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Purification, characterization and hypoglycemic activity of extracellular polysaccharides from *Lachnum calyculiforme*

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ABSTRACT

Polysaccharides produced by *Lachnum* have been found as biologically active substances in recent years. Herein, extracellular polysaccharides from *Lachnum calyculiforme* (LEP) fermenting liquor was obtained by the process of Sevag deproteination, H_2O_2 decolorization and salting out. LEP-1 was separated and sequentially purified from LEP through DEAE-Cellulose 52 and Sephadex G-100 column chromatography. The results showed that LEP-1 was detected as the homogeneous component with molecular weight of about 445.363 kDa by the high performance liquid gel permeation chromatography (HPGPC). Furthermore, we demonstrated that LEP-1 was glucose polymer by GC–MS analysis. IR and NMR spectroscopy (^1H, ^{13}C) analysis indicated that the glucose conformation was pyranose-type. Diabetic mice experiments showed that the LEP-1 had a strong hypoglycemic effect in alloxan diabetic mice with dose-dependent relationship.

1. Introduction

Modern pharmacological studies have shown that fungi polysaccharide possesses anti-oxidation, anti-radiation, hypolipidemic and hpyerglycemic activities, anti-fatigue, anti-tumor and other biological activities, which are also hotspots of research on functional factors of drugs and health food (Chen, Tsai, Lin, Khoo, & Lin, 2004; Li et al., 2006; Liu & Gu, 2007; Peng, Zhang, Zeng, & Kennedy, 2005; Tseng, Yang, & Mau, 2008).

Diabetes is the third largest threat to human health. The current treatments mainly rely on insulin injection, oral hypoglycemic drugs of sulfonylurea and biguanide and so on. However, these drugs have side effects to varying degrees. Therefore, to develop and make researches on drugs with high efficiency and low toxicity, particularly the drug extracted from natural resources with hypoglycemic activity and no side effects, is of great importance (Liu & Huang, 2007).

Lachnum belongs to Helotiales, Hyaloscyphaceae, and it is a saprophytic fungi distributed around the world (Korf, 1991). Over the years, species diversity of *Lachnum* has been extensively studied by international scholars (Cantrell & Haines, 1997; Ye & Zhuang, 2003; Zhuang & Hyde, 2001). However, the studies on its metabolites mainly focus on antimicrobial substances (Shan, Stadler, Sterner, & Anke, 1996; Stadler, Anke, & Sterner, 1995). In recent years, *Lachnum hyalopus* have been found to produce polysaccharides with strong antioxidant activity under the submerged culture condition (Ye, Li, Yang, Zhu, & Lin, 2009).

The aim of the present work was to obtain extracellular polysaccharides with homogeneous components from *Lachnum calyculiforme*, reveal its structural characterization and evaluate its hypoglycemic activity.

2. Materials and methods

2.1. Materials and reagents

The sporophores of *L. calyculiforme* YM-278 were collected in Huangshan, Anhui, China. They were separated and preserved by Microbial Resources and Application Research Laboratory of Hefei University of Technology.

DEAE-cellulose 52 was bought from Whatman Co. (Maidstone, Kent, UK); Sephadex G-100 was bought from Pharmacia Co. (Uppsala, Sweden); glucose standard was from Sigma Chemical Co. (St. Louis, MO, USA); standard glucan products were from Shodex Co. (Tokyo, Japan); chloroform, phenol, butanol and sulphuric acid were purchased from Shanghai Zhenqi Chemical Reagent Co., Ltd. (Shanghai, China); other reagents were of analytical grade; male mice (17–20 g) were purchased from Anhui Changlinhe pharmaceutical Co. (Anhui, China).

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2.2. Strain culture

The strain of *L. calyculiforme* YM-278 was inoculated in PDA culture medium for activation of 4 days at 25 °C, and stored as the seeds for shake-flask fermentation.

Fermentation medium was prepared with 3.0% glucose, 0.5% yeast extract, 0.1% potassium dihydrogen phosphate, 0.1% magnesium sulfate heptahydrate and 0.005% vitamin B1.

