obtain a polysaccharide precipitation;

(6) drying the polysaccharide precipitation to obtain the *Gracilaria lemaneiformis* polysaccharide.

Preferably, the crushing and sieving step in step (1) comprises sieving by a 20-80 mesh sieve after crushing.

Preferably, the pH of the organic acid solution in step (2) is 2.0-4.0.

Preferably, the organic acid solution in step (2) is a citric acid solution or a malic acid solution.

Preferably, the mass of the organic acid solution in step (2) is 20-40 times of the mass of the *Gracilaria lemaneiformis* powder.

Preferably, parameters of the pre-homogenization in step (2) comprise 20-50 MPa, 1 - 3 min, and 1-2 times of homogenization;

Preferably, control conditions for the dynamic high pressure microfluidization in step (2) comprise a microfluidization pressure of 120-180 MPa, an extraction temperature of 75-100°C, an extraction time of 80-160 min, and 1-2 times of extractions.

Preferably, the ultrafiltration in step (3) is performed by using an ultrafiltration membrane with a molecular weight cut-off of 1 kDa.

Preferably, a model of the ion exchange resin in step (4) is DEAE-fast flow or DEAE-52.

Preferably, the concentrated solution obtained by vacuum concentration in step (4) has a volume of 1/10 - 1/5 of the volume of the retentate.

Preferably, the ethanol in step (5) has an amount of 60% - 90% of a total volume of the concentrated solution after mixing with the ethanol,

Preferably, the standing in step (5) comprises standing at 2 - 8°C for 8 - 14 hours.

Preferably, the drying in step (6) comprises vacuum freeze drying or spray drying.

Gracilaria lemaneiformis polysaccharide prepared by the above-mentioned preparation method has a molecular weight of 20-35 kDa and a sulfate group content of 20-28% by weight.

The above-mentioned *Gracilaria lemaneiformis* polysaccharide is used in the preparation of hypolipidemic drugs or health products.

Compared with the prior art, the present invention has the following advantages and effects:

(1) The present invention uses an organic acid and dynamic high pressure microfluidization to extract *Gracilaria lemaneiformis* polysaccharide, so that the extraction rate is 3 - 5 times higher than that of the traditional water extraction method, which can greatly shorten the extraction time and reduce energy consumption.

(2) The *Gracilaria lemaneiformis* polysaccharide obtained in the present invention has a good effect of lowering blood lipids.

Brief description of the drawings

Figure 1a - 1e are the oil red o stained section photos (200×) of the liver tissues of mice in NC, HF, HF+AT, HF+LGP, HF+HGP groups, respectively.

Figure 2a - 2e are the HE stained section photos (400×) of the liver tissues of mice in NC, HF, HF+AT, HF+LGP, HF+HGP groups, respectively.

Detailed description

The present invention will be further described in detail below in conjunction with examples, but the embodiments of the present invention are not limited thereto.

Example 1

A method for preparing a *Gracilaria lemaneiformis* polysaccharide extract comprises the following steps.

(1) Wash, air-dry, crush, and sieve *Gracilaria lemaneiformis* by a 20-mesh sieve, and collect the sieved powder;

(2) Mix 50 g of the *Gracilaria lemaneiformis* powder with pH 2.0 citric acid at a mass ratio of 1:30 in a container. After pre-homogenization at 30 MPa for 2 times, 2 min for each, extract the *Gracilaria lemaneiformis* powder by dynamic high pressure microfluidization with a microfluidization pressure of 125 MPa at 90°C for 150 min for 2 times, neutralize with NaOH until the pH is neutral, and filter the mixture to obtain a filter residue and an extract solution;

(3) Perform ultrafiltration of the extract solution in step (2) by using an ultrafiltration membrane with a molecular weight cut-off of 1 kDa, discard a permeate, and collect a retentate;

(4) Separate the retentate with a DEAE-fast flow ion exchange resin, and concentrate a component with the highest hypolipidemic activity under vacuum to

obtain a concentrated solution having a volume of 1/10 of that of the retentate;

(5) Mix the concentrated solution with anhydrous ethanol until a final concentration of the ethanol is 85wt%, stand at 6°C for 12 h after mixing the solution homogenously, filter the solution and remove the supernatant to obtain a polysaccharide precipitation;

(6) Freeze dry the polysaccharide precipitation to obtain the *Gracilaria lemaneiformis* polysaccharide, which was recorded as 1# extract.

Example 2

A method for preparing a *Gracilaria lemaneiformis* polysaccharide extract comprises the following steps.

