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Antidiabetic activity of *Callicarpa nudiflora* extract in type 2 diabetic rats *via* activation of the AMPK-ACC pathway

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ABSTRACT

Objective: To evaluate the antidiabetic effect of *Callicarpa nudiflora* extract in streptozotocin-induced diabetic rats.

Methods: The chemical constituents in *Callicarpa nudiflora* extract were identified by UPLC-Q-TOF-MS. *Callicarpa nudiflora* extract (0.15 and 0.3 g/kg) was orally administered to streptozotocin-induced diabetic rats for 42 d. The effects of *Callicarpa nudiflora* extract on body weight, blood glucose, serum insulin, total cholesterol, triglyceride, LDL-C and HDL-C were investigated, and its effect on liver and pancreatic pathology was assessed by histopathological analysis. Moreover, the expression levels of adenosine 5'-monophosphate-activated protein kinase (AMPK), glucose transporter type 4 (GLUT4), phospho-AMPK/AMPK, and p-acetyl-coA carboxylase (P-ACC)/ACC in the skeletal muscles and liver were determined by reverse transcription-polymerase chain reaction, Western blotting, and immunohistochemistry.

Results: A total of 34 compounds, including 8 iridoids, 14 phenylpropanoids, and 12 flavonoids, were identified from *Callicarpa nudiflora* extract. *Callicarpa nudiflora* extract significantly reduced blood glucose and significantly restored all other biochemical parameters to near normal levels, including serum insulin, total cholesterol, triglyceride, LDL-C, and HDL-C. *Callicarpa nudiflora* extract improved insulin resistance and reversed the damage in the liver and pancreas caused by diabetes. Furthermore, *Callicarpa nudiflora* extract increased the expression levels of phospho-AMPK, GLUT4, P-ACC, and insulin receptor substrate-1 and decreased the expression level of PPAR γ in diabetic rats.

Conclusions: *Callicarpa nudiflora* extract improved oral glucose tolerance, lipid metabolism, insulin resistance, and reversed the diabetes-related damage in the liver and pancreas by activating the AMPK-ACC pathway.

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1. Introduction

Diabetes mellitus (DM) is a metabolic disease characterized by high levels of glucose in the bloodstream[1]. There are two principal types of DM: type 1, which is caused by insulin deficiency, and type 2, which is caused by insulin resistance[2]. Type 2 diabetes mellitus (T2DM) accounts for 90% of DM cases[2]. The pathophysiology of T2DM is complex, and characterized by impaired glucose uptake-induced hyperglycemia, dysregulation of insulin action, insulin resistance, and β-cell dysfunction[2]. Evidence indicates that T2DM is associated with an increased risk of myocardial infarction, heart failure, ischemic stroke and atherosclerosis-related cardiovascular diseases[3–6].

Callicarpa nudiflora (*C. nudiflora*) Hook. et Arn. is a plant belonging to the genus Callicarpa, and is widely distributed in Guangxi, Guangdong, and Hainan Provinces of China[7]. It has been extensively used as a traditional Chinese herbal medicine to treat inflammation, rheumatism, and bleeding[8,9]. Recent studies have shown that an extract of *C. nudiflora* exhibits anti-metastatic, cytotoxic, hepatoprotective, and anti-HPV activities and improves the learning and memorizing abilities of rats[10,11]. Studies have also shown that it contains various chemical constituents, including flavonoids, phenolic acids, phenylpropanoids, diterpenoids, triterpenoids, iridoid terpenoids, and sterols[12,13], and one of its main constituents, polyphenolic chemicals, has demonstrated lipid-lowering effects[14,15].

In recent years, the impact of plant extracts on the development of DM has been investigated. For example, an ethanolic extract of *Cassia nemophila* pods showed anti-diabetic activity in streptozotocin-induced diabetic rats and inhibited diabetic nephropathy *in vitro* and *in vivo*[16–18]. In this study, we investigated the effects of *C. nudiflora* extract on the body weight, lipid metabolism, glucose tolerance, insulin resistance, and liver and pancreatic pathology of diabetic rats. Moreover, the mechanism underlying this effect was further explored.

2. Materials and methods

2.1. Preparation of C. nudiflora extract

The aerial parts of *C. nudiflora* were collected from Wuzhi Mountain, Hainan, China, in July 2017 and identified by Dr. Chen Tao of Shenzhen Fairy Lake Botanical Garden, Chinese Academy of Sciences, China. Air-dried leaves of *C. nudiflora* were pulverized into a powder (approximately 80 mesh). Then, 2 kg of the powder was extracted twice with 4 L of 80% ethanol. The extracted solvent was filtered and concentrated in vacuo, yielding 230 g of extract. The obtained extract was dissolved in water and subjected to chromatography on an HP-20 macroporous adsorption resin column. The column was eluted with 95% ethanol, filtered and evaporated in vacuo, and then freeze-dried (Eyela FDU-2110; Eyela Corp, Tokyo, Japan) to obtain *C. nudiflora* extract (120 g). A voucher specimen (SZG00048161) was deposited in the Plant Herbarium of Fairy Lake Botanical Garden (Shenzhen, China).

2.2. UPLC-ESI-Q-TOF-MS analysis

The UPLC-ESI-Q-TOF-MS analysis was conducted on an ACQUITY UPLC® I-Class system coupled to a Xevo G2-XS Q-TOF mass spectrometer detector equipped with an electrospray ionization source (ESI). The ACQUITY UPLC HSS T3 (100 mm × 2.1 mm; Waters Corp., Milford, MA, USA) was used for column chromatography at a flow rate of 0.3 mL/min, column temperature of 40 ℃, and injection volume of 1 μL. The mobile phase was composed of a linear gradient of (A) 0.1% formic acid in water and (B) 0.01% formic acid in acetonitrile as follows: 0–2 min, 1%–10% B; 2–5 min, 10%–15% B; 5–8 min, 15%–20% B; 8–10 min, 20%–25% B; 10–11 min, 25%–35% B; 11–15 min, 35%–99% B; 15–19 min, 99% B; 19–20 min, 99%–1% B; 20–23 min, 1% B.

Q-TOF was performed in positive and negative ion modes. The mass scan range was less than 1 200 Da, and the scan time was 0.2 s. For the low-energy scan function, the collision energy was 6 V, which was ramped up to high energy (20–60 V). The capillary voltage was 2.0 kV in both positive and negative modes. The sampling cone voltage was 40 V, with the desolvation and source temperatures at 450 ℃ and 100 ℃, respectively. The desolvation gas flow rate was set at 600 L/h, and the cone gas flow rate 50 L/h.