2.3. Extraction of L. calyculiforme extracellular polysaccharide (LEP)

The activated strain was inoculated in a 250 mL triangular flask containing 50 mL fermentation medium, followed by 25 °C culture for 4 days at the shaking speed of 180 rpm, as the seed solution for 5-L fermentor. Basic conditions for 5-L fermentor were to be fermented for 10 days at 25 °C with stirring speed of 180 rpm.

Ninety-five percent ethanol with 3-fold volume was added into the fermenting liquor after its suction filtration and concentration. Then the solution was precipitated at 4 °C for 12 h before being centrifuged for 10 min at 4000 rpm. After supernatant was removed, sediment was dissolved with distilled water. Crude extracellular polysaccharides were obtained after freeze-drying.

2.4. Purification of LEP

The crude polysaccharides were dissolved in hot water before 1/10 volume of 30% H_2O_2 was added for bleaching or decoloring with heat preservation at 50 °C. Then proteins were removed by Sevag method (Sun et al., 2009). The samples were freeze-dried to gain LEP after 48 h of dialysis with tap water and distilled water respectively. After LEP was applied to a DEAE-52 cellulose column (1.6 cm × 60 cm) with NaCl solution of different concentrations (0, 0.1 and 0.5 M NaCl) as eluant, 15 mg of the main fraction obtained was dissolved in 0.5 mL double distilled water for chromatography with Sephadex G-100 column (1.6 cm × 60 cm), with double distilled water as eluant, elution volume of 70 mL and the flow rate of 0.2 mL/min. The samples were partly collected with each tube containing 2 mL. Then the trace detection was conducted with phenol-sulphuric acid method to measure absorbance values at 490 nm wavelength (Vinarta, Molina, Figueroa, & Farina, 2006). Elution curve was made by absorbance vs. tube number. The collected components were scanned with ultraviolet spectrum at 280 nm and 260 nm.

2.5. Determination of LEP-1 purity and its molecular weight

The purity and molecular weight of LEP-1 were determined by high performance liquid gel permeation chromatography (HPGPC). The Waters-2414 HPLC host that was equipped with Waters-1515 parallax shading monitor was used. Ultrahydrogel™2000 analytical column with double-distilled water as mobile phase, flow rate as 0.6 mL/min and column temperature as 35 °C, was adopted. The purity was judged by the peak shape. The standard curve, the elution time plotted against the logarithm of molecular weight, was made using the Dextran T standards (MW: 10,000, 40,000, 70,000, 133,000, 482,000, 2,000,000). According to the retention time of LEP-1, its molecular weight of LEP-1 was calculated by the calibration curve equation.

2.6. Acetylation of LEP-1

Five mg LEP-1 with homogeneous components was placed in 5 mL ampoule tube in which 4 mL of 2 moL/L TFA was added. The tube was sealed after N_2 was filled into it. Then, the sample was hydrolyzed at 120 °C for 3 h. After cooling, hydrolyzed liquor was evaporated to dryness at 40 °C. The residue was dissolved by 3 mL of methanol before being put into the water bath for drying, which was repeated for four to five times so as to fully eliminate TFA. The remaining residue was polysaccharide hydrolyzate.

Hydrolyzed sample was dissolved in 3 mL distilled water in which 30 mg N_aBH_4 was added for 3 h reduction at room temperature. 25% HOAc was used to neutralize excessive $NaBH_4$ with the pH controlled between 4 and 5 until no bubbles appeared. Then methanol was added for several times before decompression evaporation to dryness so as to remove byproduct, boric acid, and water, which was repeated for several times until large solid particles did not attach to the bottle wall. The sample was placed into a vacuum dryer overnight. The sample was placed in 110 °C oven to heat for 15 min so as to fully remove residual moisture. 3–5 mL acetic anhydride was added into the sample and it was sealed for 1 h reaction at 100 °C. When the sample was cool, 2–3 mL toluene was added into it for decompression evaporation so as to remove excessive acetic anhydride, which was repeated for 5 times. Finally, the sample was evaporated to dry so as to obtain LEP-1 acetylated products.