(1) Wash, air-dry, crush, and sieve *Gracilaria lemaneiformis* by a 40-mesh sieve, and collect the sieved powder;

(2) Mix 50 g of the *Gracilaria lemaneiformis* powder with pH 3.5 citric acid at a mass ratio of 1:20 in a container. After pre-homogenization at 25 MPa for 3 times, 1 min for each, extract the *Gracilaria lemaneiformis* powder by dynamic high pressure microfluidization with a microfluidization pressure of 160 MPa at 80°C for 100 min for 1 time, neutralize with NaOH until the pH is neutral, and filter the mixture to obtain a filter residue and an extract solution;

(3) Perform ultrafiltration of the extract solution in step (2) by using an ultrafiltration membrane with a molecular weight cut-off of 1 kDa, discard a permeate, and collect a retentate;

(4) Separate the retentate with a DEAE-52 ion exchange resin, and concentrate a component with the highest hypolipidemic activity under vacuum to obtain a concentrated solution having a volume of 1/8 of that of the retentate;

(5) Mix the concentrated solution with anhydrous ethanol until a final concentration of the ethanol is 68 wt%, stand at 4°C for 10 h after mixing the solution homogenously, filter the solution and remove the supernatant to obtain a polysaccharide precipitation;

(6) Freeze dry the polysaccharide precipitation to obtain the *Gracilaria lemaneiformis* polysaccharide, which was recorded as 2# extract.

Example 3

A method for preparing a *Gracilaria lemaneiformis* polysaccharide extract comprises the following steps.

(1) Wash, air-dry, crush, and sieve *Gracilaria lemaneiformis* by a 60-mesh sieve, and collect the sieved powder;

(2) Mix 50 g of the *Gracilaria lemaneiformis* powder with pH 3.0 citric acid at a mass ratio of 1:40 in a container. After pre-homogenization at 45 MPa for 1 time, 3 min for each, extract the *Gracilaria lemaneiformis* powder by dynamic high pressure microfluidization with a microfluidization pressure of 148 MPa at 96°C for 130 min for 1 time, neutralize with NaOH until the pH is neutral, and filter the mixture to obtain a filter residue and an extract solution;

(3) Perform ultrafiltration of the extract solution in step (2) by using an ultrafiltration membrane with a molecular weight cut-off of 1 kDa, discard a permeate, and collect a retentate;

(4) Separate the retentate with a DEAE-Fast flow ion exchange resin, and concentrate a component with the highest hypolipidemic activity under vacuum to obtain a concentrated solution having a volume of 1/5 of that of the retentate;

(5) Mix the concentrated solution with anhydrous ethanol until a final concentration of the ethanol is 75 wt%, stand at 2°C for 8 h after mixing the solution homogenously, filter the solution and remove the supernatant to obtain a polysaccharide precipitation;

(6) Freeze dry the polysaccharide precipitation to obtain the *Gracilaria lemaneiformis* polysaccharide, which was recorded as 3# extract.

Comparative Example 1 preparing a *Gracilaria lemaneiformis* polysaccharide extract by conventional water extraction

A method for preparing a *Gracilaria lemaneiformis* polysaccharide extract by conventional water extraction comprises the following steps.

(1) Wash, air-dry, crush, and sieve *Gracilaria lemaneiformis* by a 40-mesh sieve, and collect the sieved powder;

(2) Boil the *Gracilaria lemaneiformis* powder in a container for 120 min, and filter the mixture to obtain a filter residue and an extract solution;

(3) Perform ultrafiltration of the extract solution in step (2) by using an ultrafiltration membrane with a molecular weight cut-off of 1 kDa, discard a permeate, and collect a retentate;

(4) Separate the retentate with a DEAE-Fast flow ion exchange resin, and concentrate a component with the highest hypolipidemic activity under vacuum to obtain a concentrated solution having a volume of 1/8 of that of the retentate;

(5) Mix the concentrated solution with anhydrous ethanol until a final concentration of the ethanol is 80 wt%, stand at 0-4°C for 12 h after mixing the solution homogenously, filter the solution and remove the supernatant to obtain a polysaccharide precipitation;

(6) Freeze dry the polysaccharide precipitation to obtain the *Gracilaria lemaneiformis* polysaccharide, which was recorded as 4# extract.

Experimental Example 1 Determination of the extraction rate of polysaccharides

The extraction rate of the *Gracilaria lemaneiformis* polysaccharide in the examples and comparative example were determined.

Phenol-sulfuric acid method was used to determine the polysaccharide content in 1# - 4# extract, and the the polysaccharide extraction rate was calculated with the formula: polysaccharide extraction rate (%) = (polysaccharide content in the extract (g)/raw material weight (g))× 100.

