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Characterization and hypoglycemic effect of a polysaccharide extracted from the fruit of *Lycium barbarum* L.

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ABSTRACT

Diabetes mellitus is a group of complicated metabolic disorders characterized by high blood glucose level and inappropriate insulin secreting capacity due to decreased glucose metabolism and pancreatic β cell mass or dysfunction of β cells. Thus, improving glucose metabolism and preserving β cell mass and function might be useful for the treatment of diabetes. In this study, a novel acidic polysaccharide LBP-s-1 extracted from *Lycium barbarum* L. was obtained by purification using macroporous resin and ion-exchanged column. Monosaccharide composition analysis indicated that LBP-s-1 was comprised of rhamnose, arabinose, xylose, mannose, glucose, galactose, galacturonic acid in the molar ratio of 1.00:8.34:1.25:1.26:1.91:7.05:15.28. The preliminary structure features of LBP-s-1 were investigated by FT-IR, ^{1}H NMR and ^{13}C NMR. *In vitro* and *in vivo* hypoglycemic experiments showed that LBP-s-1 had significant hypoglycemic effects and insulin-sensitizing activity through increasing glucose metabolism and insulin secretion and promoting pancreatic β cell proliferation. Preliminary mechanisms were also elucidated.

1. Introduction

Diabetes mellitus (DM) is a group of diseases associated with various metabolic disorders, the main feature of which is chronic hyperglycemia due to insufficient insulin secreting capacity (Kuzuya et al., 2002) as a result of decreased glucose metabolism and pancreatic β cell mass or dysfunction of β cells. According to the statistics from the International Diabetes Federation (IDF) in 2012, more than 371 million people were diagnosed to have diabetes and still half of the number of people with diabetes remained undiagnosed. New data projected that the number of people with diabetes would rise to 552 million by 2030, which means that within every 10 s approximately 3 more people would be diagnosed with diabetes. The major classes of agents used to treat diabetes were insulin and its analogs, biguanide, sulfonylureas, thiazolidinediones, α-glucosidase inhibitors, glucogen like peptide 1 receptor agonists (exenatide) and dipeptidyl peptidase-4 inhibitors (Tahrani, Piya, Kennedy, & Barnett, 2010). Since that these chemical oral drugs or biological injections have a number of limitations, such as adverse effects or being of high cost for treatment, searching for more effective and safer anti-diabetic drugs is still a common goal in the field of medicinal research. Many components extracted from traditional medicine or other natural materials were reported to have hypoglycemic effect. Polysaccharides were polymers extracted from abundant resources with high effectiveness and rare adverse effects, which enabled them to stand a good chance of being novel drug candidates for diabetes.

Lycium barbarum L., a solanaceous defoliated shrubbery, is widely distributed in arid and semi-arid regions of Northwestern China. The fruit of *Lycium barbarum* L., also called Gouqizi in Chinese or wolfberry in English, is a well-known traditional Chinese medicine recorded by the ancient herbalist around 2300 years ago (Amagase & Farnsworth, 2011). Previous studies showed that the main bioactive constituents of *Lycium barbarum* L., such as polysaccharides, carotenoids and zeaxanthin, had a variety of beneficial effects on decreasing the level of blood glucose and serum lipids, anti-aging, immuno-modulating, anticancer, anti-fatigue, and male fertility-facilitating (Chang et al., 2011; Gan, Zhang, Liu, & Xu, 2003; Luo, Cai, Yan, Sun, & Corke, 2004; Luo et al., 2006; Wang,

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Chang, Inbaraj, & Chen, 2010; Wang, Yin, et al., 2010). Among all of the bioactive substances, the most well studied components are a group of water-soluble glycoconjugates (*Lycium barbarum* polysaccharides, LBP). It is reported that hypoglycemic effects of LBPs were mostly related to anti-oxidative activity, and mechanisms of hypoglycemic effects were not elucidated.

In this study, a novel acidic polysaccharide LBP-s-1 was obtained by purification using macroporous resin and ion-exchanged column. Its components and preliminary structure features were characterized by chemical and instrumental analysis. In addition, *in vitro* and *in vivo* experiments were carried out to evaluate the hypoglycemic effect of LBP-s-1, along with preliminary mechanism investigation.

2. Materials and methods

2.1. Materials

The fruit bodies of *Lycium barbarum* L. were obtained from the Agriculture and Forestry Research Institute of Ningxia Huizu Autonomous Region, People's Republic of China. Macroporous Adsorption Resin S-8 was produced by the Chemical Plant of Nankai University (Tianjin, China). The diethylaminoethyl-cellulose (DEAE-52) and dextran standards were obtained from Pharmacia Co., Ltd. (Uppsala, Sweden). Streptozotocin and Thiazolyl Blue Tetrazolium Bromide (MTT) were purchased from Sigma–Aldrich (St. Louis, USA). 1-Phenyl-3-methyl-5-pyrazolone (PMP) was purchased from Acros Organics (Geel, Belgium). Insulin, exendin 4 (Ex-4) and metformin (MET) were produced by Novo Nordisk (North Carolina, USA), TASH Biotechnology Co., Ltd. (Shanghai, China) and Beyotime (Nantong, China), respectively. All other reagents of analytical grade were purchased from Shanghai Chemical Co. (Shanghai, China).

2.2. Animals

Forty eight male C57BL/6J mice (18 ± 2 g, 8-week-old) were housed in plastic cages and maintained under standard conditions (12 h light/dark cycle; 23–25 °C; 35–60% humidity). Before and during the experiment, the mice were fed with a high fat or normal laboratory pellet diet and water was freely available. The study complied with the current ethical regulations for the care and use of laboratory animals of China Pharmaceutical University (Nanjing, China).

2.3. Cell line and culture

RIN-m5f (a rat insulinoma cell line), HepG2 (a human hepatoma cell line) and 3T3-L1 (a mouse pre-adipocyte cell line) cells were supplied by Chinese Academy of Sciences. HepG2 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco Life Technologies, NY, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS, Gibco Life Technologies, NY, USA), 2 g/L $NaHCO_3$, 100 U/mL penicillin and 0.1 g/L streptomycin. RIN-m5f cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco Life Technologies, NY, USA), 2 g/L $NaHCO_3$, 100 U/mL penicillin and 0.1 g/L streptomycin. 3T3-L1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) high glucose (Gibco Life Technologies, NY, USA) supplemented with heat-inactivated 10% FCS, 3.7 g/L $NaHCO_3$, 100 U/mL penicillin and 0.1 g/L streptomycin. The cells were maintained in monolayer culture with 5% CO_2 at 37 °C. At 90% confluence, they were trypsinized with 0.25% trypsin–0.02% EDTA in PBS solution for about 60 s and resuspended in complete culture medium.

