SUPPLEMENTARY MATERIAL

^{1}H NMR-based metabonomics of the hypoglycemic effect of polysaccharides from *Cordyceps militaris* on streptozotocin-induced diabetes in mice

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Abstract

The crude polysaccharide was extracted from *Cordyceps militaris*. Material ratio of powder and water was 1:10. The polysaccharide was successively purified by Sevag and chromatography on Sephadex G-100 column to produce a polysaccharide fraction termed CBPS-II. The average molecular weight of CBPS-II was 1.273×10^3 kDa. The study was conducted to investigate the hypoglycemic effect of *Cordyceps militaris* polysaccharide on diabetic mice. Analysis of the clinical chemistry of the serum samples included serum creatinine (CRE), urea nitrogen (BUN), triglyceride (TG) and total cholesterol (TC). Results revealed that a certain dose of polysaccharide can alleviate the symptoms of metabolic disorders of diabetes, contributing to the body to restore the normal levels. The metabolic profiling method was adopted to find the related biomarkers and the metabolic pathway of diabetes. Moreover, results showed that 100 $mg.kg^{-1}$ of *Cordyceps* polysaccharides can effectively reduce the blood glucose level of diabetic mice, thus regulating the metabolism of their energy, amino acids and intestinal microbes. The biomarkers noted in their metabolism were glucose, lactic acid, 3-hydroxy butyric acid, creatine, glutamate, valine, leucine, isoleucine and very low density lipoprotein (VLDL).

Keywords: *Cordyceps militaris*; polysaccharides; metabonomics; NMR; hypoglycemic

1. Experimental

1.1. Reagents

The strain used in this study was *C. militaris* CICC 14015, which was obtained from China Center of Industrial Culture Collection (CICC), and stored in the Key Laboratory of Food Nutrition and Safety (Ministry of Education, China), College of Food Science and Biotechnology (Tianjin University of Science and Technology, Tianjin, China). Kits for serum creatinine (CRE), blood urea nitrogen (BUN), triglyceride (TG) and total cholesterol (TC) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Streptozotocin (STZ) was bought from Sigma Chemicals Company (St. Louis, USA). D_2O was obtained from Energy Chemical Company (Shanghai, China). Papain (23.74 U/mg) was purchased from Beijing Obo Star Biotechnology Co., Ltd (Beijing, China). All other chemicals used in this experiment were analytical reagent grade and purchased from local chemical suppliers in China.

1.2. Extraction and isolation of CBPS-II

The dried fruit bodies were grinded and passed through an 80 mesh sieve with a pore diameter of 180 μm. The powder (20 g) was defatted with 95% ethanol at room temperature for 2 h with continuous stirring to remove most of the polyphenols, pigments, and monosaccharides. This treatment was repeated thrice. The residue was added to 200 mL of distilled water (1:10 w/v) and stirred for 2 h at 80 ºC to extract *Cordyceps* polysaccharide. After vacuum filtration, the filtrate was combined and condensed to 150 mL with a rotary evaporator under reduced air pressure at 60 °C. The resultant extract was disposed by precipitation with 4 times volume of 95% (v/v) ethanol, deproteinization with the Sevag method and lyophilization to produce the substance termed CBPS-I. The polysaccharide (CBPS-I) was subjected to gel filtration on a column (30 cm × 3 cm) of Sephadex G-100, eluted with deionized water at a flow rate of 0.3 mL/min and monitored using the phenol–sulfuric acid method. Then rendered the purification process elution curve and collected the single component termed CBPS-II. The molecular weight (*Mw*) distribution of polysaccharides was determined by using a HPGPC (Agilent-1200) equipped with a