The determination results of the *Gracilaria lemaneiformis* polysaccharide extract was shown in Table 1:

Table 1

Extract	1# Extract	2# Extract	3# Extract	4# Extract
Polysaccharide extraction rate(%)	23.65±0.27	19.80±0.62	20.31±0.80	5.46±0.52

Note: The data in the table are means ± standard deviations (n=3)

From the data in Table 1, it can be seen that the extraction rate of *Gracilaria lemaneiformis* polysaccharide in the method of the present invention is significantly

higher than that of the traditional water extraction method.

Experimental example 2 hypolipidemic experiment of the *Gracilaria lemaneiformis* polysaccharide

In this experiment, male Kunming mice were fed with high-cholesterol and high-fat feed to set up a hyperlipidemia model, which is used to evaluate the hypolipidemic effect of the *Gracilaria lemaneiformis* polysaccharide prepared by the aforementioned method.

1. Experimental materials

1.1 Reagents: high cholesterol and high fat feed (sucrose 20%, lard 15%, cholesterol 1.2%, sodium cholate 0.2%, casein 10%, calcium hydrogen phosphate 0.6%, stone powder 0.4%, premix 0.4% , basic feed 52.2%; total energy 4.4kcal/g; provided by Guangdong Medical Animal Center), mouse gavage syringe, disposable sterile syringe, centrifuge tube, sterile saline, atorvastatin calcium tablets (Pfizer Pharmaceutical Co., Ltd., product lot number: S90890), triglyceride test kit (Nanjing Jiancheng Biotechnology Co., Ltd., product lot number: 20180420), total cholesterol test kit (Nanjing Jiancheng Biotechnology Co., Ltd., product lot number: 20180420), low-density lipoprotein test kit (Nanjing Jiancheng Biotechnology Co., Ltd., product lot number: 20180426), high-density lipoprotein test kit (Nanjing Jiancheng Biotechnology Co., Ltd., product lot number: 20180426), free fatty acid test kit (Nanjing Jiancheng Biotechnology Co., Ltd., product lot number: 20180420) *Gracilaria lemaneiformis* polysaccharide (GLP) prepared by our laboratory according to the aforementioned preparation method.

1.2 Main experiment equipment: a low-speed centrifuge, a microplate reader, and a pipette.

1.3 Experiment animals: Kunming mice, male, weighing 22-24g, purchased from Guangdong Medical Animal Center.

2. Sample solution

The *Gracilaria lemaneiformis* polysaccharide prepared in Example 1.

3. Preparation of each sample solution:

3.1 Preparation of statin solution: suspend 7 atorvastatin calcium tablets (containing 140 mg of atorvastatin) in 350 mL of sterile normal saline to prepare a sample solution having a concentration of 0.4 mg/mL (based on the content of atorvastatin)

3.2 Preparation of a low-dose *Gracilaria lemaneiformis* polysaccharide solution: accurately weight 3.57 g of *Gracilaria lemaneiformis* polysaccharide and dissolve it in 350 mL of sterile normal saline to prepare a sample solution with a polysaccharide content of 6.0 mg/mL.

3.3 Preparation of a high-dose *Gracilaria lemaneiformis* polysaccharide solution: accurately weigh 13.37 g of *Gracilaria lemaneiformis* polysaccharide and dissolve it in 350 mL of sterile normal saline to prepare a sample solution with a polysaccharide content of 22.5 mg/mL.

4. Experimental method

4.1 Modeling stage: 102 Kunming mice, 22-24g, male, were randomly divided into two groups, wherein a group of 24 was a normal control group, fed with ordinary

feed; the other group of 78 mice was a hyperlipidemia model group (hereinafter referred to as the model group), fed with high-cholesterol and high-fat diet, unlimited water and food. After 6 weeks of feeding, 6 mice were randomly selected from each group. After fasting but water for 8 h, the blood was collected by eyeball blood sampling, and the serum was separated by centrifugation. According to the kit instructions, the contents of total cholesterol (TC), triglycerides (TG), low density lipoprotein (LDL-C), high density lipoprotein (HDL-C), and free fatty acid (FFA) were determined, respectively. The above indicators were used to judge whether the modeling is successful.

4.2 Experimental stage: After a successful model building, the model group was randomly divided into 4 groups, each with 18 mice: model control group (HF), atorvastatin intervention group (HF+AT), low-dose *Gracilaria lemaneiformis* polysaccharide intervention group (HF+LGP), high-dose *Gracilaria lemaneiformis* polysaccharide intervention group (HF+HGP). The normal control group (NC) and the model control group were given 0.1 mL/10g of sterile saline per day, and the administration groups were given 0.1 mL/10g volume of corresponding drugs per day. A total of 42 days of administration were conducted. Then, 14, 28, and 42 days after the administration, 6 mice in each group were randomly selected after fasting but watered for 8 hours. The blood was collected by eyeball blood sampling. After the mice were sacrificed by cervical vertebrae, the peridídymal fat was weighed and the liver tissue sections were taken for pathological analysis; centrifugal separation of serum within 2 hours of blood collection was performed according to the test kit instructions, and the contents of TC, TG, LDL-C, HDL-C, and FFA were determined.