2.4. Separation and purification of LBP-s-1

Dried fruits of *Lycium barbarum* (200 g) was defatted in a Soxhlet extractor with chloroform methanol solution (boiling point: 80 °C) twice and pretreated with 80% ethanol twice to remove some colored materials and small molecule materials. Dry residuals (50 g) were extracted in 80 °C hot water for three times. The concentrated extract was precipitated by three times volume of 95% ethanol. The precipitate was collected by centrifugation, washed successively with ethanol and acetone, and then dried under reduced pressure, giving crude polysaccharide powder. Crude polysaccharide powder solution was decolored by macropolorus resin S-8 at 37 °C for 1 h. The decolored and condensed solution was applied to DEAE column and eluted with 0.33 mol/L NaCl solution. Fractions were collected and enriched according to the result of phenol-sulfuric acid analysis. The collected fraction was dialyzed (MWCO 14,000) against distilled water for 48 h and precipitated by three times volume of 100% ethanol, and the precipitate was coded as LBP-s-1 and used in the following tests. The total carbohydrate content of LBP-s-1 was determined by phenol-sulfuric acid method (DuBois, Gilles, Hamilton, Rebers, & Smith, 1956) using D-glucose as standard sample. The uronic acid content was determined using *m*-hydroxydiphenyl-sulfuric method (Filisetti-Cozzi & Carpita, 1991) with galacturonic acid as standard sample.

2.5. Characterization of LBP-s-1

2.5.1. Purity and molecular weight determination

The purity and molecular weight of LBP-s-1 were determined using high performance size-exclusion chromatography (HPSEC). Sample was first dissolved in distilled water and passed through 0.45 μm filter, and then was applied to gel-filtration column (Shodex SUGAR KS-805, 8 mm ID × 300 mm, Showa Denko, Japan), eluted at a flow rate of 1.0 mL/min with deionized water and detected by a refractive index detector. Column calibration was performed using standard dextrans with different molecular weights (21,400, 41,100, 84,400, 133,800, 2,000,000 Da, respectively). The standard curve represented the linear relationship of the retention time and the logarithm of their respective molecular weights. The molecular weight of LBP-s-1 was calculated by comparing with the standard dextrans with different molecular weights.

2.5.2. Analysis of monosaccharide composition

Monosaccharide composition analysis of polysaccharide was performed using derivation with acetate by gas chromatography (GC) and pre-column derivatization with PMP by high-performance liquid chromatography (HPLC) as described previously (Englyst & Cummings, 1984; Fu & O'Neill, 1995; Yang et al., 2005).

2.5.3. Infrared spectral analysis

The infrared spectrum (IR), as KBr pellets, was recorded by a Nicolet-170X spectrophotometer in a range of 4000–400 cm^{-1}.

2.5.4. Nuclear magnetic resonance spectroscopy

^1H NMR and ^{13}C NMR spectra were recorded by Brucker DRX-500NMR Spectrometer. LBP-s-1 was dissolved in D_2O and examined at 500 MHz at 30 °C.

2.6. RIN-m5f proliferation assay and insulin-releasing capacity

2.6.1. RIN-m5f proliferation assay

RIN-m5f cells, seeded in 96-well microplate with a density of 10^4 cells/mL, were allowed to attach for 12 h at 37 °C. Cells were treated in the absence or presence of different concentrations of LBP-s-1 (15.63, 31.25, 62.5, 125, and 250 nmol/L) for 48 h. Exendin-4 (200 nmol/L) and medium were added as positive and negative

control, respectively. Cell proliferation was measured by MTT assay. MTT solution (5 g/L, 20 μL) was added to each well and incubated at 37 °C for 4 h. After adding stop solution DMSO (100 μL/well), the absorbance at 490 nm was measured by a Multiskan Spectrum Microplate Spectrophotometer (Thermo, Finland).

2.6.2. Insulin-releasing capacity of RIN-m5f

RIN-m5f cells were seeded in 12-well microplate with a density of 10^5 cells/mL and allowed to attach for 48 h in RPMI 1640 (containing 5.5 mmol/L glucose) at 37 °C. The culture medium was replaced, and cells were washed with DMEM high glucose twice.

