European Review for Medical and Pharmacological Sciences 2022; 26: 8358-8369

Potentilla bifurca flavonoids effectively improve insulin resistance

G.-Y. WANG[1], P.-Y. YAN[1], W. LIU[1], L.-K. LIU[1,2], J.-P. LI[1,2], Y. ZENG[1,2]

[1]College of Life Science, Qinghai Normal University, Xining, China
[2]Academy of Plateau Science and Sustainability, Xining, China

G. Wang and P. Yan contributed equally to this article

Abstract. – OBJECTIVE: The goal of this paper is to investigate the use of Potentilla bifurca in the treatment of type 2 diabetes.

MATERIALS AND METHODS: We analyzed the improvement effect of 3T3-L1 adipocytes under insulin resistance (IR) with the compounds of *Potentilla bifurca*.

RESULTS: The *Potentilla bifurca* can significantly improve the glycolipid metabolism disorder in 3T3-L1 adipocytes (the effect of MC compounds is very significant). It can improve insulin resistance by enhancing the glucose uptake of 3T3-L1 adipocytes, decreasing IL-6 content, and regulating the content of p-Akt/Akt, IKKβ, and p-NF-κBp65/NF-κBp65 in the IRS/PI3K/Akt signaling pathway.

CONCLUSIONS: Studies have shown that Potentilla bifurca has the ability to regulate glucolipid metabolism and can be used in the treatment of type 2 diabetes.

Key Words:
Potentilla bifurca, Insulin resistance, Type 2 diabetes, Glucolipid metabolism, Flavonoids.

Introduction

Diabetes mellitus (DM) is a systemic metabolic disease that affects the multi-organ system, such as impaired insulin secretion or action, characterized by elevated blood sugar[1,2]. The International Diabetes Federation has anticipated that the number of diabetic patients will increase to 380 million in 2025[2]. DM is divided into type 1 diabetes mellitus (T1DM), type 2 diabetes mellitus (T2DM), gestational diabetes, and other special types of diabetes. T2DM is responsible for more than 90% of diabetes cases[3]. T2DM is mainly caused by insulin resistance, and the patients are usually treated by improving insulin resistance and reducing the level of blood glucose[4]. Insulin is a well-known hormone associated with the regulation of glucose and lipid metabolism, and insulin resistance occurs when insulin receptors in insulin target tissues fail to respond appropriately to insulin[5]. Furthermore, inflammatory factors such as tumor necrosis factor-α (TNF-α) played a key role in the development of insulin resistance[6]. Studies[7] have shown that inflammatory pathways could disrupt insulin receptor substance-1 (IRS-1) and glucose transporter (GLUT), causing insulin resistance.

The current treatment of diabetes has achieved some results, but many patients still suffer from its complications and side effects. The oral administration of hypoglycemic drugs, in addition to insulin injection, is the main strategy to treat diabetes[8]. Sulfonylureas and biguanides, two commonly used medications, can help patients manage their blood sugar levels; however, they can have negative health effects and have no protective effects on pancreatic islet β-cells[9,10]. Therefore, there is still a need to develop more effective and healthy treatments for diabetes[11]. Emerging evidence suggests that most medicinal plants contain flavonoids which can improve diabetes by regulating factors associated with diabetes and its complications[12].

The therapeutic effect of flavonoids on insulin resistance is a hot topic in the world. Quercetin is a natural flavonoid with beneficial effects in the diabetic animal model[13], which in the flowers of *Edgeworthia Gardner* could significantly improve glucose tolerance in the peritoneum, the insulin level in plasma, and triglyceride (TG) content in the liver in T2DM mice[14]. *Paulownia fortunei* flowers have enough flavonoids, whose extract can attenuate Diet-Induced Hyperlipidemia and insulin resistance in obese mice[15]. Corylin, a flavonoid compound extract from *Psoralea corylifolia L*, has been shown to exert anti-inflammatory,

Corresponding Authors: Yang Zeng, MD; e-mail: zy-3@263.net
Jinping Li, MD; e-mail: 2008ljp@163.com

anticancer, anti-atherosclerotic effects, ameliorate hyperlipidemia, and insulin resistance[16]. Sea buckthorn leaves extract and flavonoid glycosides extract from sea buckthorn leaves can ameliorate adiposity, hepatic steatosis, insulin resistance, and inflammation in diet-induced obesity[5]. The chemical components from natural plant sources have become an important source of DM preventive and therapeutic drugs. The development and study of the active components of such plants can provide a new research direction for DM treatment. What's more, it can also improve the utilization value of natural plants.