2.3. Experimental animals and treatment

Six-week-old male Wistar rats purchased from Guangdong Medical Laboratory Animal Center (Guangdong, China; Certificate NO. 44007200053332) weighing 160–180 g were used in this study. After one week of acclimation, 40 rats were randomly divided into the following groups: one group (n = 8) served as a normal control was fed rodent chow and the other group used as diabetic rat models (n = 32). The diabetes model group rats were fed a high calorie and high sugar diet (66.5% standard feed, 15% sucrose, 10% lard oil, 1% cholesterol, 0.5% cholate, and 7% fresh eggs) for eight weeks, then intraperitoneally injected with streptozotocin (35 mg/kg/day). Then, the diabetic rats were randomly subdivided into four groups (n =8): (1) diabetic control; (2) positive control, diabetic rats treated with metformin (0.2 g/kg/day); (3) diabetic rats treated with low dose of *C. nudiflora* extract (0.15 g/kg/day)[19]; and (4) diabetic rats treated with high dose of *C. nudiflora* extract (0.3 g/kg/day). All treatments were administered by oral perfusion daily for 6 weeks. The body weight of every mouse was recorded daily. This study was conducted in accordance with the guidelines of the Animal Ethics Committee of Guangdong Provincial Engineering Technology Institute of Traditional Chinese Medicine (Guangdong, China; Approval NO. 048645).

2.4. Oral glucose tolerance test (OGTT)

During the last week of intervention, the rats were fasted for 12 h. Glucose solution was then orally administered (2 g/kg BW). Blood samples were collected from the tail vein at 30, 60, and 120 min post glucose administration, and fasting blood glucose (FBG) was measured by the Glucose CII-Test.

2.5. Homeostasis model assessment-insulin resistance (HOMA-IR) index

Fasting insulin in serum was measured with the rat INS ELISA kit, and the HOMA-IR index was calculated using the following equation:

HOMA-IR = FBG × fasting insulin/22.5.

2.6. Blood biochemistry

Rats were anesthetized by administration of chloral hydrate (3 mL/kg), and blood samples were obtained from the abdominal aorta. After centrifugation, the serum samples were stored at −80 ℃ until analysis. Then, triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and total cholesterol (TC) levels in serum were determined using an automatic biochemical analyzer (Hitachi 7080; Hitachi, Japan).

2.7. RNA isolation and quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Quantitative RT-PCR was performed as previous described[20]. Total RNA was isolated from tissues using TRIzol reagent (Ambion, MA, USA), and cDNA was synthesized with HiFiScript cDNA Synthesis Kit (Cwbio, China). PCR was performed using PowerUp™ SYBR™ Green Master Mix (Thermo Fisher, USA) on a QuantStudio™ 6 Flex System. Actin was used as an amplification control.

2.8. Western blot analysis

Liver and muscular tissues were homogenized in lysis buffer and centrifuged at 14 000 ×*g* for 15 min at 4 ℃. The protein concentration in the lysates was measured using the BCA Protein Assay kit (Beyotime Biotechnology, Shanghai, China). Western blotting was performed as previous described[21]. Briefly, equal protein was separated on 10% SDS-polyacrylamide gels and then transferred to PVDF membranes (Millipore, Boston, MA, USA) by electro-transfer. Following electro-transfer, the blots were blocked with 5% skim milk and incubated with the specific primary antibodies overnight at 4 ℃. After washing the blots with Tris-buffered saline containing 0.05% Tween-20 (3 times for 10 min each), the blots were incubated with secondary antibodies for 1 h at room temperature. Finally, the membranes were incubated with ECL assay reagent for signal detection (Bio-Rad, USA). Images were acquired with a ChemiDoc XRS+ (Bio-Rad) and analyzed with Image J software (NIH, USA). Tubulin was used as a loading control for the analysis of the expression of PPAR γ and glucose transporter type 4 (GLUT4). Phosphorylation of ACC and AMPK was quantified after normalization to total ACC and AMPK protein expression levels.

2.9. Histopathological analysis

Liver and pancreas tissues were fixed in 10% paraformaldehyde, embedded in paraffin, and stained with hematoxylin and eosin. After fixation with Permount mounting medium (Fisher Scientific), the tissue samples were observed and photographed under a microscope (Zeiss Universal Microscope, Axio Imager A2, Germany) with a magnification of 400×.

2.10. Immunohistochemical analysis

Sections (5 μm thickness) were obtained from liver and pancreas tissues for immunohistochemical analysis. The sections were incubated with primary antibodies against the following proteins: insulin, insulin receptor substrate-1 (IRS-1), adenosine 5'-monophosphate-activated protein kinase (AMPK), P-AMPK, and GLUT4 overnight at 4 ℃, and then with the secondary antibodies. The DAB Peroxidase Staining Kit (SK-4100; Vector labs) was used to visualize the antibody reaction.

2.11. Statistical analysis

All data were expressed as mean ± SD and statistically analyzed using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). One-way ANOVA followed by Duncan's test and Student's *t*-test were used for multiple comparisons and individual comparisons. *P* value less than 0.05 was considered statistically significant.

3. Results

3.1. Identification of the chemical constituents in C. nudiflora extract by UPLC-Q-TOF-MS

The UPLC-Q-TOF-MS profile of *C. nudiflora* extract is shown in Figure 1 with detailed mass information and characterization. Each compound was assigned according to retention time, detected *m/z*, mass error, calculated molecular, and obtained MS/MS fragment ions (Table 1) and by matching the fragmentation patterns to the literature, Sci-finder, and MassBank. A total of 34 compounds, including 8 iridoids, 14 phenylpropanoids, and 12 flavonoids, were identified or tentatively characterized.

3.2. Effect of C. nudiflora extract on the body weight, FBG, and OGTT of diabetic rats

As shown in Figure 2A, the four groups (diabetic control, metformin, low and high doses of *C. nudiflora* extract) showed no significant differences in initial body mass. However, from the fourth week of the experiment onwards, the body mass of the diabetic rats significantly decreased. Treatment with *C. nudiflora* extract at low and high doses showed markedly increased body mass on the fourth week compared with that of the diabetic control group. The diabetic rats treated with metformin significantly increased body mass on the sixth week compared with the diabetic control group (Figure 2A). These results suggested that *C. nudiflora* extract reversed the weight loss of diabetic rats.

In Figure 2B, FBG levels were significantly higher in diabetic rats than in normal control rats. On the fourth week, FBG levels in rats treated with high dose of *C. nudiflora* extract were obviously lower than those in diabetic model rats. Treatment with low dose of *C. nudiflora* extract or metformin showed significant decreases in FBG levels on the sixth week, compared with the levels in the diabetic model rats. In addition, the area under the curve for glucose indicated that administration of *C. nudiflora* extract promoted oral glucose tolerance in diabetic rats (Figure 2C and D).

3.3. Effect of C. nudiflora extract on blood biochemistry in diabetic rats

TC, TG, and LDL-C were markedly increased in diabetic model rats, while HDL-C was significantly decreased in diabetic model rats compared with those in normal rats (Figure 3A–D). *C. nudiflora* extract treatment markedly reversed the increases of TC, TG and LDL-C, and the decline of HDL-C in diabetic rats, and the levels were similar to those in the metformin-treated group (Figure 3A–D).