4.3 Data processing and analysis methods: the following experimental data are all expressed as "mean ± standard deviation". One-way ANOVA with SPSS 19.0 was used for one-way analysis of variance and significant difference analysis. Whether there is significant difference or not between the variances was determined by the LSD/Dunnett's T3 method.

5. Experimental results

5.1 After determination, the extraction rate of the *Gracilaria lemaneiformis* polysaccharides used in the experimental test was 23.65±0.27% by weight, the purity of the polysaccharide was 59.01±0.91% determined by phenol sulfuric acid method, the protein content was only 1.57±0.37wt% determined by the BCA kit, the sulfuric acid group content was 22.85±0.70wt%, and the molecular weight was 31.45±5.21kDa.

5.2 The effects of *Gracilaria lemaneiformis* polysaccharide on the contents of serum total cholesterol (TC) (mmol/L), triglyceride (TG) (mmol/L), low-density lipoprotein (LDL-C) (mmol/L), high-density lipoprotein (HDL-C) (mmol/L), free fatty acid (FFA) (mmol/L), atherosclerosis index (AI) mmol/L), and peridídymal fat mass index ($\times 10^{-2}$ g/g) were shown in Table 2-8.

Table 2

Group	time			
	0d	14d	28d	42d
NC	4.09±0.81	5.05±0.50	4.30±0.85	3.85±0.50
HF	5.99±0.58^{*a}	5.96±0.46^{*a}	6.52±1.06^{*a}	6.46±1.64^{*a}
HF+AT	6.16±0.67^{*a}	6.33±0.68^{*a}	5.45±0.96^{ab}	5.53±0.32^{*ab}
HF+LGP	6.10±0.71^{*a}	5.88±1.09^{a}	5.267±0.68^{b}	4.85±0.71^{b}
HF+HGP	6.02±0.54^{*a}	6.17±0.91^{a}	5.43±1.045^{*ab}	4.89±0.72^{b}

Note: *p<0.05, comparing the high-dose group with the normal control group; **p<0.01, comparing the high-dose group with the normal control group; different letters indicate significant differences among different high-fat groups of mice (p<0.05). Same as below.

Table 3

Group	time			
	0d	14d	28d	42d
NC	0.83±0.18	0.80±0.12	0.62±0.16	0.62±0.13
HF	1.37±0.21^{**a}	0.77±0.11^{a}	0.84±0.17^{*a}	0.83±0.17^{a}
HF+AT	1.33±0.28^{**a}	1.00±0.39^{a}	0.64±0.16^{b}	0.61±0.08^{a}
HF+LGP	1.35±0.20^{**a}	0.72±0.20^{a}	0.57±0.09^{b}	0.35±0.04^{*b}
HF+HGP	1.18±0.28^{*a}	0.74±0.18^{a}	0.64±0.14^{b}	0.45±0.04^{c}

Table 4

Group	time			
	0d	14d	28d	42d
NC	0.45±0.09	0.39±0.14	0.37±0.11	0.33±0.07
HF	0.91±0.36^{**a}	1.12±0.28^{**a}	0.66±0.20^{a}	0.64±0.24^{a}
HF+AT	1.25±0.12^{**ab}	0.71±0.12^{*b}	0.53±0.07^{a}	0.61±0.26^{a}
HF+LGP	1.37±0.20^{**a}	0.86±0.14^{**ab}	0.58±0.11^{a}	0.38±0.06^{a}
HF+HGP	1.10±0.17^{**b}	0.97±0.34^{**ab}	0.75±0.26^{a}	0.29±0.06^{a}

Table 5

Group	time			
	0d	14d	28d	42d
NC	2.90±0.83	3.36±0.77	3.81±0.51	3.90±0.52
HF	1.41±0.63^{**a}	2.88±0.31^{ab}	2.84±0.60^{*a}	2.90±0.47^{*a}
HF+AT	1.60±0.34^{*a}	3.53±0.41^{a}	2.71±0.51^{**a}	3.70±0.86^{b}
HF+LGP	1.64±0.31^{**a}	3.11±0.47^{ab}	2.91±0.73^{*a}	3.87±0.64^{b}
HF+HGP	1.63±0.44^{*a}	2.99±0.48^{ab}	2.90±0.50^{*a}	3.25±0.56^{ab}