TSK gel G4000 PWxl column (7.8 mm × 300 mm, column temperature 30 °C) and Refractive Index Detector (RID, detecting temperature 35 °C). A sample solution (20 μL) was injected and run with deionized water at 0.6 mL/min as mobile phase. The standard curve was established using T-series Dextran as the standards (T-10, T-40,T-70, T-500 and T-2000). (Liu et al. 2016; Yan 2004; Zhu et al. 2014). The average molecular weight of CBPS-II was 1.273×103 kDa. The total polysaccharide (percent by weight) content of CBPS-II was determined by the method of phenol-sulphuric acid. Prepare the mother liquor with standard glucose, which was dried to constant weight at 105°C. Various volumes of the mother liquor (0, 0.1 mL, 0.2 mL, 0.3 mL, 0.4 mL, 0.5 mL, 0.6 mL, 0.7 mL, 0.8 mL, 0.9 mL) were pipetted into test tubes respectively， and then make up to 1 mL with distilled water. Each tube was added 1.0 mL 6% phenol solution. When mixed fully, add 5 mL concentrated sulfuric acid and shake well. Heat 15 min in boiling water bath, then cooling to room temperature. The absorbance was measured at 490 nm, and the mass of glucose (g) was used as the horizontal coordinate, and the absorbance (OD) was used as the ordinate. The standard curve was drawn and the linear regression equation was regressive treatment. The total polysaccharide (percent by weight) content of CBPS-II was 89.6%, which obtained based on linear regression equation. And the content of reducing sugars are basically not detected through the method of 3,5-Dinitrosalicylic acid (DNS). The structure of CBPS-II has been identified in our previous study (Liu et al. 2016). CBPS-II was water soluble having neutral hetero-polysaccharides with few branches.

1.3. Animals and drug administration

1.3.1. Preparation of Diabetes Mellitus Model

Fifty male Kunming mice weighing 18 ± 2 g were raised in an SPF-level lab and acclimatized in cages for 7 days before drug administration. Food and tap water were provided ad libitum. Temperature and relative humidity were controlled at 23 ± 2 °C and 50 ± 10%, respectively. STZ was first dissolved in citrate buffer solution (pH=4.3) before administration. Then 0.1 M of it was administered intra-peritoneally to 40 mice. Observation of the general situations of animals included their food intake, faeces volume, hair gloss, coitus, mental state, and mortality. The control group was

intra-peritoneal administered with an equivalent volume of citrate buffer solution. After 7 days, the fasting blood glucose value of each mouse was measured with the blood glucose meter (Sinocare Inc., Changsha, China). The threshold level for diabetic mouse was set as glucose level of > 11.1 mmol/L (Cao et al. 2014). The diabetic mice were randomly divided into five groups, with 10 mice in each group: the hyperglycemia model group (DM), the normal group (CN), the low-dose CBPS-II group (L-CBPS-II), the medium-dose CBPS-II group (M-CBPS-II), and the high-dose CBPS-II group (H-CBPS-II). L-CBPS-II, M-CBPS-II and H-CBPS-II groups were injected intra-gastrically to the mice daily for three consecutive weeks. The dosages for L-CBPS-II, M-CBPS-II and H-CBPS-II groups were set as 30 mg/kg, 60mg/kg and 100 mg/kg, respectively. Meanwhile, the CN and DM group were injected with water.

1.3.2. Sample collection

On the last day, the fasting blood glucose value of each mouse was measured. Blood was drawn from the intra-orbital angular vein after slight anesthetization. After blood collection, the rats were sacrificed and disposed of properly. Blood samples (including serum and plasma) were centrifuged at 4000 rpm for 15 min. The obtained serum was used for biochemical assay, while plasma samples were stored at -80 ℃ prior to metabolomics' analysis.

1.4. Clinical biochemistry assessments

Biochemical analysis of the serum samples was performed on a Multiskan Spectrum (Thermo Corporation, USA). Blood for biochemical measurements was processed according to the specification. Biochemical parameters included serum creatinine (CRE), urea nitrogen (BUN), triglyceride (TG), and total cholesterol (TC). Data was presented as mean ± standard deviations (SD).

