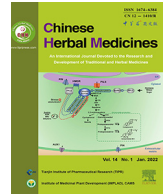




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Original Article

Anti-diabetic effects of linarin from *Chrysanthemi Indici Flos* via AMPK activationZhenji Wang^{a,b}, Zhe Bai^a, Jinghua Yan^a, Tengting Liu^a, Yingmei Li^a, Jiahui Xu^a, Xiaoqian Meng^a, Yuefeng Bi^{a,b,*}^aSchool of Pharmaceutical Sciences, Zhengzhou University, Zhengzhou 450001, China^bCollaborative Innovation Center of New Drug Research and Safety Evaluation, Zhengzhou 450001, China

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ABSTRACT

Objective: The purpose of this study is to investigate the anti-diabetic effects of linarin, a flavonoid extracted from *Chrysanthemi Indici Flos* (CIF), and its potential mechanisms.**Methods:** The effects of linarin on cell viability and glucose consumption in HepG2 cells were measured. Meanwhile, monosodium glutamate (MSG) mouse model was constructed to monitor the changes of insulin tolerance, glucose tolerance, triglyceride and cholesterol. The protein expression levels of p-AMPK, p-ACC, PEPCK and p-GS were detected by Western blot.**Results:** Linarin could increase the relative glucose consumption of HepG2 cells, improve insulin tolerance and glucose tolerance, and decrease the levels of triglyceride and cholesterol of MSG mice. Simultaneously, the expression levels of p-AMPK and p-ACC in HepG2 cells and the liver tissue of MSG mice were increased, while the expression levels of PEPCK and p-GS were decreased after treatment with linarin.**Conclusion:** Insulin resistance could be ameliorated by linarin in type 2 diabetes, and its mechanism may be related to AMPK signaling pathway.© 2021 Tianjin Press of Chinese Herbal Medicines. Published by ELSEVIER B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

Type 2 diabetes, affecting 463 million people in 2019 (Saeedi et al., 2019), is a common metabolic disease characterized by hyperglycemia, which is mainly caused by insulin resistance or insufficiencies of insulin secretion. According to the prevalence of chronic diseases and its risk factors in China, the prevalence of diabetes among people over 18 years old was 10.9% (Wang et al., 2017). Therefore, the prevention and treatment of diabetes is of great significance.

Insulin resistance (IR) is the main feature of type 2 diabetes, which leads to increased gluconeogenesis and eventually hyperglycemia. IR can be ameliorated by activating AMP-activated protein kinase (AMPK) (Miller et al., 2011), a metabolically sensitive serine/threonine protein kinase widely present in eukaryotic cell organisms (Miller et al., 2011). When AMPK is activated by phosphorylation, p-AMPK can phosphorylate glycogen synthase (GS)

and acetyl-CoA carboxylase (ACC), inhibit phosphoenolpyruvate carboxy kinase (PEPCK), thus restrain fatty acid production and gluconeogenesis (Santos et al., 2013).

Flavonoids can regulate glucose production in HepG2 cells, which is related to the activation of AMPK (Cordero-Herrera et al., 2013). Linarin is one of the main flavonoids compound in *Chrysanthemi Indici Flos* (CIF), with a content up to 6%–7%, which is an important index of quality control of CIF (Fan et al., 2017). As an effective drug for the treatment of type 2 diabetes (Bai et al., 2018), CIF extracts can improve insulin metabolism disorders. Therefore, we speculate that linarin in CIF may be the main active ingredient in anti-diabetic activity, and our previous experiment also hints this idea.

In this study, the anti-diabetic effects of linarin and its mechanism were investigated on HepG2 cells and MSG mice. The results revealed that linarin could improve insulin resistance in type 2 diabetes, and the mechanism was related to AMPK signaling pathway.

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2. Materials and methods

2.1. Chemicals and reagents

Linarin was prepared by our research group. High glucose DMEM medium, trypsin and hypersensitive ECL chemiluminescence kits were obtained from Solarbio Life Science (Beijing, China). Fetal bovine serum (FBS) was obtained from Procell Life Science & Technology (Wuhan, China). Cell counting kit-8 (CCK-8) was produced by Beyotime Biotechnology (Shanghai, China). Rosiglitazone and L-glutamate were purchased from Furen Group Pharmaceutical Co., Ltd (Henan, China). Glucose assay kit, triglyceride assay kit and cholesterol assay kit were obtained from Biosino Biotechnology and Science Inc. (Beijing, China). BCA protein concentration assay kit was purchased from Beyotime Biotechnology (Shanghai, China). Antibodies including p-AMPK, p-GS, anti-PEPCK, anti- β -Actin and goat anti-rabbit secondary antibody were purchased from Cell Signaling Technology (Danvers, MA, USA).