Table 1. The mass spectrometry data and identification of *C. nudiflora* extract.

No.	t_R (min)	Formula	Quasi-molecular ion (*m/z*)	Mass error (ppm)	MS^2 ions (*m/z*)	Molecular weight (Da)		Tentative identification	Type
						Observed	Theoretical		
1	2.39	$C_{15}H_{22}O_{10}$	407.118 [M+HCOO]$^-$	-3.7	199.060	362.120	362.121	Catalpol	I1
2	3.72	$C_{20}H_{30}O_{12}$	461.166 [M-H]$^-$	-2.0	315.108 135.045	462.173	462.174	Decaffeoyl-verbascoside	P1
3	3.94	$C_{21}H_{28}O_{13}$	487.144 [M-H]$^-$	-2.8	179.035 135.045	488.152	488.153	1-*O*-caffeoyl-(6-*O*-*L*-rhamnopyranosyl)-glucopyranoside	P2
4	4.21	$C_{15}H_{18}O_9$	341.087 [M-H]$^-$	-1.2	179.035 161.024	342.095	342.095	1-*O*-caffeoyl glucoside	P3
5	4.51	$C_{15}H_{18}O_9$	341.088 [M-H]$^-$	0.0	179.035 161.025	342.095	342.095	6-*O*-caffeoyl glucoside	P4
6	5.18	$C_{17}H_{26}O_{11}$	451.145 [M+HCOO]$^-$	-0.7	179.057 145.029	406.147	406.148	8-acetyl harpagide	I2
7	5.47	$C_9H_8O_4$	179.035 [M-H]$^-$	-1.4	135.045 134.037	180.042	180.042	Caffeic acid	P5
8	5.76	$C_{24}H_{28}O_{13}$	523.146 [M-H]$^-$	1.7	179.035 161.024	524.154	524.153	6-*O*-*trans*-caffeoyl-catalpol	I3
9	6.05	$C_{29}H_{36}O_{16}$	639.191 [M-H]$^-$	-3.6	621.181 179.035 161.025	640.198	640.200	β-hydroxy acteoside	P6
10	6.15	$C_{29}H_{36}O_{16}$	639.192 [M-H]$^-$	-1.2	621.183 179.035 161.025	640.200	640.200	β-hydroxyacteoside	P7
11	6.69	$C_{21}H_{20}O_{12}$	463.088 [M-H]$^-$	-1.1	301.035 299.019	464.095	464.096	6-Hydroxy-luteolin-7-*O*-β-*D*-glucoside	F1
12	7.17	$C_{34}H_{44}O_{19}$	755.239 [M-H]$^-$	-2.4	593.207 179.035 161.024 135.045	756.246	756.248	Forsythoside B	P8
13	7.29	$C_{25}H_{30}O_{13}$	537.163 [M-H]$^-$	2.4	523.147 179.035	538.170	538.169	6-*O*-*trans*-feruloyl catalpol	I4
14	7.68	$C_{24}H_{28}O_{13}$	523.145 [M-H]$^-$	-2.1	361.093 161.025 133.030	524.152	524.153	Nudifloside	I5
15	7.83	$C_{21}H_{20}O_{11}$	447.093 [M-H]$^-$	-1.5	285.040 284.033	448.100	448.101	Luteoloside	F2
16	7.89	$C_{27}H_{30}O_{15}$	593.152 [M-H]$^-$	1.0	285.040	594.159	594.159	Luteolin-7-*O*-neohesperidoside	F3
17	7.91	$C_{29}H_{36}O_{15}$	623.197 [M-H]$^-$	-1.7	461.168 315.110 285.041 179.036 161.025 133.030	624.204	624.205	Acteoside	P9
18	8.24	$C_{22}H_{22}O_{12}$	477.104 [M-H]$^-$	-0.8	315.051 300.026 146.966	478.111	478.111	Luteolin-3'-methoxyl-6-hydroxy-7-*O*-β-*D*-glucopyranoside	F4
19	8.30	$C_{35}H_{46}O_{19}$	769.253 [M-H]$^-$	-3.6	461.161 175.039 161.024	770.261	770.263	Alyssonoside	P10
20	8.58	$C_{29}H_{36}O_{15}$	623.198 [M-H]$^-$	-1.0	461.166 315.109 179.035 161.025 135.045	624.205	624.205	Isoacteoside	P11
21	8.70	$C_{29}H_{36}O_{15}$	623.197 [M-H]$^-$	-1.3	461.166 315.109 161.025 133.030	624.205	624.205	Parvifloroside B	P12
22	9.12	$C_{27}H_{32}O_{14}$	579.171 [M-H]$^-$	-1.9	161.025	580.178	580.179	Naringoside	F5
23	9.16	$C_{21}H_{20}O_{11}$	447.095 [M-H]$^-$	3.2	285.042 284.032 133.030	448.102	448.101	Lutedin-4'-*O*-β-*D*-glucoside	F6
24	9.20	$C_{21}H_{20}O_{10}$	431.099 [M-H]$^-$	1.7	269.044 268.038 145.030	432.106	432.106	Apigenin-7-*O*-β-*D*-glucoside	F7
25	9.33	$C_{30}H_{38}O_{15}$	637.214 [M-H]$^-$	1.0	461.167 193.051 175.040 135.045	638.222	638.221	Cistanoside C	P13

Table 1. The mass spectrometry data and identification of *C. nudiflora* extract (continued).

No.	t_R (min)	Formula	Quasi-molecular ion (*m/z*)	Mass error (ppm)	MS^2 ions (*m/z*)	Molecular weight (Da)		Tentative identification	Type
						Observed	Theoretical		
26	9.59	$C_{22}H_{22}O_{11}$	461.109 [M-H]$^-$	0.5	446.085 283.025 255.029	462.116	462.116	Chrysoeriol-7-*O*- β -*D*-glucoside	F8
27	9.79	$C_{21}H_{20}O_{11}$	447.093 [M-H]$^-$	-0.8	285.041 284.031	448.100	448.101	Luteolin-3'-*O*- β -*D*-glucopyranoside	F9
28	10.30	$C_{25}H_{30}O_{12}$	521.166 [M-H]$^-$	-0.9	297.113 195.066 163.040	522.173	522.174	6'-*O*-*trans*-*p*-coumaroyl-8-epiloganic acid	I6
29	10.48	$C_{30}H_{26}O_{14}$	609.126 [M-H]$^-$	1.8	285.041 135.045	610.133	610.132	Luteolin-4'-*O*-(6"-*trans*-caffeoyl)- β -*D*-glucopyranoside	F10
30	10.52	$C_{25}H_{30}O_{12}$	521.167 [M-H]$^-$	1.6	297.114 163.041	522.175	522.174	Unknown	I7
31	10.65	$C_{26}H_{32}O_{13}$	551.175 [M-H]$^-$	-4.3	357.118 195.065 193.050	552.182	552.184	Alboside Ⅰ	I8
32	10.94	$C_{31}H_{40}O_{15}$	651.228 [M-H]$^-$	-2.6	193.050 175.040 160.016 134.037	652.235	652.237	Martynoside	P14
33	11.73	$C_{15}H_{10}O_6$	285.041 [M-H]$^-$	1.3	179.035 151.003 133.030	286.048	286.048	Luteolin	F11
34	13.83	$C_{18}H_{16}O_7$	343.083 [M-H]$^-$	2.4	313.037 241.013	344.090	344.090	5,4'-dihydroxy-3,7,3'-trimethoxyflavone	F12

F: flavonoid; P: phenylpropanoids; I: iridoid.