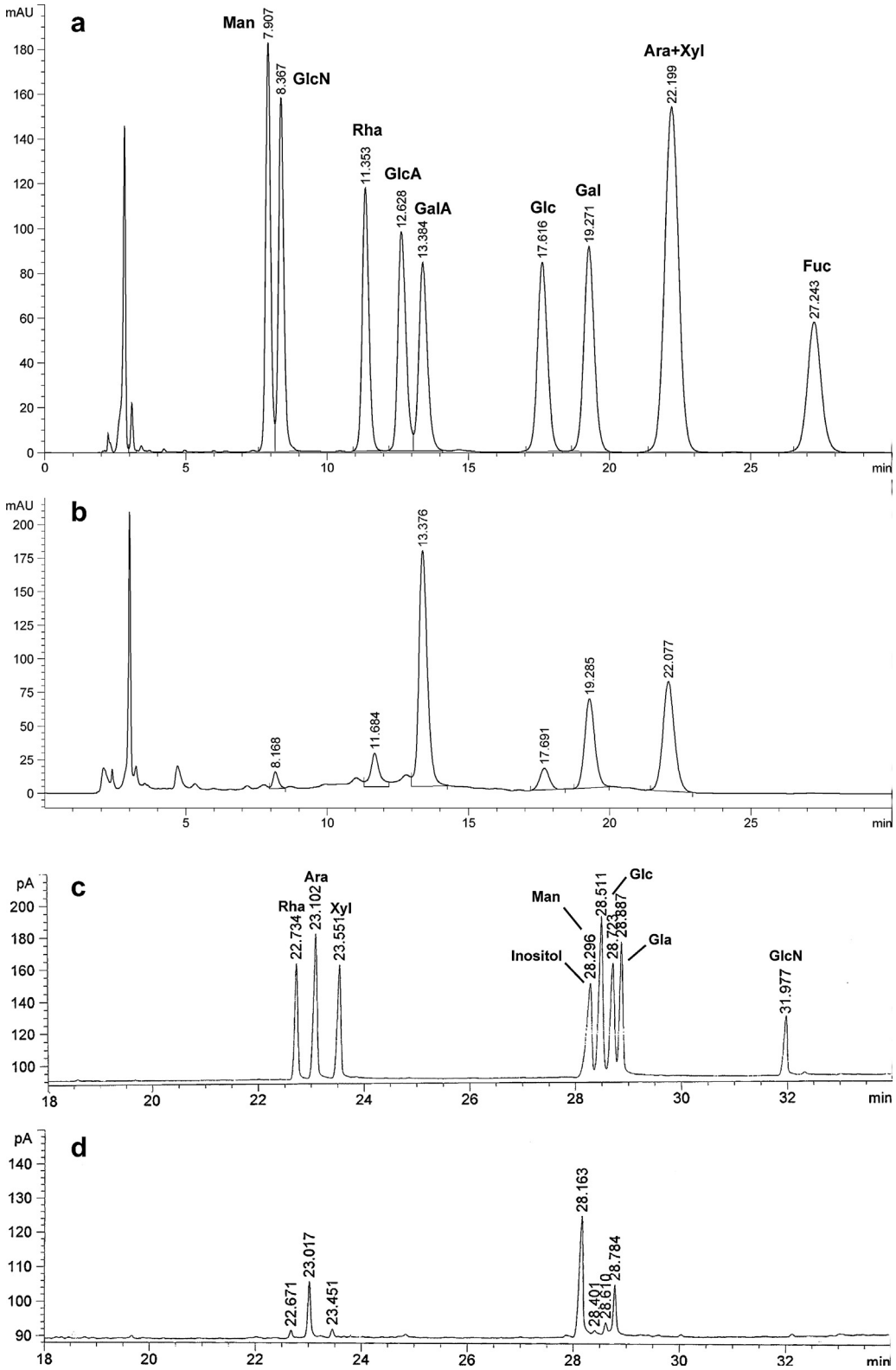

Fig. 1. GC and HPLC chromatograms of monosaccharide composition of LBP-s-1. The GC chromatograms of acetate derivative of seven standard monosaccharides (a) and component monosaccharides in LBP-s-1 (b). The HPLC chromatograms of PMP derivatives of ten standard monosaccharides (c) and component monosaccharides in LBP-s-1 (d).

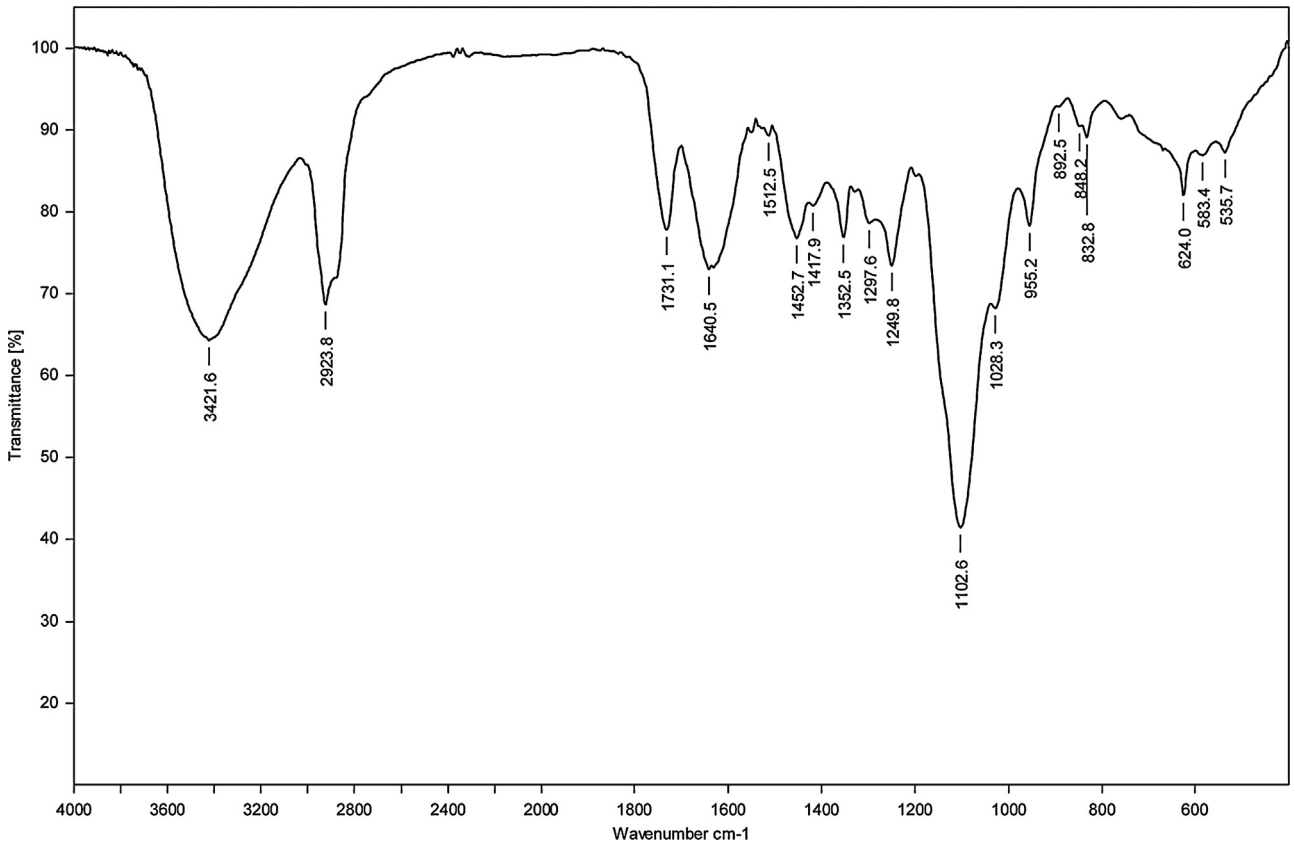

Fig. 2. IR spectrum of LBP-s-1.

Cells were incubated for 2 h at 37 °C with DMEM high Glucose medium (containing 16.7 mmol/L glucose) in the absence or presence of different concentrations of LBP-s-1 (15.63, 31.25, 62.5, 125, and 250 nmol/L). Exendin-4 (200 nmol/L) and medium were added as positive and negative control, respectively. The insulin concentrations of the cell culture supernatant of each group were determined by Insulin Elisa kit (Jiancheng, Nanjing) following the manufacturer's instruction.