2.2. Extraction and isolation

CIF was collected from Henan Province, China in October 2019. The voucher specimen was deposited at School of Pharmaceutical Sciences, Zhengzhou University, with the collection number CIF 2019. Dried flower buds (2 kg) were extracted three times with 20 L methanol at room temperature for 1.5 h. The combined extracts were evaporated in vacuum to yield 450 g of residue. The residue was extracted with ethyl acetate and acetone, successively. The acetone extracts were concentrated properly until the yellow precipitate began to appear. Then it was re-precipitated to obtain 10 g of linarin (pale yellow powder).

2.3. Cell culture and treatments

HepG2 cells line was obtained from Cell Resource Center, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China) and cultured in high glucose DMEM containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37 °C, 5% CO₂. When the confluence was over 80%, the cells were seeded in 96-well plates at (6×10^3) cells/well and incubated for 24 h, followed by 48 h with different concentrations of linarin.

2.4. Relative glucose consumption assay

After incubation with linarin, the contents of glucose in the culture medium were determined by glucose oxidase assay to study the effects of linarin on glucose consumption in HepG2 cells. The relative glucose consumption was calculated as follows: Relative glucose consumption = glucose consumption / OD value of cell viability.

2.5. Cell viability assay (CCK-8 Assay)

CCK-8 assay was used to detect cell viability. 5 μ L of the CCK-8 solution was added and incubated at 37 °C for 4 h. The OD value was measured at a wavelength of 450 nm using a Microplate reader (NI-synergy, Gene Company Limited). The OD value represented the cell viability, and the assay was repeated three times.

2.6. Animal preparations

The animals were kept in the IVC animal room of School of Pharmaceutical Science, Zhengzhou University. The experimental environment was in accordance with laboratory animal-

requirements of environment and housing facilities (GB14925-2001), and the experimental animals using certificate number were SYXK (Yu) 2010-0002. Mice were divided into five groups in standard cages with free access to food and water and maintained under a 12 h dark/light cycle (starting at 7:30 AM), 22 °C, and 55% humidity (standard conditions). Animals were observed between 9:00 a.m. and 1:00 p.m. For the laboratory biology and facility safety, the experiment fully complies with the Laboratories - General Requirements for Bio-safety (GB19489-2008).

2.7. Experimental design

According to the study of Dominique Maiter (Maiter et al., 1991), neonatal mice were injected subcutaneously with monosodium glutamate (MSG) at 4 g/kg for eight consecutive days from the second day after birth. After that, all mice were fed in the animal room. Five months later, MSG mice showed significant centripetal obesity.

Insulin tolerance test was conducted in MSG mice and the insulin-resistant mice were selected for the following experiment. MSG mice were randomly divided into five groups (10 mice in each group), and the specific groups were as follows: low, medium and high concentrations of linarin groups (25, 50 and 100 mg/kg), positive control group (rosiglitazone, 7 mg/kg), model group (0.5% tragacanth solution). Normal ICR mice were used as normal group (0.5% tragacanth solution). The linarin were prepared as suspension solution using 0.5% tragacanth, which was administered by gavage once a day for 4 weeks. After the administration, the mice were anesthetized and sacrificed, and livers were collected.

2.8. Insulin tolerance test

Insulin tolerance test was performed on the 13th and 23rd day of the administration, respectively. After fasting for 12 h, caudal vein blood was taken as the initial blood glucose concentration. Then insulin was injected subcutaneously (0.4 IU/kg) in the dorsal region. Blood was collected at 40 min and blood glucose was measured in accordance with the instructions of glucose assay (Glucose oxidase method) kit.

2.9. Oral glucose tolerance test

The oral glucose tolerance test was performed on the 18th and 28th day of the administration, respectively. The blood samples were collected from the tail as the initial blood glucose concentration. The mice were given glucose solution (2 g/kg) intragastrically. Then blood samples were collected at 30, 60 and 120 min after glucose loading, and blood glucose levels were measured.

2.10. Determination of plasma triglyceride (TG) and cholesterol (CHO) levels

The levels of TG and CHO were determined with the TG assay kit and the CHO assay kit according to the instructions.