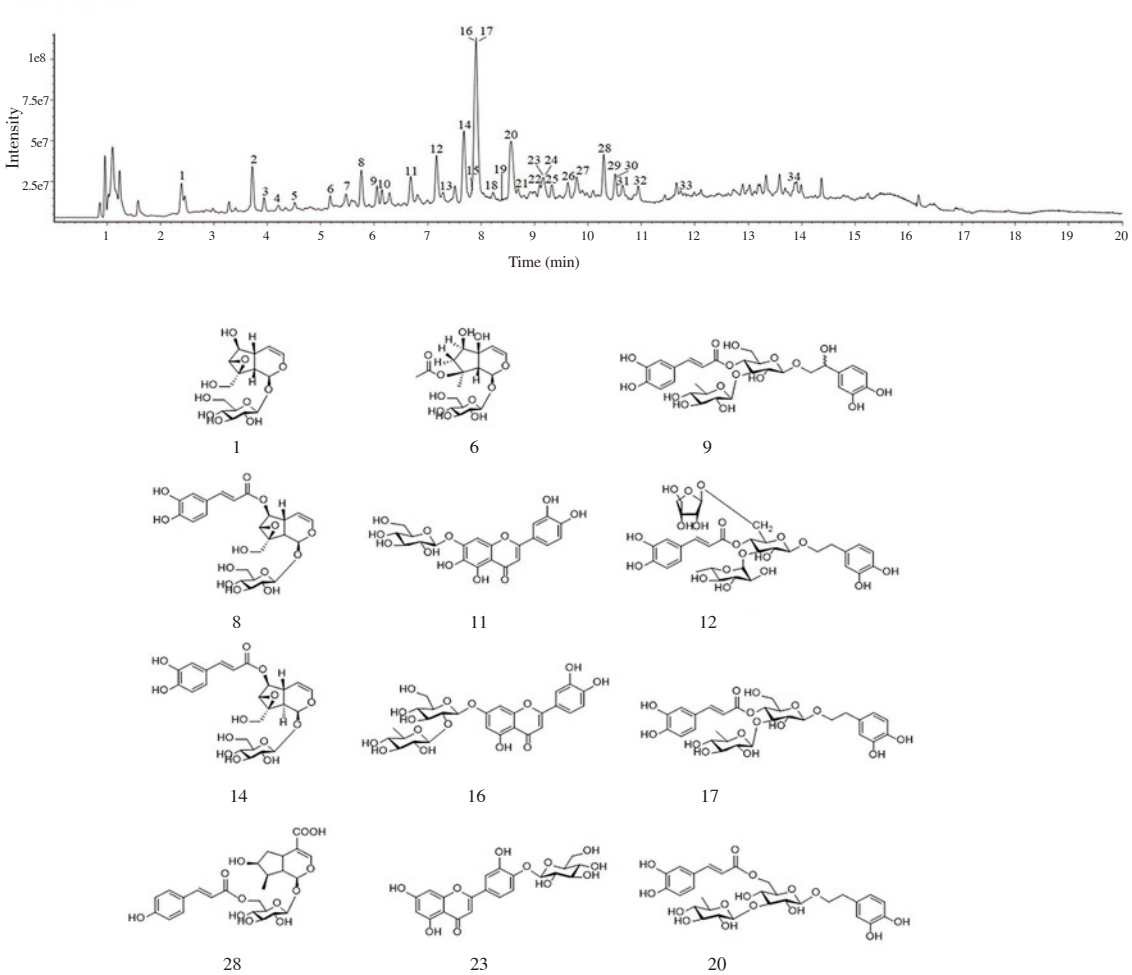

Figure 1. Total ion chromatogram of *C. nudiflora* extract in negative ion mode and the chemical structure of the characteristic compounds in its extract. Catalpol (1), 8-acetyl harpagide (6), β -hydroxy acteoside (9), 6-*O*-*trans*-caffeoyl-catalpol (8), 6-hydroxy-luteolin-7-*O*- β -*D*-glucoside (11), forsythoside B (12), nudifloside (14), luteolin-7-*O*-neohesperidoside (16), acteoside (17), 6'-*O*-*trans*-*p*-coumaroyl-8-epiloganic acid (28), lutedin-4'-*O*- β -*D*-glucoside (23), isoacteoside (20).