Table 6

Group	time			
	0d	14d	28d	42d
NC	1.31±0.31	1.52±0.25	1.11±0.15	1.13±0.23
HF	1.73±0.33^{a}	1.90±0.43^{*a}	1.56±0.19^{*a}	1.41±0.20^{*a}
HF+AT	1.94±0.55^{*a}	1.12±0.16^{*b}	1.27±0.24^{b}	1.28±0.26^{ad}
HF+LGP	1.95±0.59^{*a}	1.30±0.11^{bc}	1.37±0.20^{*ab}	0.78±0.09^{**b}
HF+HGP	1.81±0.09^{*a}	1.55±0.20^{c}	1.24±0.32^{b}	1.32±0.26^{a}

Table 7

Group	time			
	0d	14d	28d	42d
NC	0.15±0.05	0.14±0.08	0.10±0.03	0.09±0.02
HF	0.84±0.56^{**a}	0.42±0.15^{**a}	0.24±0.05^{**a}	0.20±0.07^{*a}
HF+AT	0.82±0.13^{**a}	0.22±0.05^{b}	0.19±0.01^{*a}	0.17±0.07^{*a}
HF+LGP	0.87±0.13^{**a}	0.26±0.04^{ab}	0.21±0.08^{**a}	0.11±0.04^{b}
HF+HGP	0.71±0.34^{**a}	0.38±0.08^{**a}	0.26±0.06^{**a}	0.09±0.02^{b}

Note: AI＝LDL-C/HDL-C

Table 8

Group	time			
	0d	14d	28d	42d
NC	1.15±0.36	1.08±0.30	0.92±0.52	1.21±0.46
HF	1.76±0.34^{**a}	2.10±0.61^{**a}	2.46±0.68^{**a}	2.40±0.63^{*a}
HF+AT	1.81±0.21^{**a}	2.10±0.11^{**a}	1.97±0.51^{**ac}	1.68±0.54^{b}
HF+LGP	1.78±0.38^{**a}	1.78±0.83^{*a}	1.68±0.26^{*bc}	1.28±0.16^{b}
HF+HGP	1.74±0.31^{**a}	1.67±0.63^{a}	1.60±0.56^{*bc}	1.56±0.20^{b}

Figures 1a - 1e are the oil red o stained section photos (200×) of the liver tissue sections of mice in NC, HF, HF+AT, HF+LGP, HF+HGP groups, respectively.

Figures 2a - 2e are the HE stained photos (400×) of the liver tissue sections of mice in NC, HF, HF+AT, HF+LGP, HF+HGP groups, respectively.

6. Analysis and discussion

By comparing the 0d results of the model group and the normal control group, the concentration of TC increased significantly ($p<0.05$), the concentrations of TG and LDL-C increased very significantly ($p<0.01$), and the concentration of HDL-C was significantly reduced ($p<0.05$) or very significantly reduced ($p<0.01$). This result indicated that the experimental animal model was successfully established. In terms of changes in TC concentration after 42 days of intervention, compared with the model group, the low-dose and high-dose *Gracilaria lemaneiformis* polysaccharide groups exhibited significant differences ($p<0.05$), while the atorvastatin group decreased without significant difference ($p>0.05$). In terms of TG concentration changes after 42 days of intervention, compared with the model group, the low-dose and high-dose

Gracilaria lemaneiformis polysaccharide groups exhibited significant differences ($p<0.05$). In terms of changes in LDL-C concentration after 14 days of intervention, compared with the model group, the statin group had a significant reduction ($p<0.05$), while the low-dose and high-dose *Gracilaria lemaneiformis* polysaccharide groups did not ($p>0.05$). As the intervention time increased to 42 days, the LDL-C concentration of high-dose mice tended to decrease, but there was no significant difference between groups ($p>0.05$). In terms of changes in HDL-C concentration after 42 days of intervention, compared with the model group, the atorvastatin group and the low-dose *Gracilaria lemaneiformis* polysaccharide group had a significant increase ($p<0.05$), while the high-dose *Gracilaria lemaneiformis* polysaccharide group did not ($p>0.05$). In terms of changes in FFA concentration after 42 days of intervention, compared with the model group, the low-dose *Gracilaria lemaneiformis* polysaccharide group had a significant reduction ($p<0.05$), while the atorvastatin group and the high-dose *Gracilaria lemaneiformis* polysaccharide group did not ($p>0.05$). In terms of changes in serum atherosclerosis index (AI=LDL-C/HDL-C) after 42 days of intervention, compared with the model group, the low-dose and high-dose *Gracilaria lemaneiformis* polysaccharide groups had a significant reduction ($p<0.05$), while the atorvastatin group did not ($p>0.05$). In terms of changes in the peridídymal fat mass index (peridídymal fat mass /body weight, $\times 10^{-2}$ g/g) after 42 days of intervention, compared with the model group, the atorvastatin group, the low-dose and high-dose *Gracilaria lemaneiformis* polysaccharide groups had significant reduction ($p<0.05$).