1.5. ^{1}H NMR spectral data reduction and pattern recognition

Take 400 μL of serum and add 200 μL of phosphate buffer solution (0.2M Na2HPO4 / 0.2M NaH2PO4, pH 7.0, 99.9% D_2O) transferred to tubes for 1H NMR acquisition. 1H NMR spectra of bio fluids was acquired on a 400 MHz Bruker Avance spectrometer (Bruker Biospin, Rheinstetten,Germany) operating at 400 MHz and a constant temperature of 297.1 K using a standard 1D pulse sequence (recycle delay (RD)-90_-t1-90_-tm-90_-acquire free induction decay (FID)) with water suppression

applied during RD of 8 s and mixing time (tm) of 100 ms and a 90_ pulse set at 10.50 <mu>s. Spectra were acquired using 16 scans for serum and points with a spectral width of 8196.722 Hz. The FIDs were multiplied by an exponential function corresponding to 0.366 Hz line broadening before applying fourier transformation.

All the serum spectra were phase and baseline adjusted manually, and then automatically integrated with software MestRe-C (3.6.9). The chemical shifts of spectra were referenced to the TSP at δ 0.00. The integrals of these buckets covered the region δ 0.0-6.2 and were input as variables for principal component analysis (PLS-DA). The region of δ 4.64-5.26 was excluded to eliminate the effect of water suppression. Therefore, the spectra over the ranges δ 0.0-4.64 and δ 5.26-6.0 was selected, each 0.005 ppm wide. The data was transferred into .xlsx formation (Microsoft Excel 2007, Microsoft, Redmond, WA, USA) and was normalized to the total integrals of each spectrum for partial least squares-discriminate analysis (PLS-DA).

All ^{1}H NMR spectra were submitted to PLS-DA using the software Simca-P^{+} 14.1 (Umetrics, Sweden). Score plots and loading plots, providing potential biomarkers, were visualized in the PLS-DA.

Multivariate data analysis, including principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA), were used to identify the plasma metabolites responsible for the differentiation of the treatment groups (CN, L-CBPS-II, H-CBPS-II). In our present study, metabolites that were far from the cluster in the s-plot and loading plots and with a VIP value>1.0 were chosen as biomarkers. An independent sample t-test was performed using SPSS 17.0 software to determine whether or not the chosen biomarkers were significantly different.

All the mice (animal license No. SCXK-2012-0004) were provided by Academy of Military Medical Sciences, Experimental Animal Center (Beijing, China). All procedures involving animals complied with the China National Institutes of Health Guidelines for the care and use of laboratory animals. Animal welfare and experimental procedures were carried out under the relevant laws according to the guide for the care and use of laboratory animals (Ministry of Science and Technology of China, 2006),

and were approved by the animal ethics committee of Tianjin University of Science and Technology. No human subject was involved in any of the experiment.