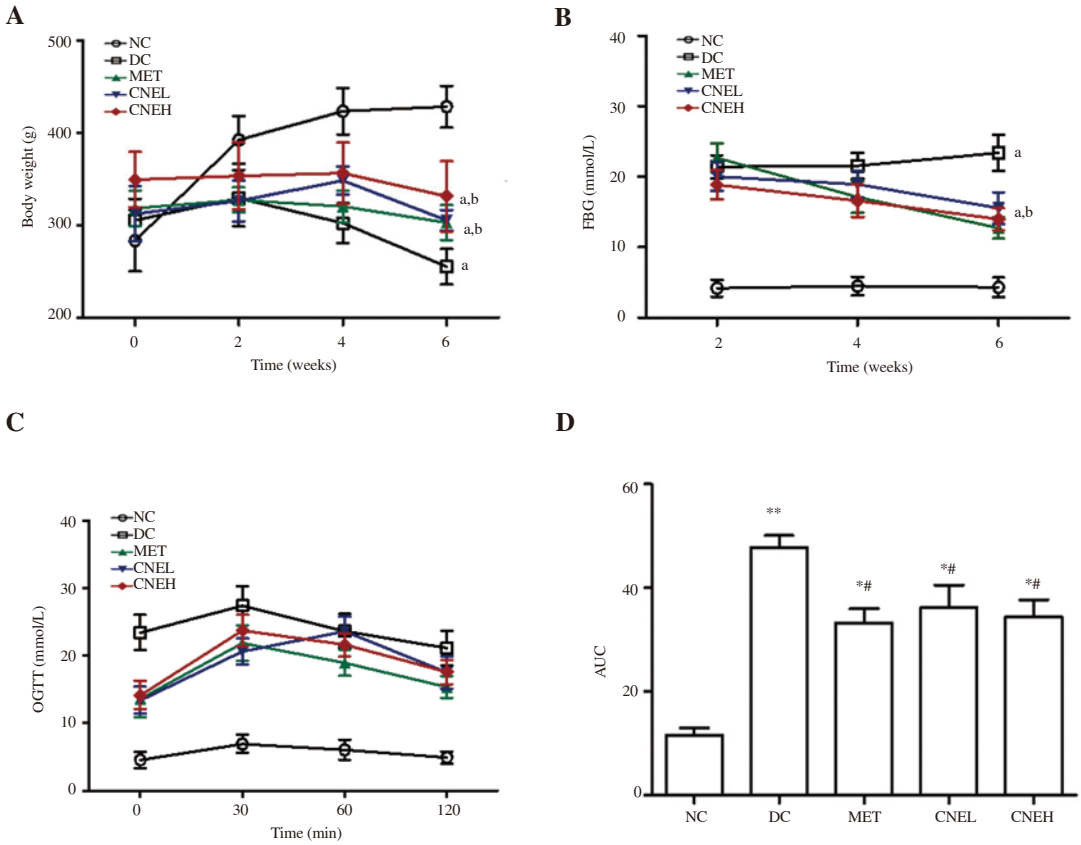

Figure 2. Effect of *C. nudiflora* extract on body weight (A), fasting blood glucose (FBG) (B), and oral glucose tolerance test (OGTT) (C, D). AUC: area under the curve for glucose in the oral glucose tolerance test. NC: normal control; DC: diabetic control; MET: diabetic + metformin (0.2 g/kg/day) group; CNEL: diabetic + low dose of *C. nudiflora* extract (0.15 g/kg/day) rats; CNEH: diabetic + high dose of *C. nudiflora* extract (0.3 g/kg/day) rats. Data are expressed as mean ± SD (n = 8). $^{a}P < 0.05$ compared with the NC group; $^{b}P < 0.05$ compared with the DC group; $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the NC group; $^{\#}P < 0.05$ compared with the DC group.

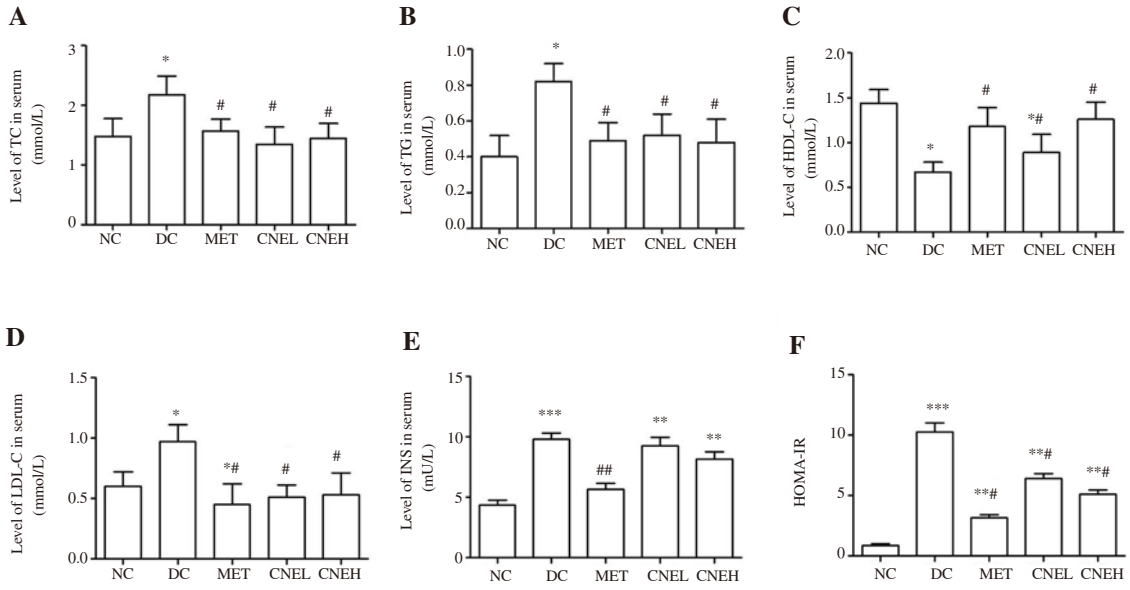

Figure 3. Effect of *C. nudiflora* extract on blood biochemistry and insulin resistance in diabetic rats. (A) total cholesterol (TC), (B) triglyceride (TG), (C) high-density lipoprotein cholesterol (HDL-C), (D) low-density lipoprotein cholesterol (LDL-C), (E) and insulin (INS) levels in the experimental groups. The homeostasis model assessment-insulin resistance (HOMA-IR) index was analyzed (F). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with the NC group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with the DC group. NC: normal control; DC: diabetic control; MET: diabetic + metformin (0.2 g/kg/day) group; CNEL: diabetic + low dose of *C. nudiflora* extract (0.15 g/kg/day) rats; CNEH: diabetic + high dose of *C. nudiflora* extract (0.3 g/kg/day) rats.

These results suggested that *C. nudiflora* extract treatment can improve serum lipid metabolism.

3.4. Effect of C. nudiflora extract on the expression of insulin and the HOMA-IR index in diabetic rats

Serum insulin and the HOMA-IR index were significantly higher in diabetic model rats than in normal rats (Figure 3E and F). In contrast, metformin caused a significant decline in serum insulin and the HOMA-IR index in diabetic model rats (Figure 3E–F). Although *C. nudiflora* extract did not affect serum insulin, it markedly reduced the HOMA-IR index in diabetic rats. In addition, immunohistochemical analysis showed that insulin expression in the pancreas of diabetic rats was significantly increased after treatment with *C. nudiflora*