2.7. Glucose consumption assay in two kinds of peripheral cells

2.7.1. Glucose consumption assay in HepG2 cells

HepG2 cells were seeded in a 96-well microplate with a density of 5000 cells/well and allowed to attach for 12 h at 37 °C. Cells were washed and replaced with DMEM high glucose containing 2% FCS. Subsequently, HepG2 cells were incubated with insulin (5×10^{-7} mol/L) for 24 h, washed once with serum-free DMEM high glucose, and then treated with LBP-s-1 at different concentrations (15.63, 31.25, 62.5, 125, and 250 nmol/L). The concentrations of glucose in cell culture supernatant of each group were determined by Glucose assay kit (Jiancheng, Nanjing) following the manufacturer's instruction. MTT assay was introduced here to evaluate cell viability. Metformin (20 μmol/L) and medium were added as positive and negative control, respectively.

2.7.2. Glucose consumption assay in 3T3-L1 cells

Mouse 3T3-L1 pre-adipocytes were seeded in a 96-well microplate with a density of 5000 cells/well and allowed to confluence. Confluent cells were differentiated in medium A (DMEM high glucose with 10% FBS, 10 mg/mL insulin, 1 mmol/L dexamethasone, and 0.5 mmol/L isobutyl-1-methylxanthine) for 2 days. Culture was replaced with fresh medium A for another 2 days. Likewise, medium B (DMEM high glucose with 10% FBS and 10 mg/mL insulin) was added to replace medium A for 2 days twice. Afterwards, cells were cultured in DMEM high glucose plus 10% FBS with insulin (5×10^{-7} mol/L) for 24 h, washed once with serum-free DMEM high glucose, and then treated with LBP-s-1 at different concentrations(15.63, 31.25, 62.5, 125, and 250 nmol/L). Metformin (20 μmol/L) and medium were added as positive and negative control, respectively. The concentrations of supernatant glucose and MTT assay were tested as above.

2.8. Hypoglycemic activity of LBP-s-1 in vivo

2.8.1. Establishment of diabetes mellitus mice model

All of the mice were randomly divided into two groups: (Group A, $n = 8$, Group B, $n = 40$), Group A was fed with ordinary forage while Group B was fed with high fat forage. The mice were acclimatized in the new environment for a week. At the end of the 8th week, group B was treated with intraperitoneal injection of 50 mg/kg streptozotocin (STZ, in 0.1 mol/L citrate-buffered saline, pH 4.5) for seven days, the results of fasting blood glucose (FBG) were used to appraise the success of the model constructions. After the FBG remained stable for one week, Group B of successful mice were then randomly divided into five groups: diabetes group (MOD, $n = 8$), diabetes + LBP-s-1 low dose group (LBP-L, $n = 8$), diabetes + LBP-s-1 middle dose group (LBP-M, $n = 8$), diabetes + LBP-s-1 high dose group (LBP-H, $n = 8$), diabetes + metformin group (MET, $n = 8$), group A was taken as control (CON, $n = 8$). Group LBP-L, LBP-M, LBP-H and MET were administered with oral LBP-s-1 125, 250 and 500 mg/kg/day or metformin 200 mg/kg/day by gastric perfusion while group CON and group MOD were administered with the same volume of vehicle (physiological saline) for 7 weeks.

2.8.2. Analysis of insulin sensitivity and activity of hexokinase and pyruvate kinase

Insulin sensitivity was evaluated by oral glucose tolerance test (OGTT) and HOMA-IR index (Homeostasis model assessment-insulin resistance index, HOMA-IR = FPG (fasting plasma glucose) [mmol/L] × FINS (fasting insulin) [mU/L]/22.5). OGTT was performed after 16 h overnight fast. Glucose (2 g/kg) was administered by gastric perfusion and blood samples were collected from the orbital sinus at 0, 15, 30, 60 and 120 min respectively and tested using Glucose assay kit. At the end of 17th week, blood from the orbital sinus was collected to test insulin concentration by Insulin Elisa kit (Jiancheng, Nanjing). All of the mice were sacrificed, and livers were removed quickly for testing the activity of pyruvate kinase and hexokinase using the pyruvate kinase and hexokinase kit, respectively (Jiancheng, Nanjing).

2.9. Statistical analysis

All values were expressed as mean ± standard error (SD). Statistical significance was determined by analysis of variance (ANOVA) followed by Student's *t* test. Differences of $p < 0.05$ were considered statistically significant.

3. Results and discussion

3.1. Homogeneity, molecular weight, carbohydrate content, and monosaccharide composition of LBP-s-1

After hot water extraction from dry *Lycium barbarum* L. powder and purification through macropolorus resin S-8 and DEAE-cellulose-32 column, LBP-s-1 was obtained with a final yield of 0.69%. LBP-s-1 was considered as a novel polysaccharide according to the new purification process and results of monosaccharide composition and structure characterization.

HPSEC analysis of LBP-s-1 (Data not shown) showed a single and symmetric peak, which indicated that LBP-s-1 was homogeneous and purified. The total carbohydrate content of LBP-s-1 was 97.82%. No significant absorbance was observed at 260 nm or near 280 nm in the UV spectrum. The protein content was 1.85% determined by Bradford's method. Optical rotation value was +102.5° for LBP-s-1.

The average molecular weight of LBP-s-1 was determined by high-performance gel permeation chromatography. The average molecular weight of LBP-s-1 was estimated to be 1.92×10^6 Da based on the equation of the standard curve. GC is a preferred method in quantitative and qualitative determination of monosaccharide composition because of its accuracy, efficiency and convenience. According to the retention time of alditol acetate derivatives in GC (Fig. 1), LBP-s-1 consisted of six different monosaccharides, including rhamnose, arabinose, xylose, mannose, glucose, galactose, with the molar ratio of 1.00:8.34:1.25:1.26:1.91:7.05. However, the derivative method used in GC analysis was not suitable for uronide-containing polysaccharides. Because uronic acids have unusual resistance to acid hydrolysis, and lactonization and decarboxylation would occur if uronic acids were liberated from the polymers. Therefore, PMP derivatization was carried out to determine uronic acid qualitatively. The result showed that galacturonic acid was a major component of LBP-s-1. The galacturonic acid content was 46.30%, determined colorimetrically by the *m*-hydroxydiphenyl-sulfuric method. Given the information above, we found that LBP-s-1 consisted of rhamnose, arabinose, xylose, mannose, glucose, galactose, galacturonic acid with the molar ratio of 1.00:8.34:1.25:1.26:1.91:7.05:15.28.