2.11. Western blot analysis

HepG2 cells and liver tissues of mice were homogenized in the lysis buffer (with protein phosphatase inhibitor) for 30 min on ice, followed by centrifugation (12000 rpm, 4 °C, 15 min), and the supernatant were collected. The protein concentration was measured by BCA protein quantification kit. The protein samples were subjected to sodium dodecyl sulfate - polyacrylamide gel electrophoresis and then transferred onto polyvinylidene fluoride (PVDF) membrane, followed by blocking with 3% BSA and incubating with the respective primary antibodies overnight at 4 °C and

peroxidase-conjugated secondary antibody for 2 h at room temperature. The primary antibodies were: p-AMPK (CST, #2535), p-ACC (CST, #11818), p-GS (CST, #3891), PEPCK (Abcam, ab187145) and β -Actin. Finally, the protein bands were visualized with a high sensitivity ECL chemiluminescence kit and autoradiographic film.

2.12. Statistical analysis

All data were presented as mean \pm SD. Using SPSS 20.0 software, One-way ANOVA test and multiple comparisons between groups were used to analyze the data. $P < 0.05$ were considered to be statistically significant.

3. Results

3.1. Structure identification and assaying

Linarin (Fig. 1) (mp 266–267 °C) was identified by TLC ($R_f = 0.52$, yellow fluorescence with $AlCl_3$ at 365 nm). 1H NMR (400 MHz, DMSO d_6) (Fig. 2A) δ : 12.91(1H, s, OH-5), 3.86 (3H, s, H-OCH₃), 5.05 (1H, d, $J = 7.5$ Hz, glc-H-1), 3.16–3.86 (10H, m), 4.54 (1H, brs), 1.07 (3H, d, $J = 6.5$ Hz, H-CH₃), 6.45 (1H, d, H-6), 6.78 (1H, d, H-8), 6.94 (1H, s, H-3), 7.14 (2H, d, $J = 9.0$ Hz, H-3', 5'), 8.04 (2H, d, $J = 9.0$ Hz, H-2', 6'). ^{13}C NMR (100 MHz, DMSO d_6) (Fig. 2B) δ : 182.5 (C-4), 164.4 (C-2), 163.4 (C-7), 162.9 (C-4'), 161.6 (C-5), 157.4 (C-9), 128.9 (C-2',6'), 123.1 (C-1'), 115.2 (C-3', 5'), 105.9 (C-10), 104.3 (C-3), 101.0 (C-6), 100.4 (glc-1), 100.1 (rha-1), 95.2 (C-8), 76.7 (glc-3), 76.3 (glc-5), 73.5 (glc-2), 72.5 (rha-4), 71.2 (glc-4), 70.8 (rha-2), 70.1 (rha-3), 68.8 (rha-5), 66.6 (glc-6), 56.0 (4'-OCH₃), 18.3 (rha-6), all of these are correspond with the published literature data.

The purity ($\geq 95\%$) of the linarin was determined by the High Performance Liquid Chromatography (HPLC) fingerprint system (Fig. 2C). Samples were obtained from solution of linarin in DMSO at 100 mmol/L (storage conditions: 37 °C), with an interval of 7 d, and the stability of linarin for 28 d was evaluated. We found the concentration of linarin was the same (RSD = 0.18%).

3.2. Effects of linarin on relative glucose consumption in HepG2 cells

Based on the preliminary experiments, we selected the concentrations of linarin at 5, 10, and 15 $\mu\text{mol/L}$ to determine the cell viability and relative glucose consumption of HepG2 cells. (Fig. 3). Linarin could significantly increase the relative glucose consumption without affecting the cell viability at 10 and 15 $\mu\text{mol/L}$.

3.3. Effects of linarin on IR in MSG mice

The fasting blood glucose levels, reduction rate of blood glucose and body weight of mice were detected (Fig. 4), and then the insulin-resistant MSG mice were randomly divided into five groups as described in Section 2.7.

Insulin tolerance test was performed on the 13th and 23rd day after administration to detect the effects of linarin on improving IR after insulin administration. Surprisingly, 40 min after insulin injection, 100 mg/kg of linarin significantly reduced blood glucose

levels compared with the model group on the 13th and 23rd day (Fig. 5).

The oral glucose tolerance test was performed on the 18th and 28th day. After intragastric administration of glucose solution, the blood glucose level of the model group was significantly higher than that of the normal group, and the peak blood glucose levels of each drug group were lower than that of the model group (Fig. 6). Unexpectedly, we found that the hypoglycemic effects of high-dose linarin group was similar to that of positive drug on the 18th and 28th day.

3.4. Effects of linarin on plasma TG and CHO in MSG mice

The effects of linarin on plasma TG and CHO were examined in MSG mice (Fig. 7). On the 13th day of administration, we did not find that linarin had significant effects on TG and CHO, but after 23 d of administration, we were surprised to find that 100 mg/kg of linarin began to reduce the contents of TG and CHO in plasma compared with the model group.