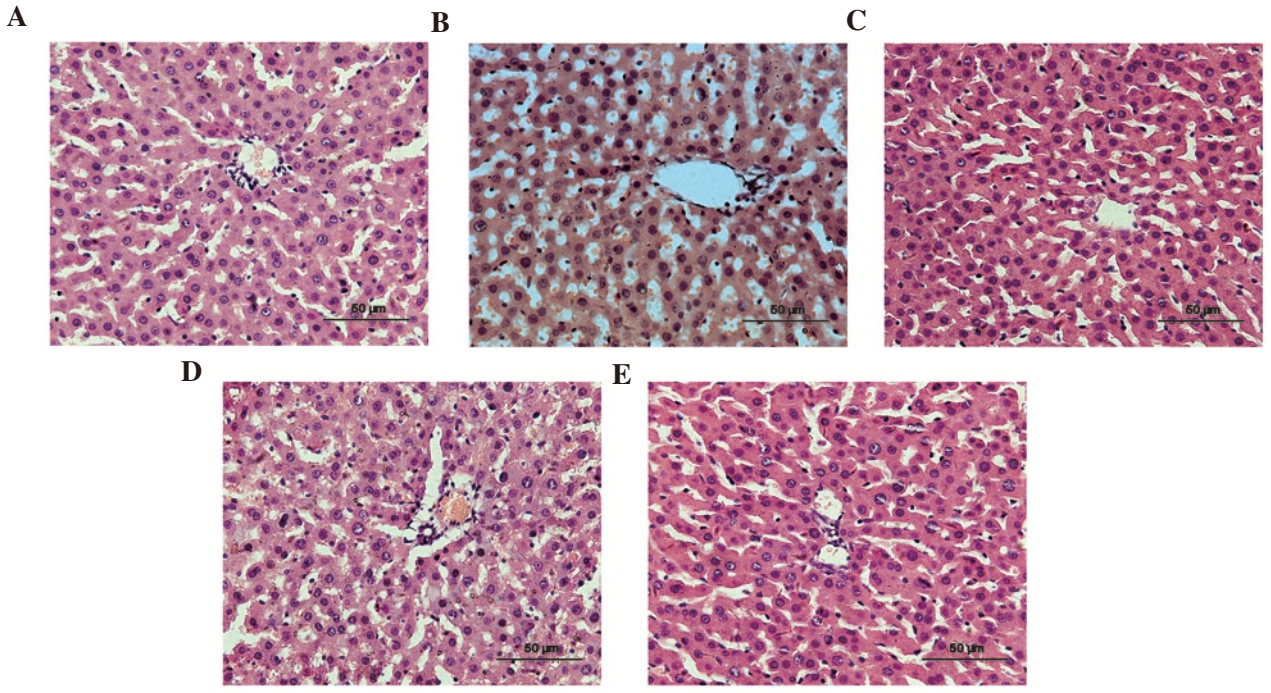

Figure 4. Histological changes in the liver tissues of experimental groups. *C. nudiflora* extract alleviated the lesion of liver tissue (Figure 4D & 4E). (Magnification = 400×, Scale bar = 50 μm). A: normal control; B: diabetic control; C: metformin; D: low dose of *C. nudiflora* extract; E: high dose of *C. nudiflora* extract.

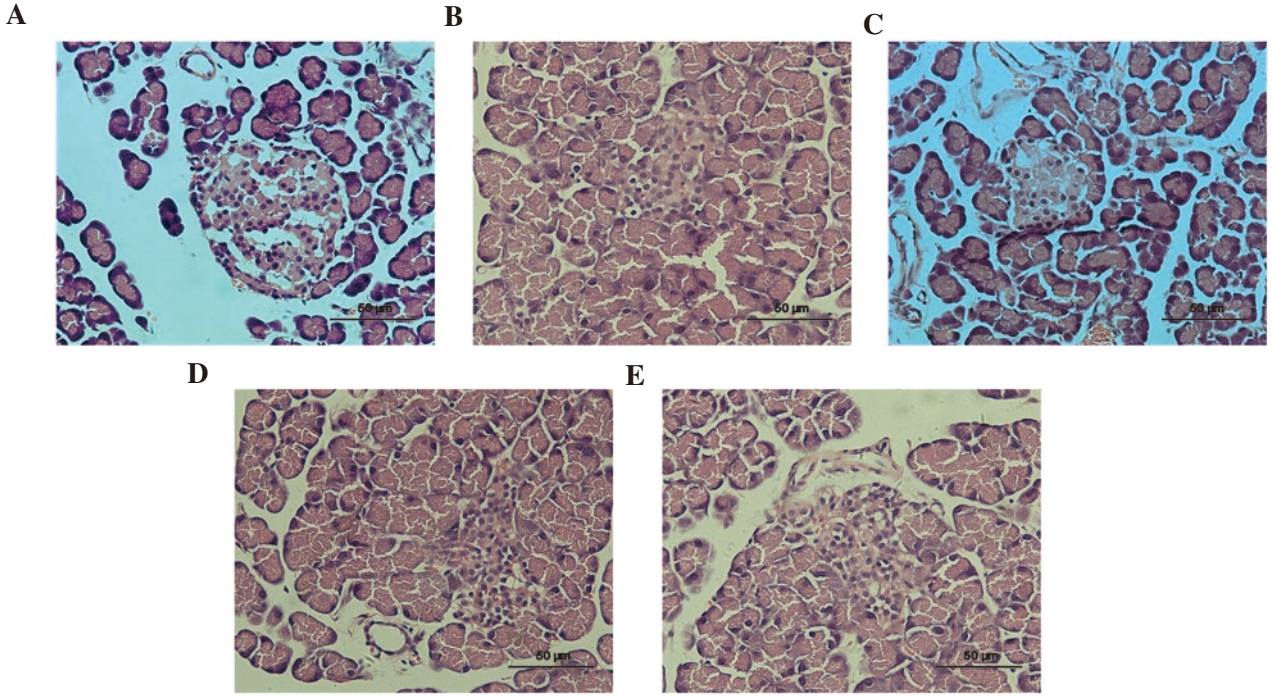

Figure 5. Histological changes in the pancreatic tissues of the experimental groups. *C. nudiflora* extract protected the pancreatic tissue (Figure 5D & 5E). (Magnification = 400×, Scale bar = 50 μm). A: normal control; B: diabetic control; C: metformin; D: low dose of *C. nudiflora* extract; E: high dose of *C. nudiflora* extract.

extract and metformin (Figure 7A). IRS-1 expression in the liver was further examined by immunohistochemical staining, and the results suggested that *C. nudiflora* extract and metformin significantly promoted the expression of IRS-1 in the livers of diabetic model rats (Figure 7B). These results indicated that *C. nudiflora* extract as well as metformin could improve the insulin resistance of diabetic rats.