2.7. GC–MS analysis of LEP-1 acetyl product

Acetyl product was dissolved with proper quantity of chloroform before being washed with equal volume of distilled water for 4 times and dried with anhydrous sodium sulfate. After the filtration, the sample was concentrated to 0.1 mL for GC–MS analysis. The conditions for GC–MS analysis were as follows: capillary column was HP-5 (30 m × 0.25 mm × 0.25 μm); column temperature was programmed to rise from 50 °C to 250 °C with 10 °C/min; injector temperature was 260 °C; He flow rate was 1 mL/min; ion source was EI, 70 eV; molecular weight range was 35–650.

2.8. Infrared analysis

One mg homogeneous polysaccharide (LEP-1) was ground and mixed with KBr before being tableted. Nexus670-type Fourier was adopted to transform infrared spectroscopy, with the scanning range of 4000–400 cm^{-1}.

2.9. NMR spectroscopy

The LEP-1 (50 mg) was put in a 5-mm NMR tube and dissolved in 1.0 mL 99.96% D_2O. Spectra were recorded at 300 K on a Bruker AV-500 spectrometer operating at 500.13 MHz for ^1H NMR and 125.76 MHz for ^{13}C NMR. Chemical shifts are given in ppm.

2.10. Establishment of alloxan diabetic mice model

Male mice (17–20 g) were kept for environmental adaptation for l week. After being weighed and fasted 12 h (except for water), the mice were intraperitoneally injected with 2% alloxan (200 mg/kg). Orbital blood was taken out for the determination of fasting blood glucose with blood glucose meter (Roche Diagnostics GmbH, Germany) 72 h later. The mice with blood glucose value above 11.12 mmol/L were regarded as diabetic mice model. The model mice manifested decrease in activity, rarefaction and lackluster of hair and increase in water and food intake, indicating the model was successful.

2.11. Determination of hypoglycemic activity of LEP-1

Forty alloxan diabetic mice were randomly divided into 4 groups (Chen, Li, Wu, Ren, & Zhang, 2008), that is, a metformin hydrochloride group (positive control), 2 LEP-1 groups and a diabetic control group, with 10 mice each group; 10 normal mice were as a non-diabetic control group.

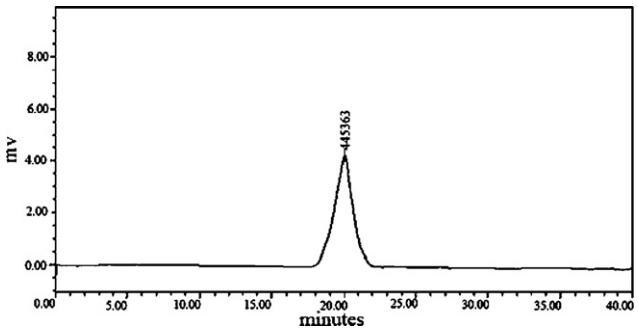

Fig. 1. LEP-1 high performance liquid gel permeation chromatogram.

The administration dosage of metformin hydrochloride was 193.3 mg/kg; the lavaged dosage of LEP-1 was 100 mg/kg, 400 mg/kg respectively for two groups; equal volume of 0.9% normal saline was given for the diabetic control group and non-diabetic control group. Intragastric administration was conducted at 8:30 every morning, once a day for 2 weeks. During the experiment, animals got free access to food and water. The food intake was measured each day, and body weight each week.

The last intragastric administration was carried out after 10 h fast. 2 h later, the blood was collected from the orbit of mice to detect the blood glucose concentration with blood glucose meter.

Forty normal mice were treated according to the above method of group experiments.

2.12. Statistical analysis

The results obtained for each group of mice tested were expressed as the mean ± SD ($n = 10$). A statistical analysis of the data was performed by Student's t-test. Differences were considered statistically significant when $p < 0.05$.