From the results of oil red o staining of the liver tissue sections of different groups of mice in Figs. 1a - 1e, it can be seen that the NC group showed normal liver morphology, with only a few liver cells containing good orange-stained lipid droplets; the HF group was stained with a diffuse and punctate dark orange red color, wherein a small piece of the necrotic area was dark orange-red, indicating that a large number of liver cells contained large lipid droplets and the necrotic lipid droplets were fused; the HF+AT group was stained to show a diffuse, punctate or small droplet-like figure, compared with the model group, the lipid droplets were reduced, though not significantly; the HF+LGP group showed a focal light orange color, indicating that the number of hepatocytes containing lipid droplets and the size of the lipid droplet were significantly reduced compared with the model group, which achieved an outstanding lipid reduction effect; the HF+HGP group showed a nest-piece-like spotted orange color, wherein the lipid droplets were reduced or smaller than the model group, which also achieved obvious lipid reduction.

From the results of HE staining of the liver tissue sections of different groups of mice in Fig. 2a - 2e, it can be seen that the hepatocytes of the NC group were arranged regularly without steatosis, inflammation or necrosis; the hepatocytes of the HF group were arranged disorderly with fatty vacuoles (arrow 1) of different numbers and sizes in the cytoplasm; severe steatosis, severe hepatocyte watery degeneration (arrow 2), and inflammatory cell focal infiltration (arrow 3) were also found, suggesting that the model group had accumulated a large amount of lipid in the liver and had moderate inflammation and necrosis; the hepatocytes in the HF+AT group were arranged slightly regular with fatty vacuoles (arrow 1), hepatocyte watery degeneration (arrow 2), and a small amount of inflammatory cell (arrow 3), suggesting that the statin intervention group had moderate steatosis and mild inflammation; the hepatocytes in the HF+LGP group were arranged regularly and no fatty vacuoles were seen, but there were multiple focal inflammatory cells infiltration, indicating that the lipid accumulation has been suppressed significantly after the intervention of low-dose

Gracilaria lemaneiformis polysaccharides, but the inflammation was aggravated possibly due to a later stage of body repair; the liver cells in the HF+HGP group were arranged regularly, and there were a few fatty vacuoles (arrow 1). Compared with the model group, the volume of vacuoles was significantly reduced with mild steatosis without inflammation, which indicated that the liver fat accumulation in the HF + HGP group was significantly improved, compared with the model group.

The above experimental results show that low-dose (polysaccharide content of 6.0 mg/mL) *Gracilaria lemaneiformis* polysaccharide may greatly reduce TC, TG, FFA and AI indexes of hyperlipidemia mice and increase HDL-C; high-dose (polysaccharide content of 22.5mg/mL) *Gracilaria lemaneiformis* polysaccharide may greatly reduce TC, TG and AI indexes of hyperlipidemia mice. In summary, *Gracilaria lemaneiformis* polysaccharide shows significant hypolipidemic effect and it can effectively reduce the risk of atherosclerosis.

Experimental example 3 Comparison of side effects caused by *Gracilaria lemaneiformis* polysaccharide and atorvastatin on liver injury

1. Experimental materials

1.1 Reagents: Atorvastatin calcium tablets (Pfizer Pharmaceutical Co., Ltd., product batch number: S90890), glutamate pyruvate transaminase (GPT) kit (Nanjing Jiancheng Biotechnology Co., Ltd., product batch number: 20180420), glutamate oxaloacetate transaminase (GOT) kit (Nanjing Jiancheng Biotechnology Co., Ltd., production batch number: 20180420), *Gracilaria lemaneiformis* polysaccharide (GLP) prepared by our laboratory.

1.2 Experimental equipment: a low-speed centrifuge, a microplate reader, a pipette,

1.3 Experimental animals: Kunming mice, male, 22-24g, purchased from Guangdong Medical Animal Center.

2. Preparation of experimental samples

The *Gracilaria lemaneiformis* polysaccharide prepared in Example 1.

3. Preparation of experimental samples

3.1 Preparation of positive control atorvastatin solution: suspend 7 atorvastatin calcium tablets (containing 140 mg atorvastatin) in 350 mL of sterile normal saline to prepare a sample solution with a concentration of 0.4 mg/mL (atorvastatin content).

3.2 Preparation of low-dose *Gracilaria lemaneiformis* polysaccharide solution: 3.57 g of *Gracilaria lemaneiformis* polysaccharide was accurately weighed and dissolved in 350 mL of sterile normal saline to prepare a sample solution with a polysaccharide content of 6.0 mg/mL.