The *Potentilla bifurca* (*Potentilla bifurca var. humilior R.*), which is a genus of more than 500 species worldwide, is a variety of the *Potentilla* of the Rosaceae family. It is an annual or perennial herb that grows widely in northern temperate regions[17]. Mainly distributed in Inner Mongolia, Hebei, Shanxi, Gansu, Qinghai, Ningxia, Xinjiang, Sichuan, Tibet, and other regions in China. Traditional Chinese medicine records that it has the treatment of dysentery, hemorrhoid bleeding, blood vomiting, pharyngitis, cough, and other effects. However, limited information is available on hypoglycemic and hypolipidemic effects of *Potentilla bifurca* right now. Therefore, the objective of this study was to explore the pharmacological activity of the flavonoids extracted from *Potentilla bifurca* in the treatment of T2DM.

Materials and Methods

Chemical Reagents

The 3T3-L1 preadipocytes were purchased from the Procell Life Science & Technology Co., Ltd (Wuhan, China). In addition, the following chemicals were used in this study: Dulbecco's Modified Eagle Medium (DMEM), Penicillin-Streptomycin Liquid (100×), Phosphate Buffered Saline (PBS), 0.25% Trypsin-EDTA Solution, Recombinant Human Insulin (Wuhan Procell Life Science & Technology Co., Ltd, Wuhan, China); Fetal Bovine Serum (FBS, Biological Industries, Shanghai, China); Oil Red O (ORO), Dex, IBMX, Cell Lysis Buffer, Western Blot Primary Antibody Dilution Buffer, Western Blot Secondary Antibody Dilution Buffer (Dalian Meilun Biotechnology Co., Ltd, Dalian, China); 4% Paraformaldehyde (Biotopped Life Sciences, Shanghai, China); Cell Counting Cit-8 (CCK-8), Triglyceride Determination Kit, Glucose Assay Kit (Nanjing Jiancheng Bioengineering Research Institute Co., Ltd, Nanjing, China); Mouse Interleukin-6 ELISA Kit (Abclonal, Wuhan, China); Anti-p-Akt (Ser308) antibody, Anti-Akt antibody, Anti-IKKβ antibody, Anti-p-NF-κBp65 antibody, Anti-NF-κBp65 antibody, β-actin, (Abcam, Shanghai, China); Goat Anti-Mouse IgG -HRP, Goat Anti-Rabbit IgG-HRP (Abgent, Beijing, China); Prestained Color Protein Marker, BCA Protein Assay Kit (Thermo Sciences, Shanghai, China).

Statistical Analysis

The data obtained from the experiments were statistically analyzed using the IBM SPSS software version 21.0 for Microsoft (SPSS Corp., Armonk, NY, USA), and one-way ANOVA was used to compare the means between groups. The final results of the experiments were expressed as mean±standard deviation; $p<0.05$ and $p<0.01$ indicated that the data were statistically significant, and the differences were significant and extremely significant, respectively.

Extraction of Six Compounds

The *Potentilla bifurca*, which was used in the experiment, was a wild plant collected from the Qinghai-Tibet Plateau. The collection of this plant complies with the legal requirements of the "Regulations on the Protection of Wild Plants of the People's Republic of China". The specimen is currently preserved in the Herbarium of the School of Life Sciences, Northwest A&F University, specimen number WUK 0452265, which was identified by Professor Yang Jinxiang of Tongji Medical College of HUST.

The six compounds used in the experiment were prepared by the research group and their preparation methods are as follows: the dried *Potentilla bifurca* powder was extracted three times with 95% methanol at 65°C, followed by extractions with petroleum ether and ethyl acetate based on polarity, and purified using SephadexLH-20, MCI-CHP20P, and RP-18, to obtain twenty-eight compounds. Previous research has shown that the total flavonoids of *Potentilla bifurca* could regulate the expression of key enzymes and hormones in glucolipid metabolism. On this basis, six flavonoid compounds were chosen for activity research and analysis.