3. Results and discussion

3.1. Extraction and purification of polysaccharide

Crude extracellular polysaccharide was extracted from 5 L (4 batches) of fermenting liquor in the fermentor. After deproteination, decolorization and dialysis, extracellular polysaccharide from *L. calyculiforme* (LEP) was obtained and then separated and sequentially purified through DEAE-Cellulose 52 and Sephadex G-100, giving two elution peaks which were named as LEP-1 and LEP-2. And the LEP-1 with homogeneous components was only chosen to be analyzed on its monosaccharide composition, characterization and hypoglycemic activity.

LEP-1 became white powder after freeze-drying. Then the sample was tested at 280 nm and 260 nm in UV spectrum, showing no absorption peak which indicated that no protein and nucleic acid remained in the powder.

3.2. Characterization of LEP-1

Purity evaluation of polysaccharide plays a significant role in the studies on polysaccharide. Because of the extremely complex structure of polysaccharide, the purity standard of small molecular compounds cannot be used to determine its purity. The general pure polysaccharide is not uniform at the micro level because it is only homogeneously distributed within a certain molecular weight range (Liu & Gu, 2007). LEP-1, the main components of pure polysaccharide, was detected through high performance liquid gel permeation chromatography and eluted with Ultrahydrogel^{TM}2000 analytical column. The elution peak (Fig. 1) was single, symmetric and narrow, with the feature of homogeneous distribution. The calibration curve equation is: $\text{Log MW} = 12.7 - 0.337x$, $R^2 = 0.9948$, where MW denotes the molecular weight of the standard dextran and x is the retention time. The molecular weight of LEP-1 was calculated to be 445.363 kDa by this equation.

LEP-1 was fully hydrolyzed and transformed into acetyl derivatives before GC–MS analysis. There was a major peak on total ion chromatogram (TIC) (Fig. 2), with the retention time of 19.006 min. The mass spectrum of the peak is shown in Fig. 3. Its retention time and mass spectrum data were completely consistent with that of acetyl derivatives from standard glucose, indicating that the acetyl derivative was N-acetylglucosamine (GlcNAc). It is demonstrated that monosaccharide composition of LEP-1 is glucose, that is to say, the LEP-1 is polymerized by glucose. It has been reported that polysaccharides of some other fungi are also glucan, such as *Coriolus unicolor* (Teng, Zhang, Wang, & Zhou, 2007), but they show differences in molecular weight.

LEP-1 was scanned in the range of 4000–400 cm^{-1} for three or more times. The obtained infrared spectrum is shown in Fig. 4, indicating that the LEP-1 had the typical features of polysaccharides. In the spectrum, there appeared stretching vibration of an absorption peak O–H at 3399.32 cm^{-1}, which was mainly caused by the (association) stretching vibration of polysaccharide glycoside hydroxyl; there appeared stretching vibration of an absorption peak C–H at 2929.40 cm^{-1}; the absorption peak at 1650.40 cm^{-1} displayed the –CHO stretching vibration or N–H deviational vibration; peak at 1417.46 cm^{-1} displayed C–O stretching vibration; the absorption peaks between 1250 and 950 cm^{-1} indicated that glucose conformation of LEP-1 was the pyranose type; the peak at 923 cm^{-1} displayed the non-symmetric ring stretching vibration

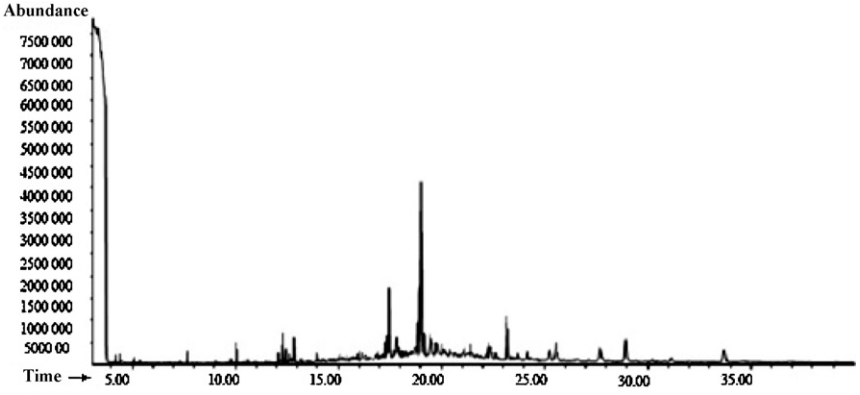

Fig. 2. The total ion chromatogram of LEP-1 acetyl derivatives.