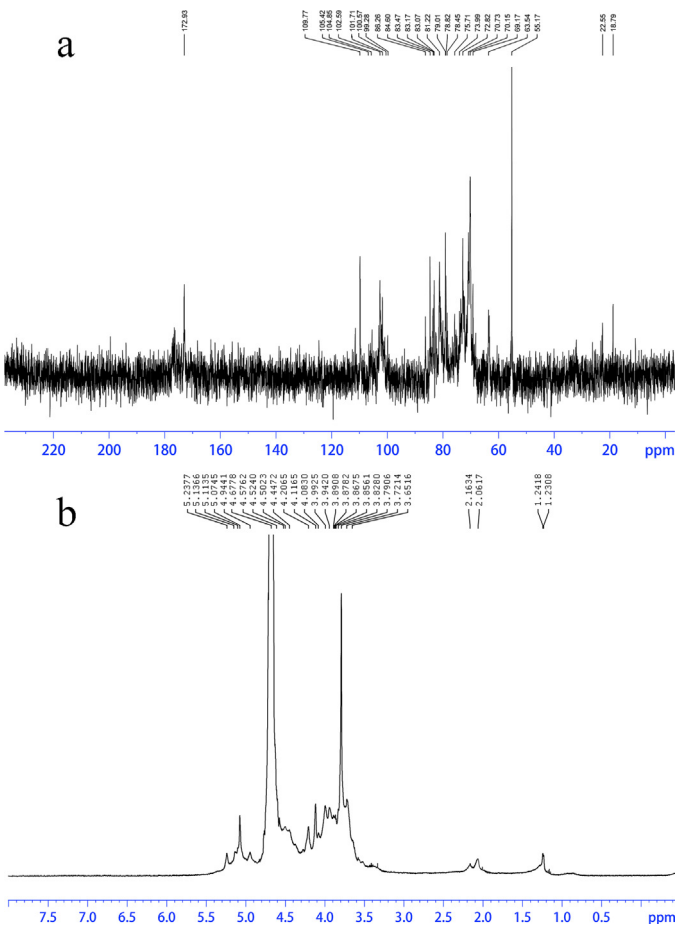

Fig. 3. ^{1}H NMR and ^{13}C NMR spectra of LBP-s-1. LBP-s-1 was dissolved in D_2O and examined on a Varian 500 NMR spectrometer. Numerical value is in d (ppm).

3.2. Structure characterization of LBP-s-1

In Fig. 2, the IR spectrum of LBP-s-1 showed typical absorption peaks of polysaccharides. The strong band at 3421.6 cm^{-1} was attributed to the hydroxyl stretching vibration of the polysaccharide. The band at 2923.8 cm^{-1} was due to C-H stretching vibration of CH_2. The broad band at 1640.5 cm^{-1} was due to the bound water. A strong band at 1731.1 cm^{-1} and a weak band at 1417.9 cm^{-1} were assigned to the absorbance of the carboxylic group (COO^-) (Nejatzadeh-Barandozi & Enferadi, 2012). The bands at 1297.6 and 1452.7 cm^{-1} were corresponded to symmetrical deformations and angle vibrations of COH groups. The absorption at 1352.5 cm^{-1} was possibly due to non-symmetrical and symmetrical CH_3 bending, which indicated the presence of rhamnose. Two intense stretching peaks at 1028.3 and 1102.6 cm^{-1} were ascribed to the presence of C—O bonds and furanose ring (Ghasemlou, Khodaiyan, Jahanbin, Gharibzahedi, & Taheri, 2012) in the monosaccharide blocks of LBP-s-1. The bands at 832.8, 851.4 and 892.5 cm^{-1} confirmed the co-existence of the α and β-glycosidic bonds and pyranose ring in the monosaccharide blocks of LBP-s-1. Given that the moderate absorbance at 832.8 and 848.2 cm^{-1} and the weak absorbance at 892.5 cm^{-1}, α-glycosidic bond was dominant in LBP-s-1, which was in agreement with the result of optical rotation value.

The ^{13}C NMR spectrum of LBP-s-1 (Fig. 3) showed the anomeric peaks between δ 99.28 and δ 109.77 ppm, indicating there were both α (δ 99–102 ppm) and β (δ 104–105 ppm) anomeric configuration of pyranose and furanose (δ 107–109 ppm) existing in LBP-s-1. This result was in agreement with that of IR spectrum. The signal at 172.93 ppm was due to C6 of galacturonic acid