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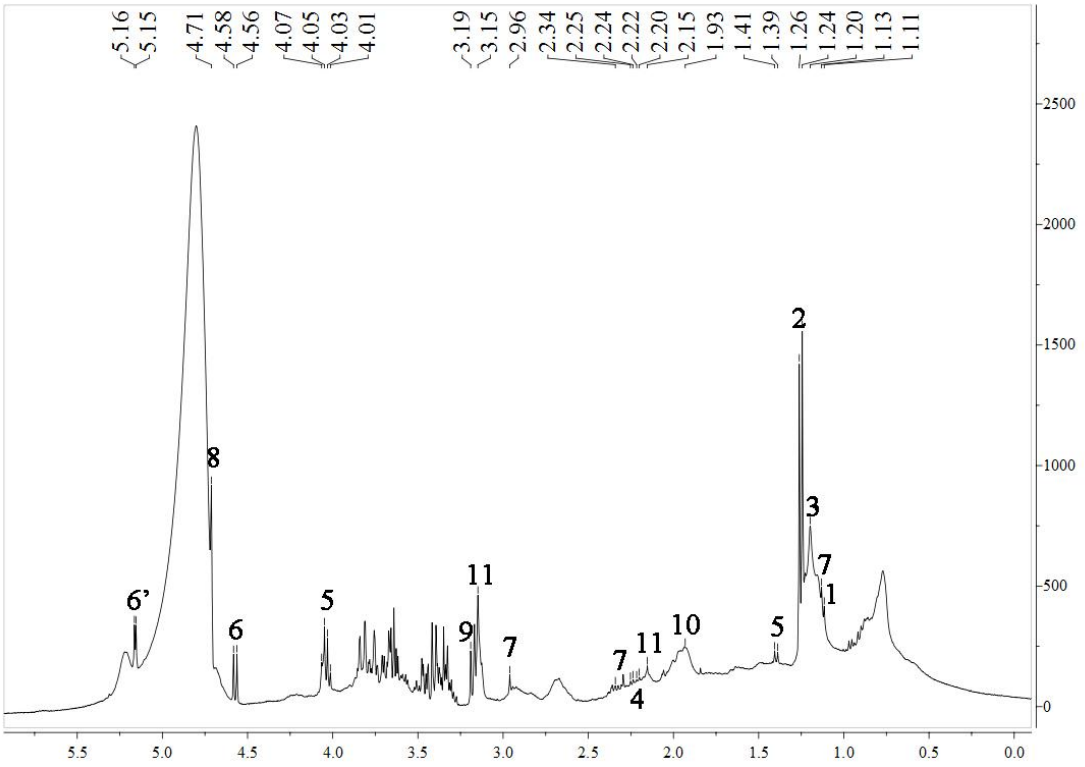
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