3.5. Effects of linarin on protein expression of p-AMPK, p-ACC, PEPCK and p-GS

In order to explore the mechanism of linarin on diabetes mellitus, we used Western blot to detect the expression of enzyme proteins which related to glycolipid metabolism in AMPK signaling pathway. Compared to the control group, when 10 and 15 $\mu\text{mol/L}$ linarin was administered, the expression of p-GS and PEPCK decreased, while the expression of p-AMPK and p-ACC increased in HepG2 cells (Fig. 8). The expression of p-GS and PEPCK was increased in MSG mice, and this augmentation was decreased after administration of linarin. The expression of p-AMPK and p-ACC was reduced in MSG mice, and this reduction was added after administration of linarin (Fig. 9).

4. Discussion

It was reported that linarin has antipyretic, analgesic, anti-inflammatory (Pan et al., 2019) and anti-oxidative effects (Dembinska-Kiec et al., 2008). Many drugs play their antidiabetic role effectively through anti-inflammatory and anti-oxidative effects (Yi et al., 2008). The results confirmed that linarin has anti-diabetic effects. In our research, compared with the normal group, the relative glucose consumption, insulin tolerance, glucose tolerance, triglyceride and cholesterol of the model group were significantly changed, indicating that linarin had regulatory effect.

We tested the cytotoxicity of linarin in HepG2 cells. The cell shrank and died in varying degrees: When the concentration of linarin was higher than 20 $\mu\text{mol/L}$, cells showed different degrees of atrophy and death, while when the concentration was lower than 20 $\mu\text{mol/L}$, it showed cytotoxicity. Based on this result, we found linarin could increase the relative glucose consumption of HepG2 cells at 10 and 15 $\mu\text{mol/L}$, which suggested that linarin could improve the ability of liver cells to use glucose to improve diabetes.

MSG mice was a typical type 2 diabetes model (Bahadoran et al., 2019) with IR, obesity, glucose and lipid metabolism disorders, and was used for the evaluation of a variety of anti-diabetic drugs. We established MSG mice model to research the antidiabetic effects of linarin. The pre-test showed that 2000 mg/kg of linarin was not toxic to mice. The doses of linarin used in insulin resistant MSG mice were set based on the data of HepG2 cells and pre-test in mice. We found that the blood glucose concentration of normal group reduced by about 50% after insulin administration, but the reduction rate of blood glucose was only about 20% in model

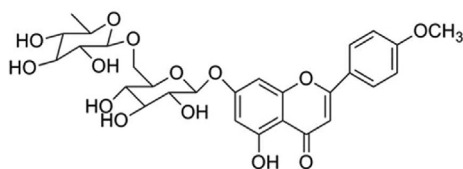


Fig. 1. Structure of linarin.

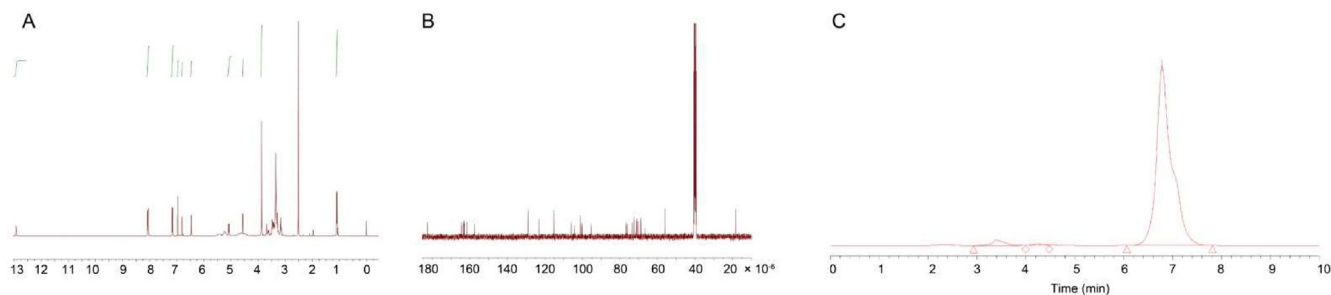


Fig. 2. ¹H NMR (A), ¹³C NMR (B) and HPLC (C) for structure identification of linarin.