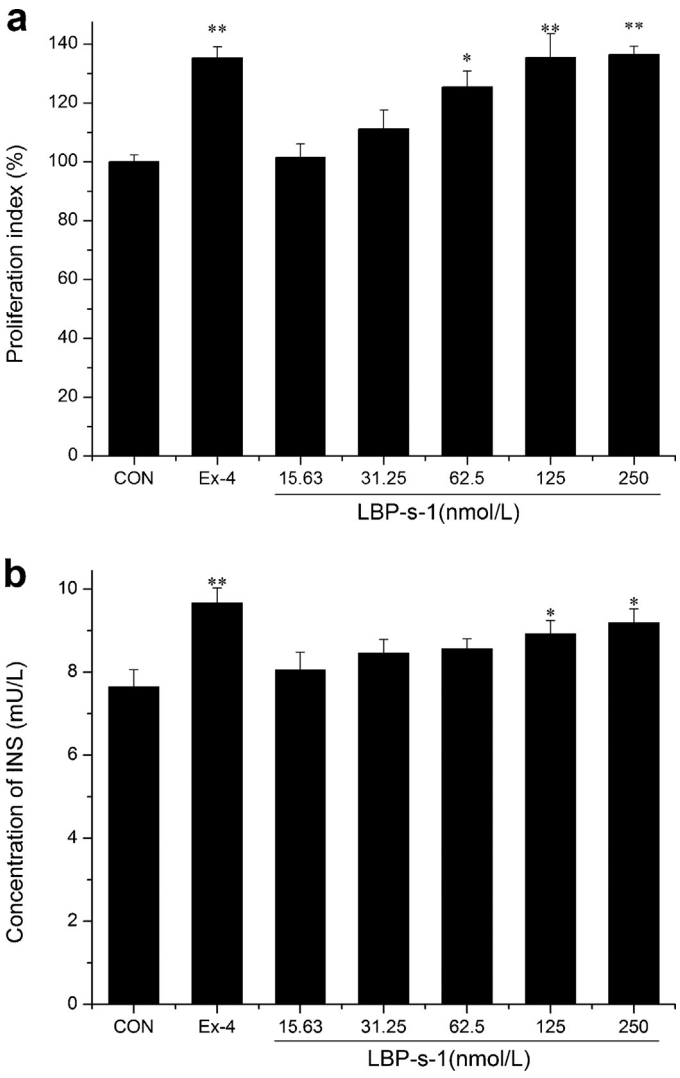

Fig. 4. Effects of LBP-s-1 on RIN-m5f proliferation index and insulin secretion. Cells were treated with Ex-4 (200 nmol/L) and different concentrations of LBP-s-1 (from 15.63 to 250 nmol/L) for 48 h, and then proliferation index was estimated by MTT method (a). Cells were treated with Ex-4 and different concentrations of LBP-s-1 for 2 h, and then concentration of insulin was estimated by Elisa (b). Values are the mean ± SD, ($n = 6$). $^{*}p < 0.05$, $^{**}p < 0.01$ vs the control group.

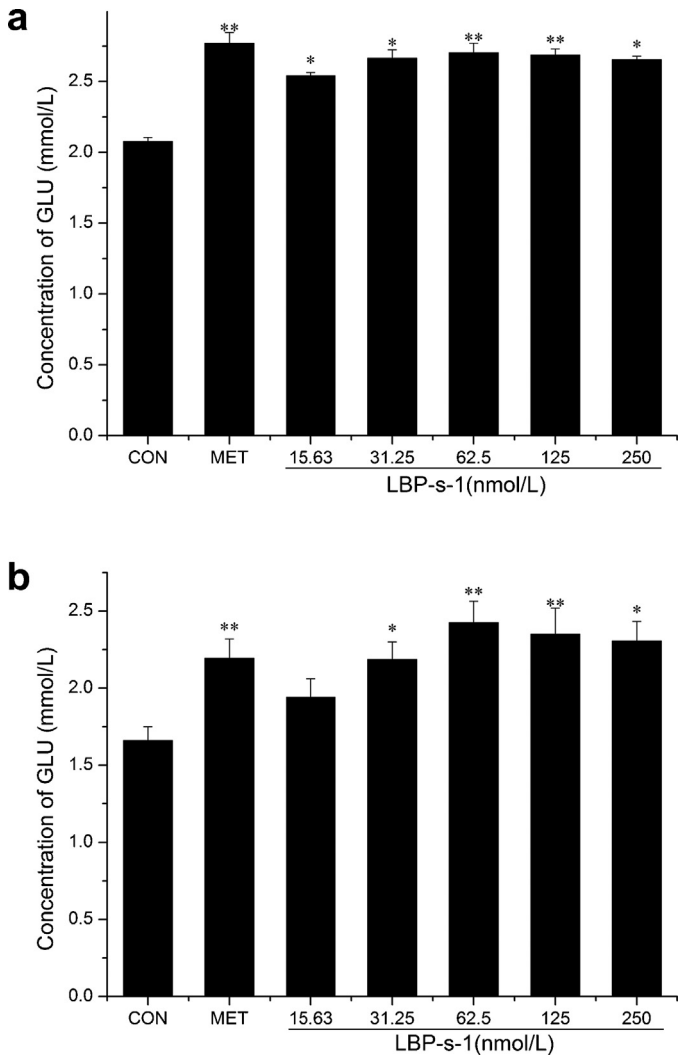

Fig. 5. Effects of LBP-s-1 on glucose utilization of HepG2 and 3T3-L1 cells. HepG2 (a) and 3T3-L1 cells (b) were pretreated with 5×10^{-7} mol/L insulin and then incubated with different concentrations of LBP-s-1 for 24 h. The glucose concentrations in cell culture supernatant of each group were determined by Glucose assay kit. Values are the mean ± SD, ($n = 6$). $^{*}p < 0.05$, $^{**}p < 0.01$ vs the control group.

(Yang, Zhou, & Liang, 2009). Signals at δ 99.28, 100.57, 101.71, 102.59, 104.85, 105.42 and 109.77 ppm were assigned to anomeric carbons of α-glucopyranose, α-xylopyranose, α-rhamnpyranose, α-mannopyranose, β-galactopyranose, β-galacturionic acid and α-arabinofuranose residues, respectively (Ghasemlou et al., 2012; Ha, Viëtor, Jardine, Apperley, & Jarvis, 2005; Wang, Chang, et al., 2010; Wang, Yin, et al., 2010; Wu, Gao, Tsim, & Tu, 2005). The signals from δ 55.17 to δ 86.26 were attributed to C2–C6 of the residues. The two small signals at δ 18.79 and δ 22.55 ppm was assigned to CH_3 of rhamnose (Ha et al., 2005).

The ^{1}H NMR spectrum of LBP-s-1 showed nine anomeric signals between δ 5.24 and δ 4.45 ppm. Signals at δ 5.24, 5.14, 5.11, 5.07 and 4.94 ppm were assigned to anomeric protons of α-rhamnpyranose, α-xylopyranose, α-mannopyranose, α-arabinofuranose and α-glucopyranose, respectively (Zdorovenko et al., 2012). Signals at δ 4.58, 4.52, 4.50 and 4.45 ppm were due to H1 of β-galacturionic acid and β-galactopyranose (Habibi, Heyraud, Mahrouz, & Vignon, 2004). The signals from δ 1.23 to δ 4.21 ppm were attributed to H2–H6 of the residues.

LBP-s-1 has an extremely complicated structure features due to its composition of seven monosaccharides. Rhamnose, xylose, mannose and glucose only comprise less than 5% of LBP-s-1 respectively, which leads to the difficulty in structural characterization in details. In this part, several typical peaks in spectra of IR, ^{1}H NMR and ^{13}C NMR confirmed the monosaccharide composition. It was concluded that LBP-s-1 consisted of furan and pyran ring with both α and β anomeric configuration.