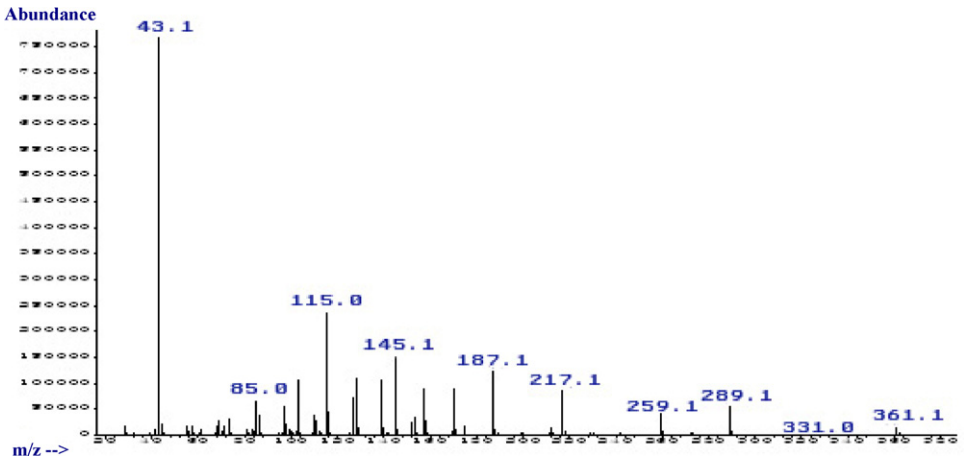

Fig. 3. The mass spectrum of peak with retention time of 19.006 min in the total ion chromatogram of LEP-1 acetyl derivatives.

of D-glucopyranosyl (Chen, Xie, Nie, Chang, & Wang, 2008; Liu & Gu, 2007).

All the NMR chemical shifts of LEP-1 are shown in Figs. 5 and 6. In the ^{1}H NMR of LEP-1, signal at around from 3.57 to 4.14 ppm were assigned the glycosidic ring (Zou, Zhang, Yao, Niu, & Gao, 2010). Of the two types of anomeric protons, signals derived from β-anomeric protons appear in the 4–5 ppm (Cui, 2005). The anomeric proton signals at δ4.660 in the ^{1}H NMR indicated that LEP-1 had β-glycopyranosidic linkages (Kim et al., 2000). In the anomeric carbon region signal at δ102.79 could be attributed to C-1 of →3)-β-D-Glcp-(1→ (He, Shao, Men, & Sun, 2010). C-3 and C-6 signals at δ73.85, δ62.97 respectively were due to the presence of β-glucopyranose residues (He et al., 2010; Roy et al., 2009). The presence of C-1 signal indicated that all sugar should be in pyran ring, as the resonance of furan ring should be round 107–109 ppm (Wang et al., 2010). The results of NMR analysis indicated that LEP-1 was (1 → 3)-β-D-glucan, which was consistent with the results of GC–MS and IR data.

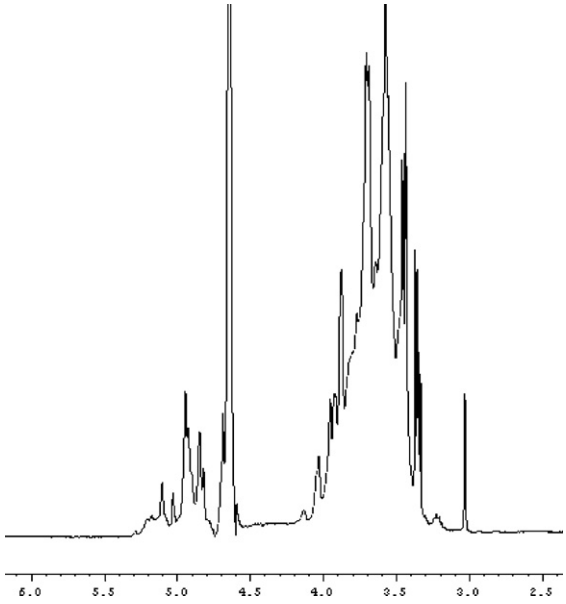

Fig. 5. ^{1}H NMR spectra of LEP-1 at 27 °C.