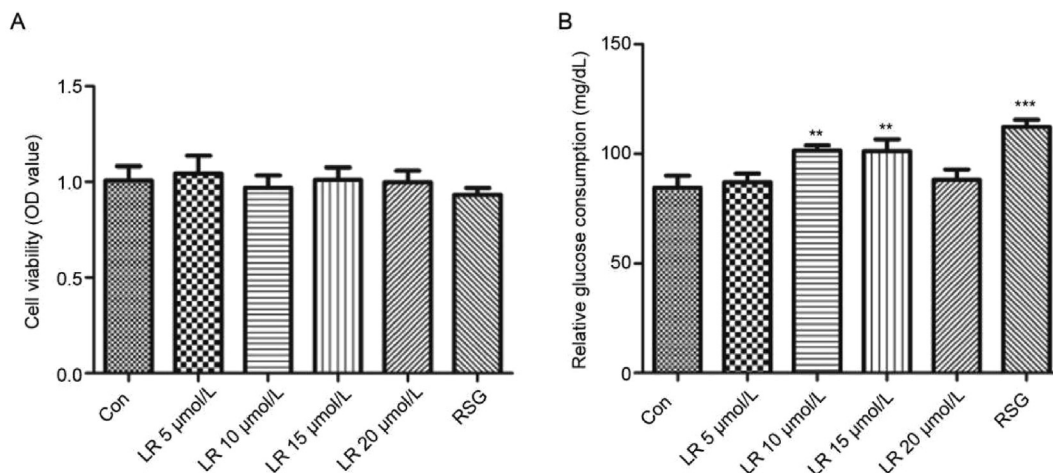


Fig. 3. Effects of linarin on relative glucose consumption in HepG2 cells (mean ± SD, n = 5). (A) Cell viability of HepG2 cells. (B) Relative glucose consumption effects of linarin at concentrations of 5, 10, and 15 μmol/L. **P < 0.01, ***P < 0.001 vs. control group.

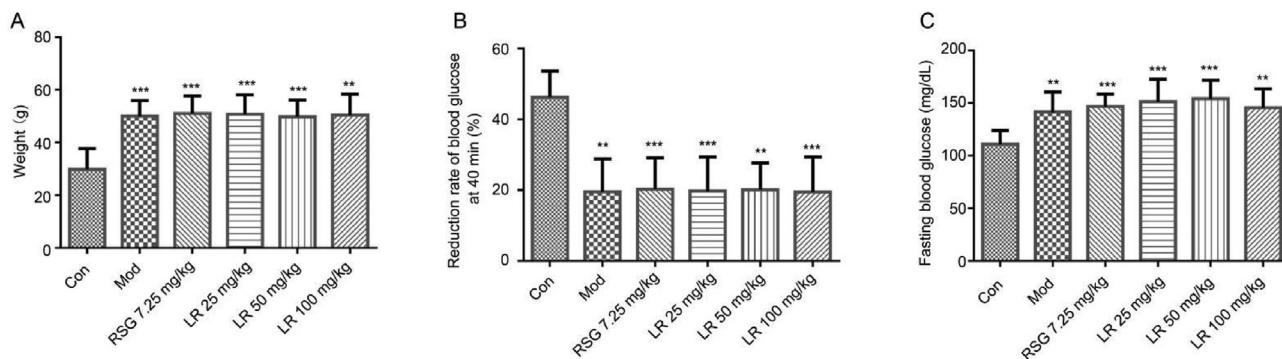


Fig. 4. Detection of body weight (A), reduction rate of blood glucose (B) and fasting blood glucose levels (C) of mice (mean ± SD, n = 10). **P < 0.01, ***P < 0.001 vs normal group.

group, which also revealed that MSG mice perform severe insulin tolerance.

Interestingly, linarin increased the percentage of blood glucose reduction at 40 min after insulin injection and significantly improved IR in MSG mice. Linarin could markedly reduce the levels of TG and CHO, which had great significance for evaluation of anti-diabetic drugs. Therefore, linarin could lessen the increase of blood glucose levels, meanwhile it showed a certain degree of improvement in glucose tolerance in MSG mice.

AMPK was known as a “cellular energy receptor”, which played a crucial role in regulating the metabolism of glucose, fat and protein (Ramamurthy & Ronnett, 2012). In the regulation of glucose metabolism, after the activation of AMPK, the expression and local-

ization of glucose transporter (GLUT4) in skeletal muscle cell are increased to promote glucose uptake (Russell et al., 1999). In the regulation of lipid metabolism, p-AMPK promotes phosphorylation of ACC (acetyl-CoA carboxylase) and inhibits fatty acid synthesis (ACC is the fatty acid synthesis rate-limiting enzyme) (Miller et al., 2011).