3.3. *RIN-m5f proliferation assay and insulin-releasing capacity*

The pancreatic β cell mass and the capacity of β cells to secrete insulin are controlled under physiological conditions according to the metabolic demand of the body, which is important to sustain homeostasis of blood glucose. Failure of the pancreas to provide appropriate insulin secreting capacity due to decreasing β cell mass or disfunction of β cells will result in deterioration of diabetes. β cell proliferation assay and glucose-induced insulin secretion assay were employed here to evaluate LBP-s-1's capacity of preserving β cell mass. Exendin-4, an agonist of glucagon-like peptide-1 receptor was used as positive control for the reported effects of stimulating β cell proliferation, inhibiting β cell apoptosis and enhancing glucose-induced insulin secretion (Arakawa et al., 2009).

RIN-m5f cell line was derived from a transplantable islet-cell tumor of an inbred NEDH (New England Deaconess Hospital) rat strain. It was considered as one ideal cell line for the study of β cell physiology because of its ability of insulin gene expression, insulin processing and glucose-stimulated insulin secretion (GSIS) (Hohmeier & Newgard, 2004). With the increase of the concentration of LBP-s-1, RIN-m5f cells grew in number dose-dependently (Fig. 4a). The proliferation index was up to 135% at LBP-s-1

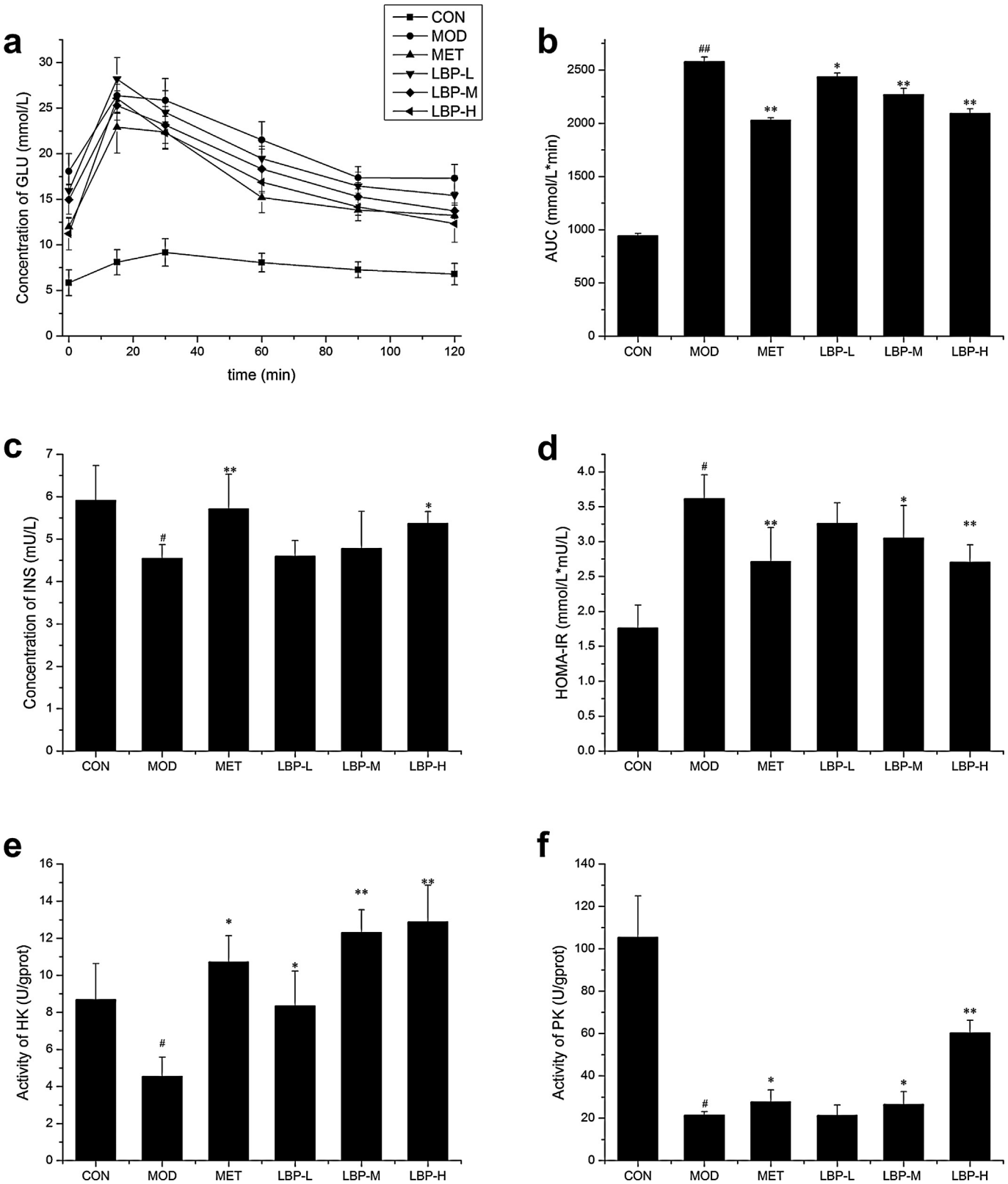

Fig. 6. Hypoglycemic effects of LBP-s-1 in the diabetic mice model. Effects of LBP-s-1 on blood glucose level (a) and serum insulin level (b). HOMA-IR index of each group in the diabetic mice (c). Effects of LBP-s-1 on the activity of Hexokinase (d) and pyruvate kinase (e) in the liver of the diabetic mice. Values are the mean ± SD, (n = 6–8). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs the normal control group (CON). $^{*}p < 0.05$, $^{**}p < 0.01$ vs the diabetes mellitus group (MOD).

concentration of 125 and 250 nmol/L, which nearly equalled to the value of Ex-4. Insulin secretion of RIN-m5f cells treated with LBP-s-1 also increased progressively and the two higher concentration groups showed significant enhancement of insulin secretion (Fig. 4b). Under physiological conditions, viability and function of β cell are regulated by various metabolic demands including peripheral insulin resistance, obesity, hyperglycemia and oxidative stress. Ex-4 could stimulate β cell neogenesis and increase insulin secretion through a series of transduction signaling to interact with some genes correlated with proliferation (Arakawa et al., 2009).