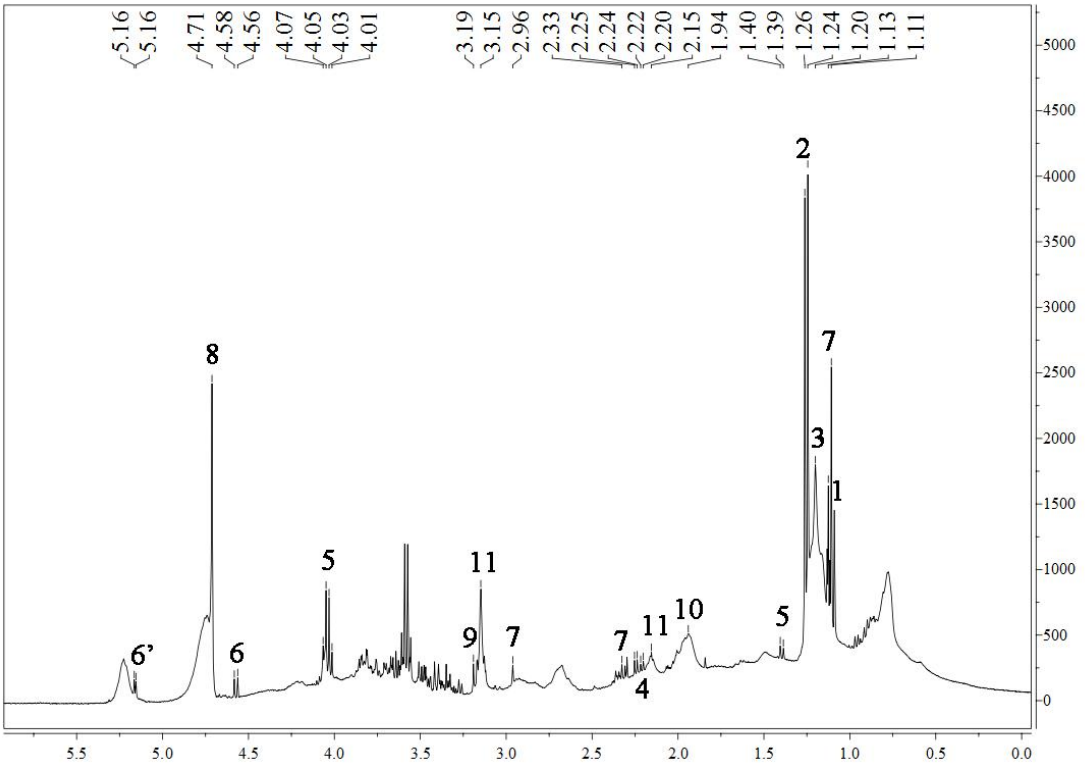
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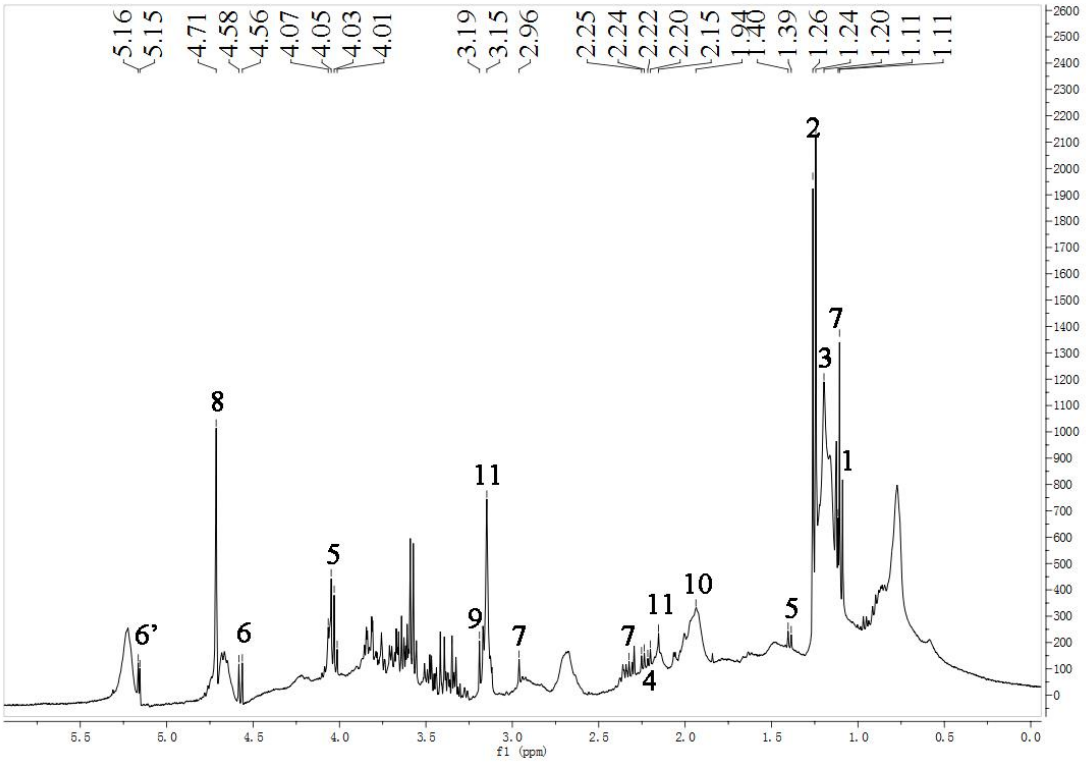
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