Our study confirmed that the expression levels of p-AMPK and p-ACC were lower in the model group than those in the normal group. Linarin remarkably increased the expression levels of p-AMPK and p-ACC in HepG2 cells at the concentrations of 15 and 10 μmol/L in HepG2 cells or at concentrations of 50 and 100 mg/kg in MSG mice. The upregulation of p-ACC could inhibit β-hydroxy-β-methylglutaryl coenzyme A (HMG-CoA) reductase

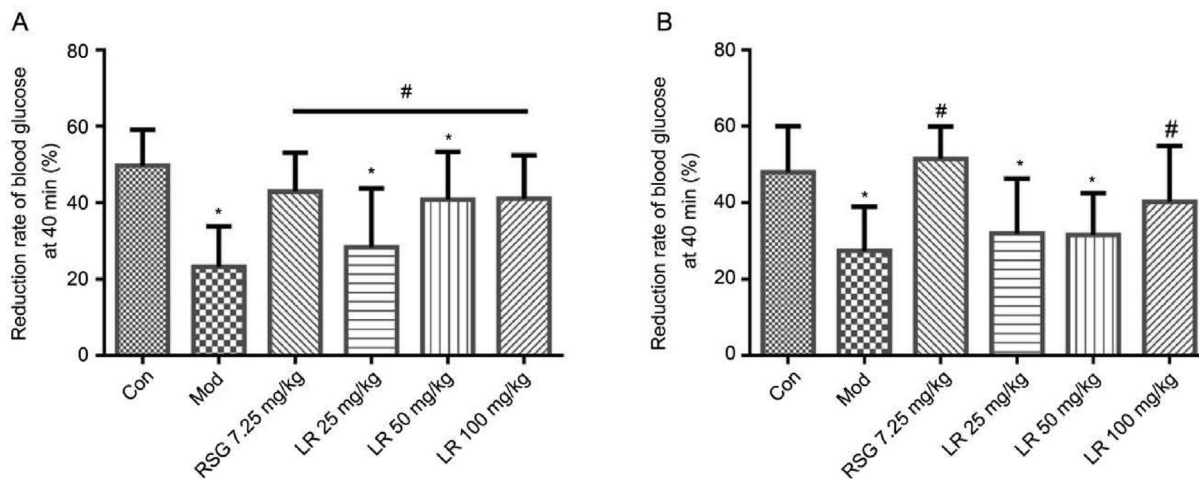


Fig. 5. Effects of linarin on blood glucose level in MSG mice (mean ± SD, n = 10). (A) Reduction rate of blood glucose after insulin administration for 40 min on the 13th day. (B) Reduction rate of blood after insulin administration for 40 min glucose on the 23th day. *P < 0.05 vs normal group, #P < 0.05 vs model group.

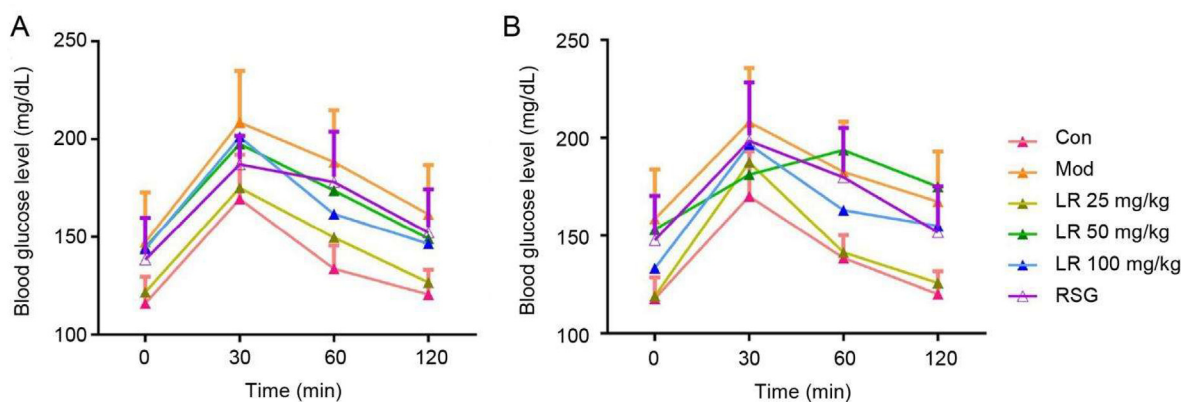


Fig. 6. Effects of linarin on oral glucose tolerance in MSG mice on the 18th (A) and 28th day (B) (mean ± SD, n = 10).