3.3. Hypoglycemic activity of LEP-1

Alloxan diabetic mice are popular animal diabetic model used in hypoglycemic experiments. Some studies have shown that a variety of fungal polysaccharides have hypoglycemic activity (Hwang et

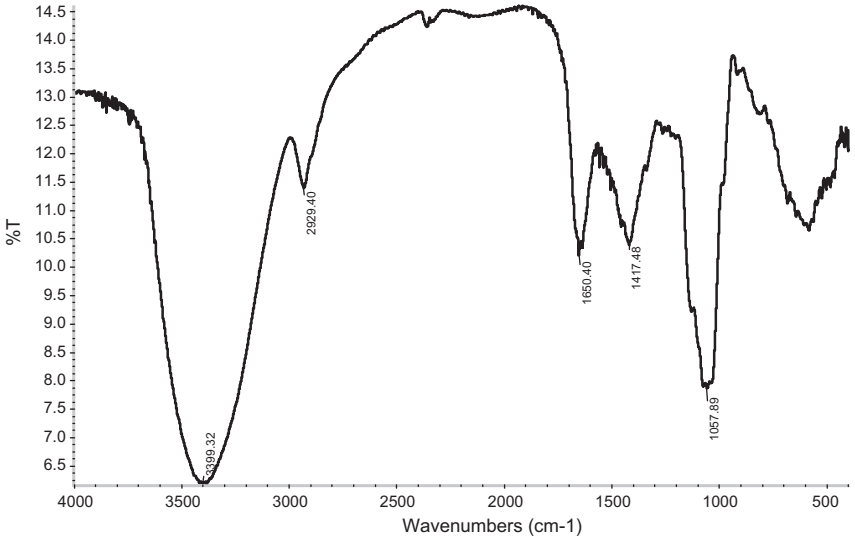

Fig. 4. LEP-1 infrared spectrogram.

Table 1
Effects of LEP-1 on blood glucose, body weight and food intake in alloxan diabetic mice. Intragastric administration of different dosage LEP-1 was conducted in alloxan diabetic mice for two weeks. Values are mean ± SD.

Group	Dosage (mg/(kg bw))	Blood glucose level (mmol/L)		Body weight (g/mouse)		Food intake (g/mouse/day)
		Initial values	14 days later	Initial values	14 days later	
Non-diabetic control	0	6.94 ± 1.23	6.75 ± 1.06	22.03 ± 0.34	26.59 ± 0.53	4.21 ± 0.70
Diabetic control	0	14.65 ± 1.65[d]	14.89 ± 1.21[d]	18.02 ± 0.56[d]	16.03 ± 0.49[d]	5.76 ± 0.15[d]
LEP-1	100	14.34 ± 1.36[d]	13.28 ± 1.02[a,d,e]	17.92 ± 0.33[d]	18.51 ± 0.89[a,d,e]	5.26 ± 0.09[a,d,e]
LEP-1	400	14.45 ± 3.28[d]	12.48 ± 2.01[b,d,e]	17.97 ± 0.57[d]	22.24 ± 0.21[b,d]	5.08 ± 0.13[b,c]
Metformin hydrochloride	193.3	14.22 ± 2.68[d]	9.26 ± 1.68[b,d]	18.07 ± 0.43[d]	21.97 ± 0.34[b,d]	4.62 ± 0.14[b,c]

[a] $p < 0.05$, significantly different from the diabetic control group.
[b] $p < 0.01$, highly significantly different from the diabetic control group.
[c] $p < 0.05$, significantly different from the non-diabetic control group.
[d] $p < 0.01$, highly significantly different from the non-diabetic control group.
[e] $p < 0.05$, significantly from the Metformin hydrochloride group (the positive control).