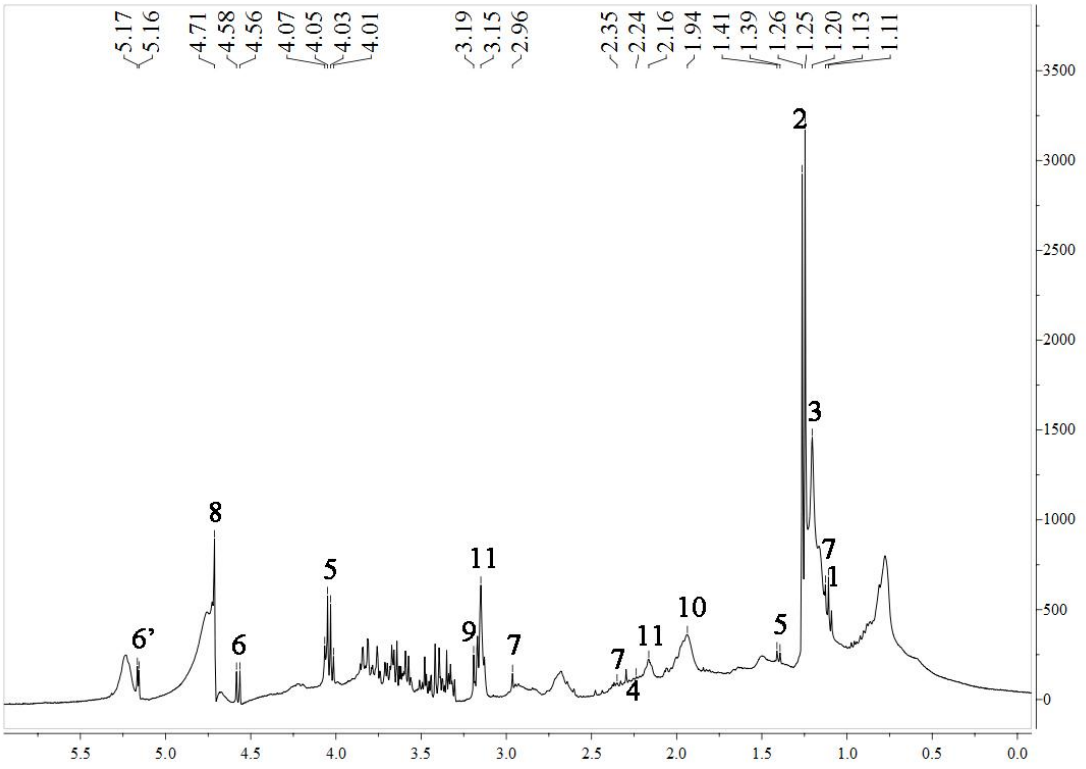
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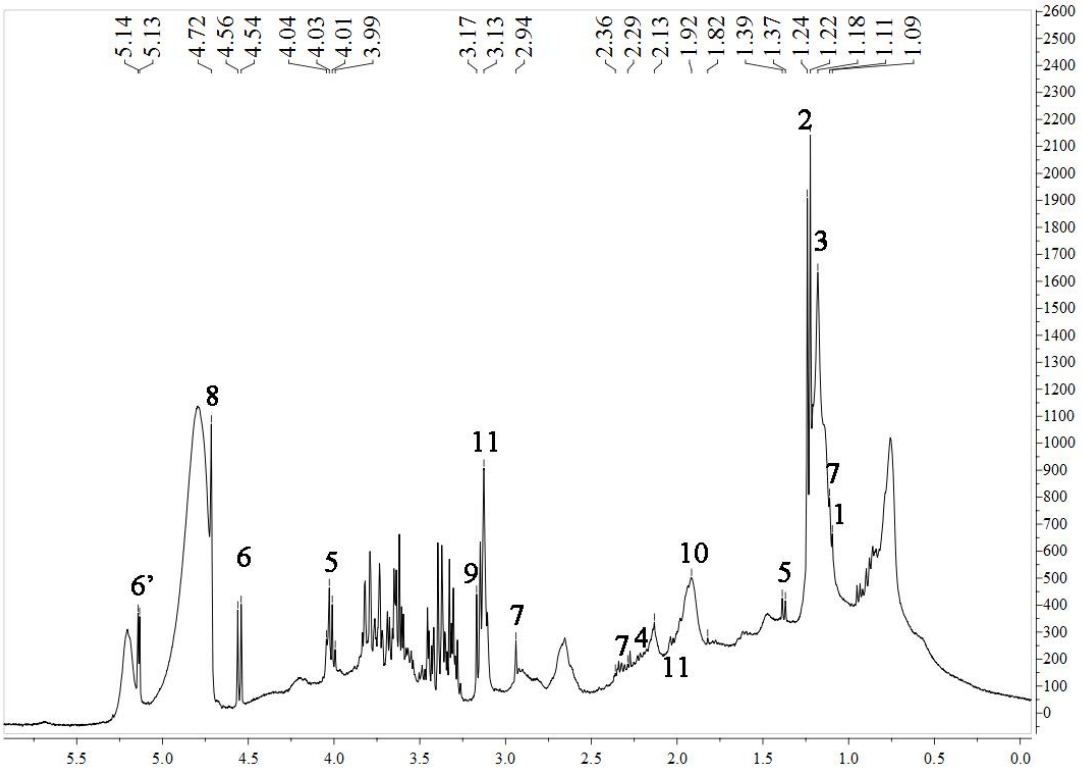