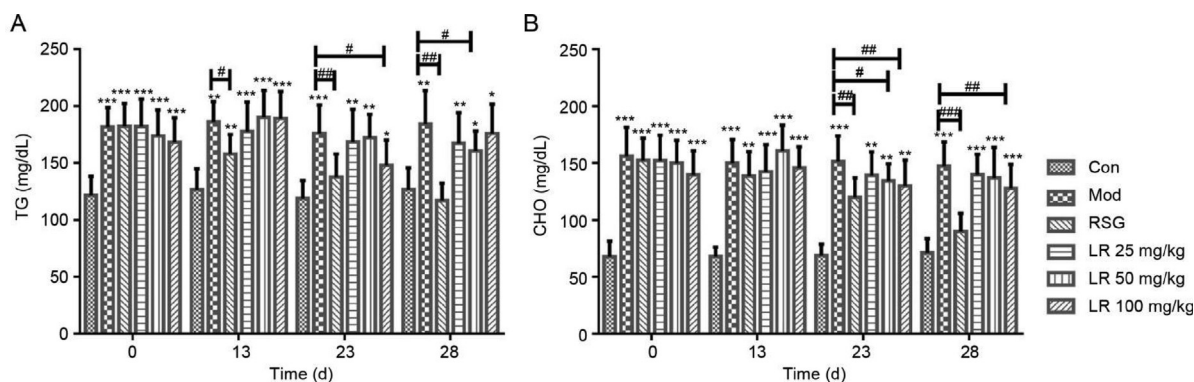


Fig. 7. Effects of linarin on plasma TG (A) and CHO (B) levels in MSG mice (mean ± SD, n = 10). *P < 0.05, **P < 0.01, ***P < 0.001 vs normal group; #P < 0.05, ##P < 0.01, ###P < 0.001 vs model group.

(Hardie & Pan, 2002), reduce the synthesis of CHO, phosphorylate hormone-sensitive lipase (HSL), and promote the decomposition of TG into fatty acids.

p-AMPK could activate 6-phosphate fructokinase-2 (PFE-2) and then increase the glycolysis (Russell et al., 1999), while inhibit the phosphorylation of GS (glycogen synthase) (Hunter et al., 2011) and PEPCK (phosphoenolpyruvate carboxykinase) (Inoue & Yamauchi, 2006), reduce glycogen production and gluconeogenesis.

Liver PEPCK is the key rate-limiting enzyme of gluconeogenesis (Stark et al., 2013). GS was the rate-limiting enzyme of glycogen synthesis, and their activities were essential for steady regulation of blood glucose (Nielsen et al., 2001). Studies had shown that the activation of AMPK would eventually lead to GS inactivation and reduced glycogen synthesis. This may be due to another AMPK-related signaling pathway that can activate GS. Researcher (Cordero-Herrera et al., 2013) found that in the PI-3K/AKT