3.3 Preparation of high-dose *Gracilaria lemaneiformis* polysaccharide solution: 13.37 g of *Gracilaria lemaneiformis* polysaccharide was weighed and dissolved in 350 mL of sterile physiological saline to prepare a medicinal solution sample with a polysaccharide content of 22.5 mg/mL.

4. Experimental method

4.1 Modeling stage: 102 Kunming mice, 22-24g, male, were randomly divided into two groups, wherein a group of 24 was a normal control group, fed with ordinary feed; the other group of 78 mice was a hyperlipidemia model group (hereinafter referred to as the model group), fed with high-cholesterol and high-fat diet, unlimited water and food. After 6 weeks of feeding, 6 mice were randomly selected from each group. After fasting but water for 8 h, the blood was collected by eyeball blood sampling, and the serum was separated by centrifugation. According to the kit instructions, the contents of total cholesterol (TC), triglycerides (TG), low density

lipoprotein (LDL-C), high density lipoprotein (HDL-C), and free fatty acid (FFA) were determined, respectively. The above indicators were used to judge whether the modeling is successful.

4.2 Experimental stage: After a successful model building, the model group was randomly divided into 4 groups, each with 18 mice: model control group (HF), atorvastatin intervention group (HF+AT), low-dose *Gracilaria lemaneiformis* polysaccharide intervention group (HF+LGP), high-dose *Gracilaria lemaneiformis* polysaccharide intervention group (HF+HGP). The normal control group (NC) and the model control group were given 0.1 mL/10g of sterile saline per day, and the administration groups were given 0.1 mL/10g volume of corresponding drugs per day. A total of 42 days of administration were conducted. 14, 28, and 42 days after the administration, 6 mice in each group were randomly selected after fasting but water for 8 hours. The blood was collected by eyeball blood sampling. According to the kit instructions, the activities of glutamate pyruvate transaminase (GPT) and glutamate oxaloacetate transaminase (GOT) were determined, respectively.

4.3 Data processing and analysis methods: the following experimental data are all expressed as "mean ± standard deviation". One-way ANOVA with SPSS 19.0 was used for one-way analysis of variance and significant difference analysis. Whether there is significant difference or not between the variances was determined by the LSD/Dunnett's T3 method.

5. Experimental results

5.1 After determination, the extraction rate of the *Gracilaria lemaneiformis* polysaccharides used in the experimental Example was 23.65±0.27% by weight, the purity of the polysaccharide was 59.01±0.91% determined by phenol sulfuric acid method, the protein content was only 1.57±0.37 wt% determined by the BCA kit, the sulfuric acid group content is 22.85±0.70 wt%, and the molecular weight is 31.45±5.21 kDa.

5.2 According to the experimental results of Experimental Example 2, compared with the normal control group, the concentration of TC increased significantly ($p<0.05$), while the concentrations of TG and LDL-C increased very significantly ($p<0.01$). The concentration of HDL-C had a significant decrease ($p<0.05$) or very significant decrease ($p<0.01$), respectively. This result shows that the model has been successfully established.

5.3 Effects of *Gracilaria lemaneiformis* polysaccharide on glutamate pyruvate transaminase (GPT) content (U/L) and on glutamate oxaloacetate transaminase (GOT) content (U/L) are shown in Table 9, and Table 10.

Table 9

Group	time			
	0d	14d	28d	42d
NC	15.39±3.68	19.03±7.00	15.65±5.40	10.99±3.45
HF	$26.25\pm4.00^{**a}$	$42.55\pm11.24^{**a}$	$31.38\pm7.16^{**a}$	$24.57\pm5.30^{**a}$
HF+AT	$25.35\pm1.73^{**a}$	$36.32\pm3.81^{**ac}$	$27.17\pm6.89^{**a}$	$20.86\pm5.62^{**ab}$
HF+LGP	$25.00\pm1.98^{**a}$	26.66 ± 4.16^{bc}	$25.11\pm4.59^{*a}$	$18.25\pm2.22^{**b}$
HF+HGP	$23.25\pm1.98^{**a}$	19.86 ± 6.52^{dc}	20.59 ± 4.63^{b}	$18.82\pm4.24^{**b}$

Note: *p<0.05, comparing the high-dose group with the normal control group; **p<0.01, comparing the high-dose group with the normal control group; different letters indicate significant differences among different high-fat groups of mice (p<0.05). Same as below.