al., 2005; Wang, Huang, & Sun, 2005). The effects of LEP-1 on blood glucose, body weight and food intake in alloxan diabetic mice are shown in Table 1. After the continuous administration for 14 days with the dose of 400 (mg/(kg bw)) of LEP-1, the blood glucose in alloxan diabetic mice significantly reduced from 14.45 mmol/L to 12.48 mmol/L ($p < 0.01$); when LEP-1 dose was 100 (mg/(kg bw)), the blood glucose decreased from 14.34 mmol/L to 13.28 mmol/L ($p < 0.05$); the blood glucose of metformin hydrochloride group (positive control group) was also significantly decreased ($p < 0.01$); the blood glucose in LEP-1 group and metformin hydrochloride group were decreased significantly comparing with the diabetic control group. The body weights in LEP-1 group and metformin hydrochloride group were increased when compared with the diabetic control group, but the body weight in the diabetic control group decreased gradually. After establishment of alloxan diabetic model, the diabetic mice had a significantly increased food intake. 14 days later, food intakes in LEP-1 group and metformin hydrochloride group were decreased significantly comparing with the diabetic control group. When compared with the non-diabetic control group, the blood glucose, body weights and food intakes in LEP-1 group and metformin hydrochloride group were significantly different.

In addition, there was not significant difference in blood glucose between normal control group and experimental groups (metformin hydrochloride group and LEP-1 group) before and after the experiment, indicating that the LEP-1 had little impact on blood glucose level of normal mice. And the body weight in the normal control group increased gradually, the effect of LEP-1 and metformin hydrochloride on body weight and food intake in normal mice was not significantly different from the normal control group (data not shown).

Alloxan chemical agent have damaging effect on insulin producing β cell of pancreas, damaging the structure of cell membrane, and leading to high glucose level by hypoinsulinism (Zou, Zhang, Yao, Niu, & Gao, 2010). Our results demonstrated that LEP-1 had a strong hypoglycemic effect on alloxan diabetic mice with dose-dependent, as it can possibly repair the damage of the pancreatic β cells and promote insulin synthesis. LEP-1 had significant hypoglycemic effects on blood glucose level in diabetic mice while little effects on normal mice, which was similar to the characteristics of hypoglycemic activity of polysaccharide from *Inonotus hispidus* recently reported (Li & Hu, 2010).

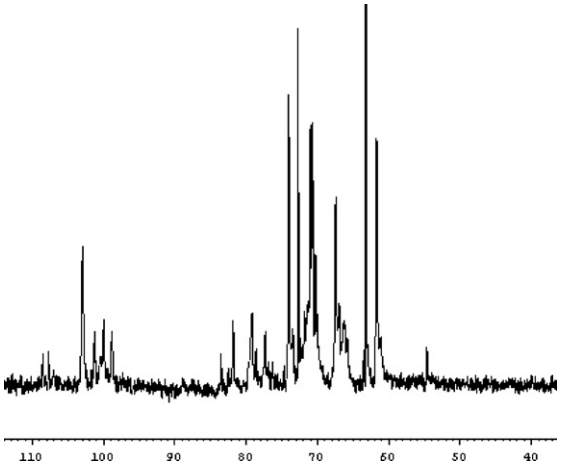

Fig. 6. ^{13}C NMR spectra of LEP-1 at 27 °C.

4. Conclusion

Through high performance liquid gel permeation chromatography, LEP-1 was detected as the homogeneous component, with the molecular weight of about 445.363 kDa. GC–MS analysis showed that LEP-1 was polymerized by glucose. IR and NMR spectra showed that LEP-1 had typical characteristics of polysaccharide with pyranoid conformation of sugar ring. Hypoglycemic activity experiment showed that LEP-1 had a significant hypoglycemic effect on alloxan diabetic mice ($p < 0.01$) with dose-effect relationship, but it had little influence on blood glucose level in normal mice. All these results indicated that extracellular polysaccharides of *L. calyculiforme*, LEP-1, was expected to be developed into hypoglycemic drugs or health food without toxicity and side effects.

Acknowledgments

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