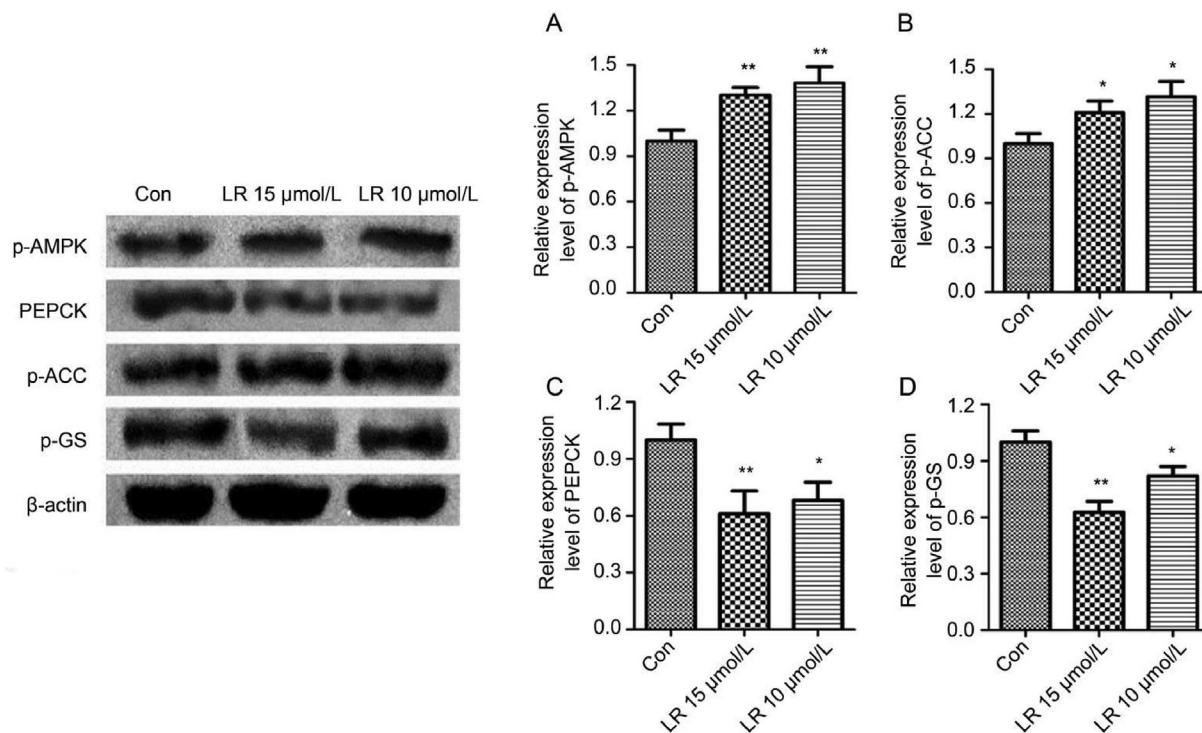


Fig. 8. Effects of linarin on protein expression of p-AMPK(A), p-ACC(B), PEPCK (C) and p-GS(D) in HepG2 cells (mean ± SD, n = 3). *P < 0.05, **P < 0.01 vs control group.

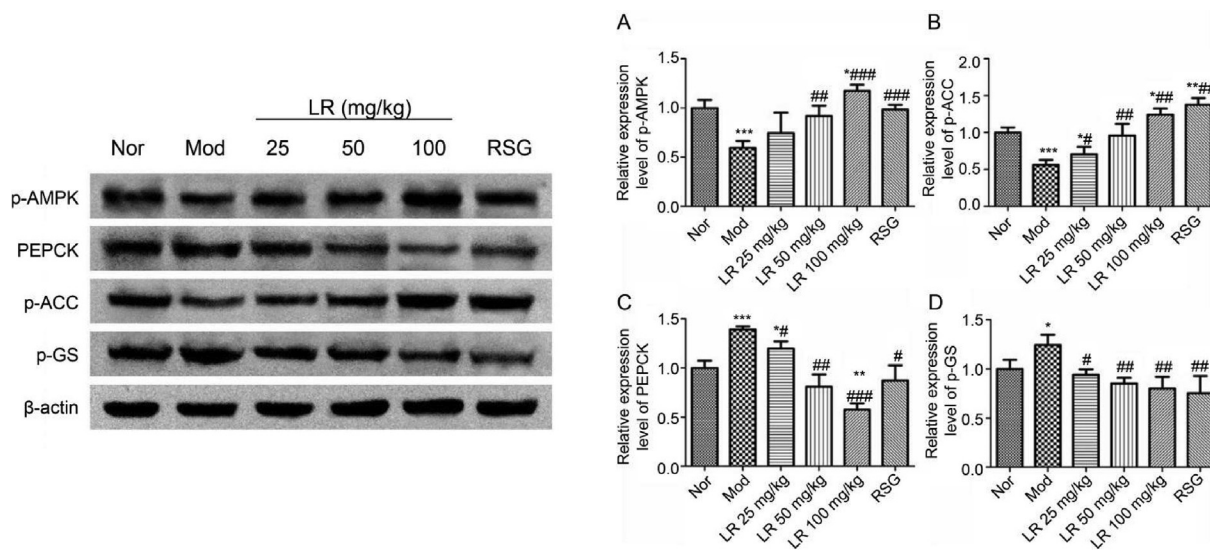


Fig. 9. Effects of linarin on protein expression of p-AMPK (A), p-ACC (B), PEPCK (C) and p-GS (D) in liver of MSG mice (mean ± SD, n = 10). *P < 0.05, **P < 0.01, ***P < 0.001 vs normal group; #P < 0.05, ##P < 0.01, ###P < 0.001 vs model group.

signaling pathway, activated AKT phosphorylated the glycogen synthase kinase-3β (GSK-3β), which further promoted the activation of GS and reduced the expression of p-GS, thus ultimately increasing glycogen synthesis. In addition, in the regulation of PEPCK, the activated AKT could inhibit gluconeogenesis and lower blood glucose (Liu et al., 2019), which was consistent with the effects of AMPK.

5. Conclusion

The present study demonstrates that linarin can increase the relative glucose consumption of HepG2 cells, improve insulin tol-

erance and glucose tolerance of MSG mice, and decrease triglyceride and cholesterol levels. Moreover, it plays anti-diabetic effects by regulating AMPK signaling pathway.

This article may provide reference for further investigation of the hypoglycemic mechanism of linarin, and new ideas for the future study to prevent diabetes using linarin.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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