e

Figure S1 ^{1}H NMR spectra of serum from the CN (a), DM (b), L-CBPS-II (c), M-CBPS-II (d) H-CBPS-II group (e).

Key:1,VLDL; 2, isoleucine; 3, leucine; 4, 3-HB; 5, lactate; 6, glucose; 7, valine; 8, creatine; 9, TMAO; 10, Acetate; 11, taurine.

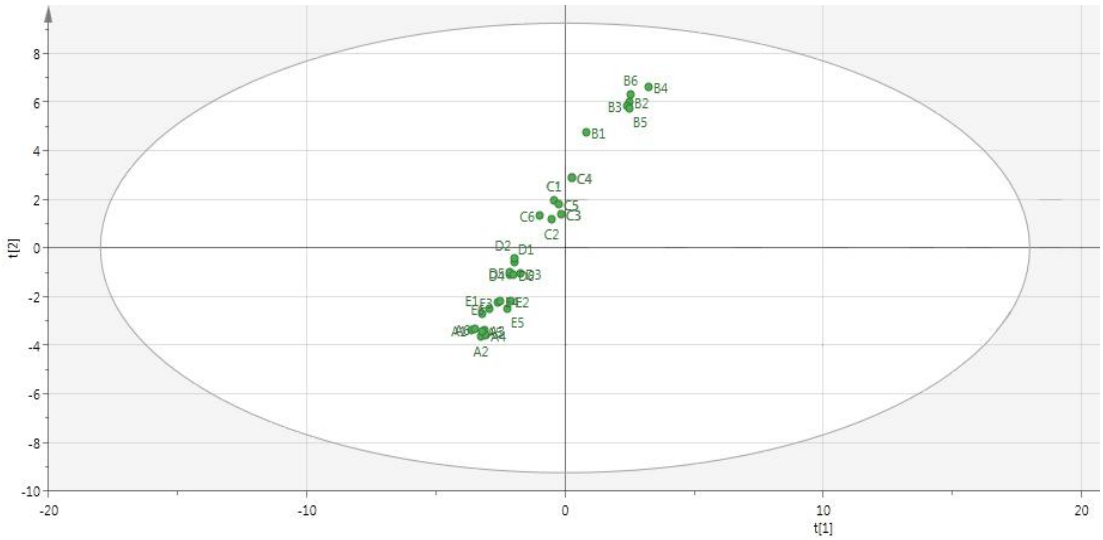

Figure S2 PCA score plot of the CN, DM, L-CBPS-II, M-CBPS-II and H-CBPS-II group ; DM-group(B) L-CBPS-II group .(C). M-CBPS-II group (D). H-CBPS-II (E). CN (A).