Table 10

Group	time			
	0d	14d	28d	42d
NC	30.21±5.75	21.57±4.05	21.81±8.27	15.33±4.26
HF	$42.79\pm6.98^{**a}$	28.89 ± 3.05^{a}	27.42 ± 4.20^{a}	$20.76\pm1.48^{**a}$
HF+AT	34.84 ± 8.29^{a}	25.12 ± 4.27^{a}	19.49 ± 3.11^{ab}	16.50 ± 1.45^{b}
HF+LGP	36.70 ± 6.20^{a}	22.43 ± 2.95^{a}	18.76 ± 2.46^{ab}	$11.48\pm1.36^{**c}$
HF+HGP	34.88 ± 6.38^{a}	27.06 ± 5.37^{a}	17.42 ± 3.58^{b}	17.41 ± 2.34^{b}

6. Analysis and discussion

In terms of changes in GPT activity after 42 days of intervention, compared with the normal control group, the high-fat group had a very significant increase (p<0.01). Compared with the model control group, the low-dose and high-dose groups significantly decreased (p<0.05). But there was no significant difference between the atorvastatin group and the *Gracilaria lemaneiformis* polysaccharide groups (p>0.05). In terms of the changes in GOT activity after 42 days of intervention, compared with the normal control group, the model control group had a very significant increase (p<0.01); compared with the model control group, the atorvastatin group and the high-dose and low-dose *Gracilaria lemaneiformis* polysaccharide all had a significant reduction (p<0.05); compared with the atorvastatin group, the low-dose gracilaria lemaneiformis polysaccharide group had a significant reduction (p<0.05).

The above results indicate that there is a difference between the side effects of *Gracilaria lemaneiformis* polysaccharide and atorvastatin in liver injury, and low-dose (polysaccharide content of 6.0 mg/mL) *Gracilaria lemaneiformis* polysaccharide is safer than atorvastatin.

The above-mentioned embodiments are preferred embodiments of the present invention, but the embodiments of the present invention are not limited by the above-mentioned embodiments, and any other changes, modifications, substitutions, combinations, and simplifications made without departing from the spirit and principle of the present invention all should be equivalent replacement methods, and they are all included in the protection scope of the present invention.

1. A method for preparing a *Gracilaria lemaneiformis* polysaccharide with significant hypolipidemic activity, comprising the following steps:

(1) washing, air-drying, crushing and sieving *Gracilaria lemaneiformis* to obtain *Gracilaria lemaneiformis* powder;

(2) adding an organic acid solution to the *Gracilaria lemaneiformis* powder; after pre-homogenization, extracting the *Gracilaria lemaneiformis* powder by dynamic high pressure microfluidization, neutralizing with a base and filtering to obtain a filter residue and an extract solution;

(3) performing ultrafiltration of the extract solution in step (2), discarding a permeate, and collecting a retentate;

(4) separating the retentate with an ion exchange resin, and concentrating a component with the highest hypolipidemic activity under vacuum to obtain a concentrated solution;

(5) mixing the concentrated solution with ethanol, standing and filtering to obtain a polysaccharide precipitation;

(6) drying the polysaccharide precipitation to obtain the *Gracilaria lemaneiformis* polysaccharide;

wherein the *Gracilaria lemaneiformis* polysaccharide has a molecular weight of 20-35 kDa and a sulfate group content of 20-28% by weight.

2. The method according to claim 1, characterized in that, the crushing and sieving step in step (1) comprises sieving by a 20-80 mesh sieve after crushing.

3. The method according to claim 1, characterized in that, the pH of the organic acid solution in step (2) is 2.0-4.0; the organic acid solution is a citric acid solution or a malic acid solution; and the mass of the organic acid solution in step (2) is 20-40 times of the mass of the *Gracilaria lemaneiformis* powder.

4. The method according to claim 1, characterized in that, parameters of the pre-homogenization in step (2) comprise 20-50 MPa, 1 - 3 min, and 1-2 times of homogenization; and control conditions for the dynamic high pressure microfluidization in step (2) comprise a microfluidization pressure of 120-180 MPa, an extraction temperature of 75-100°C, an extraction time of 80-160 min, and 1-2 times of extractions.

5. The method according to claim 1, characterized in that, the ultrafiltration in step (3) is performed by using an ultrafiltration membrane with a molecular weight cut-off of 1 kDa.

6. The method according to claim 1, characterized in that, a model of the ion exchange resin in step (4) is DEAE-fast flow or DEAE-52; and the concentrated solution obtained by vacuum concentration in step (4) has a volume of 1/10 - 1/5 of the volume of the retentate.

7. The method according to claim 1, characterized in that, the ethanol in step (5) has an amount of 60% - 90% of a total volume of the concentrated solution after mixing with the ethanol; and the standing in step (5) comprises standing at 2 - 8°C for 8 - 14 hours.

8. The method according to claim 1, characterized in that, the drying in step (6) comprises vacuum freeze drying or spray drying.

9. Use of the *Gracilaria lemaneiformis* polysaccharide according to any of claims 1 to 8 in the preparation of hypolipidemic drugs or health products.