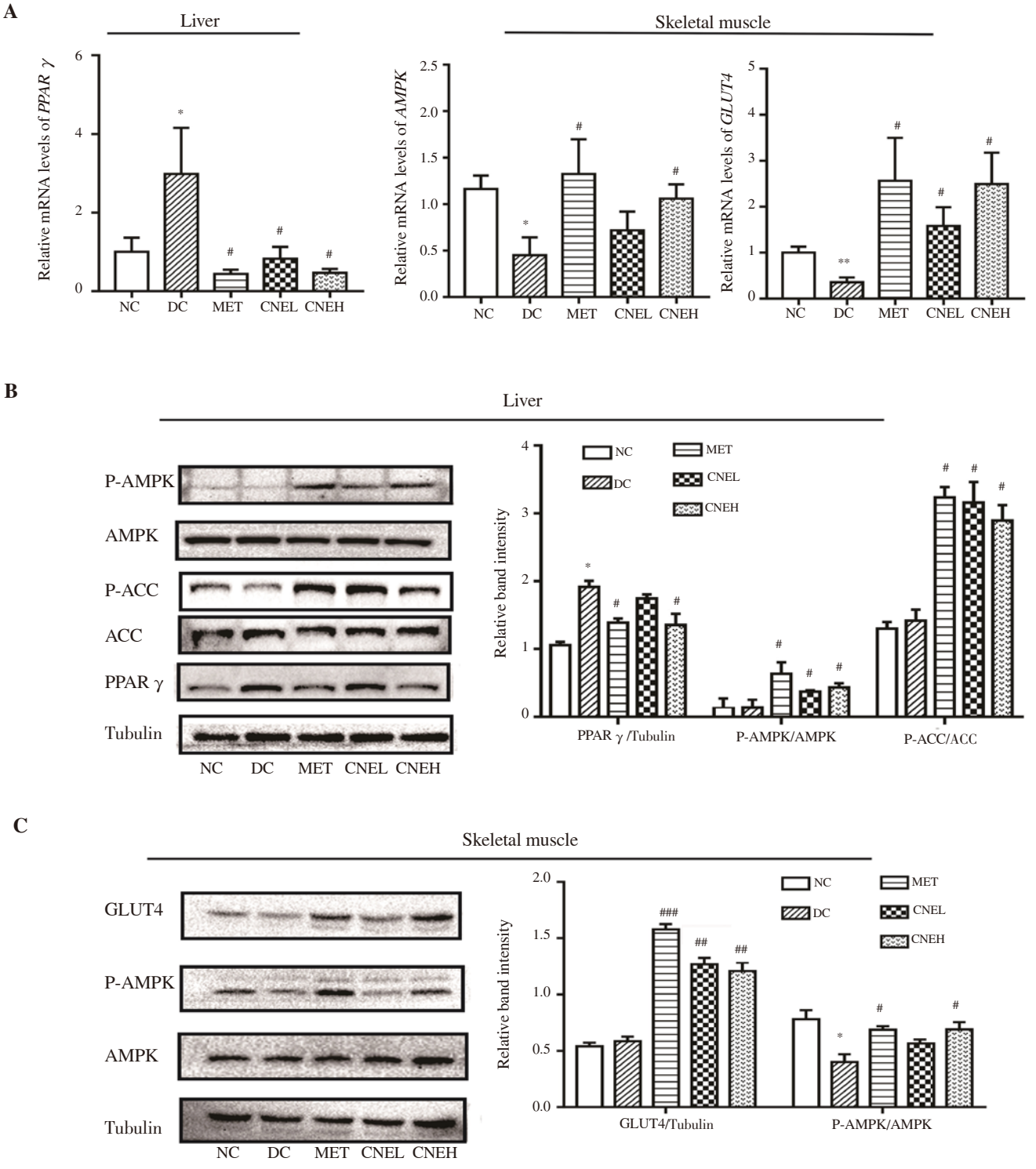

Figure 6. The effect of *C. nudiflora* on GLUT4 expression and the AMPK-ACC signaling pathway in the skeletal muscle and liver of diabetic rats. Levels of *PPAR γ* in liver tissue and *AMPK* and *GLUT4* mRNA in skeletal muscle as measured by RT-PCR (A). Expressions of AMPK, P-AMPK, ACC, P-ACC, and PPAR γ proteins in liver tissue (B), and AMPK, P-AMPK, and GLUT4 proteins in skeletal muscle (C) as measured by Western blotting. NC: normal control; DC: diabetic control; MET: diabetic + metformin (0.2 g/kg/day) group; CNEL: diabetic + low dose of *C. nudiflora* extract (0.15 g/kg/day) rats; CNEH: diabetic + high dose of *C. nudiflora* extract (0.3 g/kg/day) rats. $^{*}P < 0.05$ and $^{**}P < 0.01$, compared with the NC group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$, compared with the DC group.

3.5. Effect of C. nudiflora extract on liver and pancreas pathology in diabetic rats

As shown in Figures 4 and 5, diabetic rats showed severe hepatic and pancreatic impairment. However, the cellular architecture of the liver in rats treated with *C. nudiflora* extract and metformin was normal. A significant decrease in pancreatic islet size was observed in the histopathologic sections of diabetic control rats, and the islets were dispersed in the acini. In contrast, the islets were intact in the pancreatic acini of rats treated with *C. nudiflora* extract and metformin. The histopathology analysis indicated that *C. nudiflora* extract restored the liver and pancreatic functions of diabetic rats.

3.6. Effect of C. nudiflora extract on GLUT4 protein expression and the AMPK-ACC signal pathway in the skeletal muscle and liver of diabetic rats