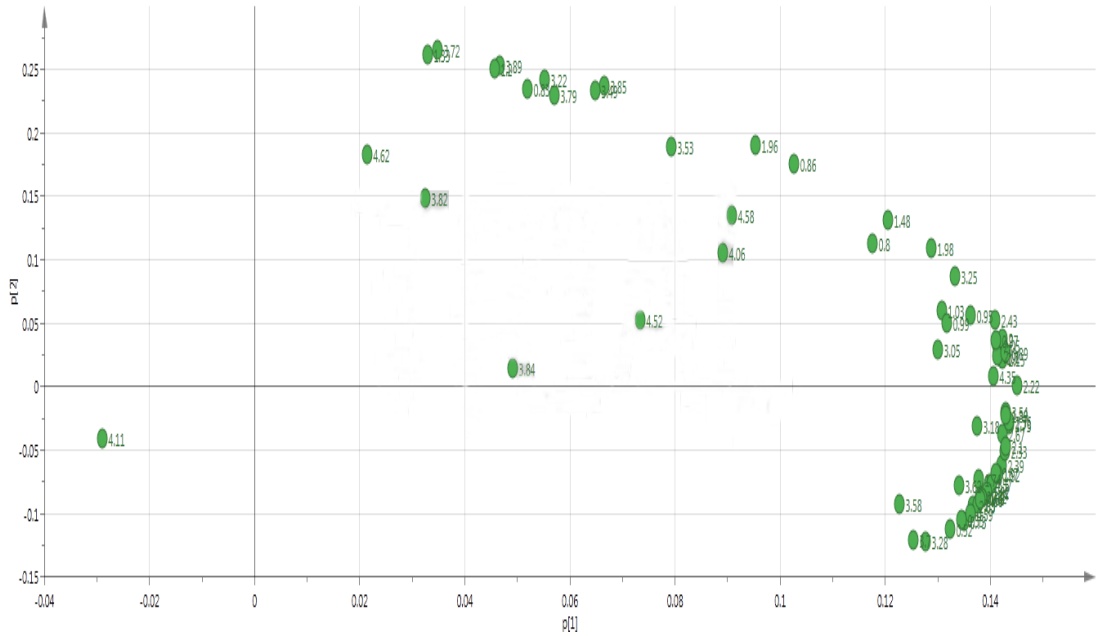

Figure S 3 Loading plot of PLS-DA model among the CN, DM, L-CBPS-II, M-CBPS-II and H-CBPS-II group

Table S1 Effects of different doses of *Cordyceps* polysaccharides on blood glucose in diabetic mice

group	CN	DM	L-CBPS-II	M-CBPS-II	H-CBPS-II
Blood sugar level（mmol/）	5.10±0.20	20.19±1.22**	19.63±1.10	15.50±0.79#	14.16±1.65##

Compared with the control group: *p＜0.05，** p＜0.01; Compared with the diabetic group : $^{\#}p$＜0.05，$^{\#\#}p$＜0.01

Table S2 Effects of Cordyceps militaris polysaccharides on the clinical biochemical parameters of diabetic mice

biochemistry assessments	TC （mmol/L）	TG （mmol/L）	BUN （mmol/L）	CREL-CBPS-II （mmol/L）
CN	4.54±0.44	0.96±0.05	6.39±0.42	0.29±0.10
DM	6.52±0.33**	3.49±0.33**	9.39±0.25**	0.85±0.15**
L-CBPS-II	6.13±0.40$^{\#}$	3.02±0.19	8.98±0.19	0.81±0.12
M-CBPS-II	5.86±0.17$^{\#\#}$	2.74±0.20$^{\#}$	8.42±0.24$^{\#}$	0.73±0.37
H-CBPS-II	5.42±0.16$^{\#\#}$	1.67±0.11$^{\#\#}$	7.24±0.29$^{\#\#}$	0.56±0.16$^{\#\#}$

Compared with the control group: *p＜0.05，** p＜0.01; Compared with the diabetic group : $^{\#}$ p＜0.05，$^{\#\#}$ p＜0.01

Table S3 ^{1}H NMR chemical shifts and assignments for serum metabolites along with their change trend

metabolin	chemically shift (ppm) and multiplicity	Trend in CN vs. DM	Trend in DM vs. L-CBPS-II	Trend in DM vs. H-CBPS-II
Very low density lipoprotein	1.11 (s)	↑**	↓	↓##
Leucine	1.20 (s)	↑*	-	↓#
Isoleucine	1.23 (d)	↑*	-	↓#
Valine	1.11 (d), 2.32 (m), 2.96 (s)	↑*	-	↓#
3-hydroxybutyric acid	2.42 (dd)	↑**	↓	↓##
Lactic acid	1.39 (d), 4.04 (q)	↑**	↓	↓##
Acetate	1.94 (s)	↑**	↓	↓#
Creatinine	4.71 (s)	↑**	↓	↓#
Taurine	2.15 (t), 3.15 (t)	↑*	-	↓#
Oxidation of trimethylamine (TMAO)	3.19 (s)	↓*	-	↑#
α-glucose	4.56 (d)	↑**	↓	↓##
β- glucose	5.15 (d)	↑**	↓	↓##

Multiplicity: s; Unimodal: d; Double peak: t; Triplet: q ; Quartet: m;

↑: Compound content increased ; ↓: Compound content decreased

*P < 0.05, **P < 0.01 Compared with the normal control group #P < 0.05, ##P <0.01 Compared with the diabetic model group