MDG-1, a polysaccharide from *Ophiopogon japonicas* was reported previously of remarkable hypoglycemic effects in the KKAy mice by upregulating the expression of phosphoinositide 3-kinase p85 subunit (PI3K), protein kinase B (Akt), insulin receptor (InsR), insulin receptor substrate-1 (IRS-1) and Glucose transporter type 4 (Glut-4) and downregulating the expression glycogen synthase kinase 3β (GSK3β) (Wang et al., 2012). Other polysaccharides were also reported to exert hypoglycemic effect by PI3K/Akt pathway. Whether LBP-s-1 works through this pathway needs to be further confirmed.

The results suggested that LBP-s-1 was capable of stimulating neogenesis and increasing insulin secretion of a pancreatic β cell line, RIN-m5f, which might lead to decreasing high blood glucose level and alleviating insulin resistance.

3.4. Glucose consumption assay in HepG2 and 3T3-L1 lines

To further elucidate the hypoglycemic effect of LBP-s-1, glucose utilization in two kinds of peripheral cells was investigated. Since that the liver and adipose tissue are the primary organs for metabolism of glucose, HepG2 and 3T3-L1 lines were employed to investigate glucose uptake by treatment with LBP-s-1. Metformin is a biguanide that has been widely used for the therapy of diabetes mellitus, the major hypoglycemic effects of which are reducing hepatic glucose, increasing insulin-stimulated glucose uptake in skeletal muscle and adipocytes (Sogame, Kitamura, Yabuki, & Komuro, 2011). Therefore, metformin was chosen as the positive control. The results of MTT assay (data not shown) showed that there was no significant difference between each group, which meant that LBP-s-1 had no influence on the viability of both HepG2 and 3T3-L1 cells. After treated with different concentrations of LBP-s-1, consumption of glucose by HepG2 and 3T3-L1 cells increased remarkably and reached to the maximum at the concentration of 62.5 nmol/L. Glucose uptake of LBP-s-1 in 3T3-L1 line was even higher than that of metformin in all of the concentration groups except the lowest concentration group (Fig. 5).

Results of these two *in vitro* activity tests showed that hypoglycemic effects of LBP-s-1 were achieved by promoting the proliferation of pancreatic β-cell and increasing glucose uptake in two peripheral tissues. These results indicated that LBP-s-1 would possibly possess a remarkable hypoglycemic effect *in vivo*.

3.5. Hypoglycemic effect of LBP-s-1 in vivo

The successful diabetes mellitus mice model was confirmed by the results of FBG at the end of 10th week (data not shown). Treatment with LBP-s-1 (125, 250, 500 mg/kg/day) for 7 weeks significantly decreased the level of FBG compared with the MOD group. The OGTT results showed that the MOD group significantly impaired glucose tolerance and LBP-s-1 and metformin treated groups obviously reduced the concentrations of blood glucose at all the time points. Compared with the MOD group, the blood glucose area under curve (AUC) was significantly lower following the administration of LBP-s-1 and metformin. In addition, the level of insulin from LBP-s-1 treated groups was higher than that of MOD group. Among three LBP-s-1 treatments, the LBP-H group had significant increment. Moreover, the HOMA-IR indexes of the LBP-s-1 treated groups were much lower than that of the MOD group (Fig. 6). These data indicated that treatment with LBP-s-1 alleviated insulin resistance by decreasing blood glucose and preserving pancreatic β cells for sufficient insulin secretion.

In order to determine whether the reduction in blood glucose level by LBP-s-1 treatment is related to increased glucose utilization, key enzymes of glucose utilization in the livers were measured. A significant reduction in the activity of hexokinase (HK) and pyruvate kinase (PK) was observed in the diabetic animals when compared to normal controls. LBP-H group resulted in ~2.81- and ~2.83-fold increases in the activity of HK and PK when compared to the control group (Fig. 6) while the DM mice treated with metformin only showed a rise of ~2.35- and ~1.29-fold. LBP-s-1 at high dose was proved to be more effective than metformin.

Insulin resistance is closely associated with decreased glucose utilization and down-regulation of hepatic glycolytic enzymes expression (Agius, 2007), such as hexokinase and pyruvate kinase. Therefore, increasing the activity of HK and PK is important in alleviating insulin resistance through increasing glucose utilization. HK and PK are key enzymes in glucose metabolism, with glucokinase and L-type pyruvate kinase mainly distributed in liver. HK is considered to be a marker in regulating glucose hepatic release/uptake according to glycaemia level, and PK is a rate-limiting enzyme in aerobic oxidation of glucose. Deletion of liver HK would lead to mild hyperglycaemia but decreased glucose utilization and glycogen synthesis (Fueger et al., 2007). The pivotal role of HK in controlling blood glucose has made it attractive as a potential drug target for the treatment of diabetes mellitus. A previous study on regulation of L-type PK gene expression by dietary fructose in normal and diabetic rats confirmed that some carbohydrates could slightly up-regulate PK gene transcription and markedly up-regulate PK gene transcription accompanied with insulin treatment (Matsuda, Noguchi, Takenaka, Yamada, & Tanaka, 1990). The possible mechanism of LBP-s-1 at high dose more effective than metformin was due to increasing insulin secretion by LBP-s-1 itself, which meant that the increase of insulin secretion by LBP-s-1 would synergistically result in the increase of PK activity.

The results indicated that LBP-s-1 worked effectively on diabetes through increasing glucose utilization to decrease blood glucose and promoting β cell proliferation for appropriate insulin secretion simultaneously. Given that diabetes mellitus is featured on multiple target genes, LBP-s-1 might be a promising anti-diabetic compound for the treatment of diabetes mellitus.

4. Conclusion

The results of GC, PMP and chemical colorimetric method demonstrated that LBP-s-1 was a heteropolysaccharide consisting of rhamnose, arabinose, xylose, mannose, glucose, galactose, galacturonic acid in the molar ratio of 1.00:8.34:1.25:1.26:1.91:7.05:15.28. Several typical peaks in spectra of IR, ^{1}H NMR and ^{13}C NMR confirmed the monosaccharide composition. *In vitro* and *in vivo* experiments on hypoglycemic effects proved that LBP-s-1 alleviated insulin resistance by preserving β cell mass and increasing insulin secretion and glucose utilization. The underlying mechanisms of these results were promoting β cell proliferation and increasing activity of key enzymes of glucose metabolism. As a natural product, LBP-s-1 was confirmed nontoxic at a high dosage. Effectiveness and safety enabled LBP-s-1 to be a potential drug candidate for diabetes mellitus. The molecular mechanisms of hypoglycemic activity of LBP-s-1 and structure–function relationship of LBP-s-1 will be further clarified in the following work.

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