As shown in Figure 6A and B, similar to metformin, *C. nudiflora*

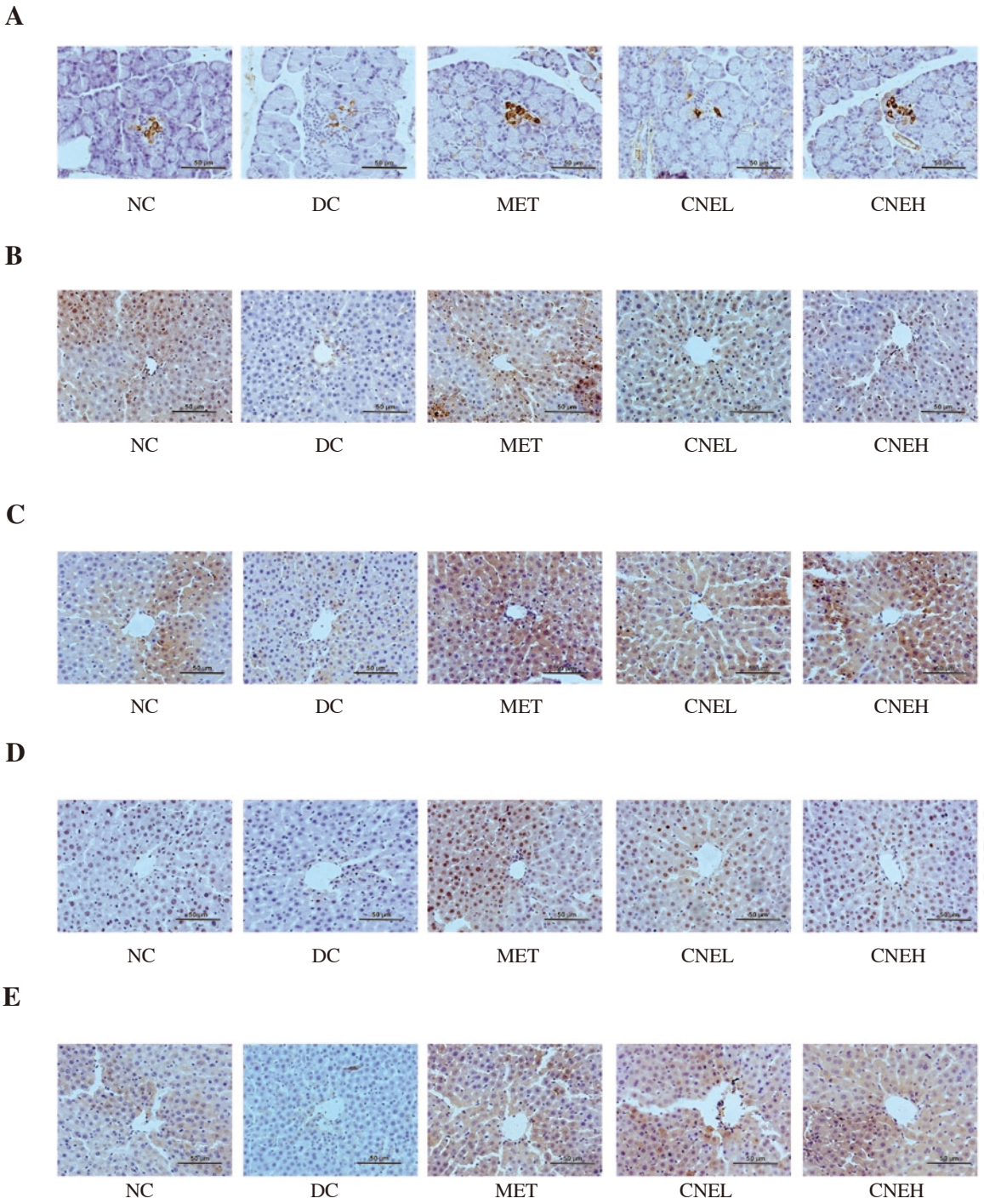

Figure 7. Immunohistochemistry of insulin (A), IRS-1 (B), AMPK (C), P-AMPK (D) and GLUT4 (E). NC: normal control; DC: diabetic control; MET: diabetic + metformin (0.2 g/kg/day) group; CNEL: diabetic + low dose of *C. nudiflora* extract (0.15 g/kg/day) rats; CNEH: diabetic + high dose of *C. nudiflora* extract (0.3 g/kg/day) rats. Magnification = 200×, Scale bar = 50 μm.

extract effectively inhibited the increase in *PPAR γ* mRNA and protein levels in the liver tissue of diabetic rats. In addition, the expression levels of P-AMPK/AMPK and P-ACC/ACC did not differed between diabetic rats and normal rats (Figure 6B), while these levels were significantly higher in the *C. nudiflora* extract and metformin treated groups. Compared with the expression levels in normal control rats, the mRNA expression levels of *AMPK* and *GLUT4* in the skeletal muscle of diabetic rats were markedly decreased, while the expression of GLUT4 protein had no changes compared with the normal control. However, treatment with *C. nudiflora* extract remarkably reversed the decreases in the mRNA expression levels of *AMPK* and *GLUT4* in the skeletal muscle of diabetic rats (Figure 6A). Similarly, GLUT4 protein expression and AMPK phosphorylation were also increased after treatment with extract or metformin in the skeletal muscle of diabetic rats (Figure 6C), which was verified by the immunohistochemical analysis (Figure 7C–E).

4. Discussion

Diabetes is a metabolic disease in which glucose is not appropriately processed, resulting in hyperglycemia[22]. In this study, FBG and OGTT were high in streptozotocin-induced diabetic rats, confirming the establishment of diabetes[23]. We showed that *C. nudiflora* extract reduced FBG and OGTT in diabetic rats, indicating that *C. nudiflora* extract can ameliorate hyperglycemia in diabetic rats.

FBG is correlated with dyslipidemia, and dysregulated blood lipid metabolism is an important factor relating T2DM mellitus with angiopathy[24]. T2DM is often characterized by elevated levels of TC, TG, and LDL-C, and decreased level of HDL-C[25]. *C. nudiflora* extract treatment inhibited the increases of TC, TG and LDL-C, and the decline of HDL-C in diabetic model rats, indicating that *C. nudiflora* extract treatment reversed the diabetic-induced disturbances in lipid profile and exhibited antilipidemic activity.

T2DM is also characterized by inadequate insulin secretion and/or action[26]. Insulin resistance was shown to inhibit insulin-stimulated glucose transport and metabolism in skeletal muscle and adipose tissue and suppress hepatic glucose output[27]. In this study, serum insulin and the HOMA-IR index were significantly increased in diabetic rats. The high-glucose and high-fat diet and streptozotocin induced severe pancreatic impairment, β-cell depletion, and dysfunction. However, *C. nudiflora* extract treatment restored the impaired pancreatic function of diabetic rats and decreased insulin levels in the pancreas and serum and the HOMA-IR index. IRS-1 is of great importance for mediating the metabolic action of insulin, and reduced IRS-1 levels contribute to impaired glucose metabolism and decreased muscle mass[28]. Treatment with *C. nudiflora* extract increased IRS-1 expression in the liver tissue of diabetic rats. To be brief, *C. nudiflora* extract could improve the insulin resistance of diabetic rats.

Increasing evidence suggests that the AMPK-ACC signaling pathway is important in glucose metabolism and insulin-resistant diabetes[29–31]. Genetic and pharmacological studies have shown that AMPK is required for maintaining glucose balance[32]. AMPK activation results in improved insulin sensitivity and maintenance of glucose homeostasis[33]. Hence, activating AMPK is regarded as an effective pathway for diabetes therapy[28]. ACC, the rate-limiting enzyme in fatty acid biosynthesis, is also a therapeutic target for diabetes[34]. AMPK stimulated the phosphorylation of ACC, reduced the activity of ACC and fatty acid synthesis[30]. In streptozotocin-stimulated diabetic rats, the levels of P-AMPK and P-ACC were reduced[35]. In our study, the P-AMPK/AMPK and P-ACC/ACC ratios were significantly increased in the group treated with *C. nudiflora* extract at both low and high doses, which was similar to those in the metformin treatment group, suggesting that the AMPK-ACC signaling pathway is involved in the antidiabetic effect of *C. nudiflora* extract.

GLUT4 is an important protein that transports glucose into cells, and is regarded as a therapeutic target for T2DM treatment[36]. Consistent with previous reports, GLUT4 was decreased in streptozotocin-stimulated diabetic rats[37]. It was reported that P-AMPK upregulated GLUT4 expression and promoted its transport to the plasma membrane[38]. Our results showed that *C. nudiflora* extract at low and high doses upregulates *GLUT4* mRNA and protein expression in the skeletal muscle of diabetic rats. Thus, *C. nudiflora* extract enhances GLUT-4 translocation through the AMPK-ACC signaling pathway in diabetes.

In summary, *C. nudiflora* extract lowered blood glucose levels and mitigated insulin resistance in diabetic rats. Moreover, *C. nudiflora* extract activated the IRS-1 and AMPK-ACC pathways, ameliorating insulin resistance and the clinical signs of diabetes. Therefore, *C. nudiflora* extract could be used for the treatment of diabetes.

Conflict of interest statement

The authors declare no competing interest.

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Authors' contributions

WYM performed mechanism research and animal experiment with LPM. BY contributed to data analysis. MZ performed drug extraction and UPLC-Q-TOF analysis. SXF conducted UPLC-Q-TOF data analysis, performed experimental design and critical revision of the article. LPT performed the *in vivo* experiment.

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