Cell Culture and IR Stability Investigation

The cell differentiation test was treated using the method[18] with some modifications. At

37°C and 5% CO_2, 3T3-L1 preadipocytes were cultured in DMEM with 10% FBS and 1% Penicillin-Streptomycin liquid. To induce 3T3-L1 adipocytes, 3T3-L1 preadipocytes were cultured in inducer I (complete medium containing insulin, DEX, and IBMX with final concentrations of 10 $\mu g \cdot mL^{-1}$, 1 $\mu mol \cdot L^{-1}$ and 0.5 $mmol \cdot L^{-1}$) for 48 h, then cultured for 48 h with inducer II (complete medium containing only insulin with a final concentration of 10 $\mu g \cdot mL^{-1}$), and then the culture was continued by replacing a piece of complete medium every 1 to 2 days. Starting with the addition of inducer I, the cells were left to differentiate until 8th day, when the cell morphology was observed by microscopy and identified by ORO.

Based on the above-mentioned induced differentiation, IR modeling of the cells was performed. On 8th day, after cell differentiation, the cells were divided into a model group and a blank group. The model group was supplemented with complete medium containing 10^{-6} $mol \cdot L^{-1}$ of insulin, and the blank group was supplemented with equal amounts of complete medium without insulin. The glucose content in the supernatant of the medium was measured using GOD-POD method at 24 h, 48 h, 72 h of modeling. According to the formula calculate the glucose consumption of each group.

$$\text{Glucose content} = S_1/S_2 \times S_3 \quad (1)$$

$$\text{Glucose consumption} = P_1\text{-}P_2 \quad (2)$$

Where S_1, S_2 were the absorbance of the group with samples and standard respectively, S_3 was the standard concentration. Where P_1, P_2 were the glucose concentration of primary and post-test, respectively.

The established IR-3T3-L1 adipocytes were divided into two groups and cultured in insulin-free, high-glucose DMEM. One group was stimulated with 10^{-7} $mol \cdot L^{-1}$ insulin for 30 min at 24 h, 48 h and 72 h, and the other group was not stimulated. The GOD-POD method was used to detect the glucose content in the culture supernatant and calculate the glucose consumption of each group, to observe the stability of IR-3T3-L1 adipocytes, and to investigate whether the established IR-3T3-L1 cell model could maintain the IR state stably for a long time.

Cytotoxicity Test

Cytotoxicity test was measured using the method[19] with some modifications. The groups were grouped according to blank group and experimental group. 3T3-L1 preadipocytes were seeded (1×10^4 cells/well) in 96-well plates and cultured in an incubator for 24 h at 37°C, 5% CO_2. The old culture medium was poured off after and replaced with PBS. The cells were washed twice, and then 100 µL of complete culture solution containing *Potentilla bifurca* compounds (100 $\mu mol \cdot L^{-1}$, 10 $\mu mol \cdot L^{-1}$, 1 $\mu mol \cdot L^{-1}$) was added. After 24 h of intervention, 10 µL of CCK-8 solution was added to each well. After 1 h of incubation, the absorbance of each well was measured at 450 nm wavelength of the microplate reader. Cell viability was calculated and the concentrations of compounds were determined for experiments.

$$\text{Cell viability} = A_e/A_b \times 100\% \quad (3)$$

Where A_e and A_b were the absorbance of the group with experimental and blank group.

Effects of Potentilla Bifurca Compounds on Lipid Droplet Accumulation

The lipid droplet accumulation was assayed in accordance using the method[20] with some modifications. 3T3-L1 preadipocytes were seeded (1×10^5 cells/well) in 24-well plates and set up a normal group, a model group and an experimental group. After the cells were cultured in an incubator at 37°C, 5% CO_2 until they were fully fused, the normal group was not induced, and the model and experimental groups were induced to differentiate according to the methods in 2.3. 10 $\mu mol \cdot L^{-1}$ of *Potentilla bifurca* compounds were added along with the inducer to intervene, and on the 8th day after the end of induction, the cells were stained with ORO and placed under the microscope for observation and photograph.

Effects of Potentilla Bifurca Compounds on Glucose Uptake in IR-3T3-L1 Adipocytes

Glucose uptake was assayed in accordance using the method[21] with some modifications. The 3T3-L1 preadipocytes at logarithmic growth stage were seeded (2×10^6 cells/well) in 6-well plates to establish a blank group, a model group and an experimental group, and after the cells were completely fused, induced to differentiate according to the methods in 2.3. On the 8th day of induction differentiation, the blank group continued to be cultured with complete medium, and the model and experimental groups were mod-

eled with 10^{-6} mol·L^{-1} of insulin. After reaching the optimal modeling time, the blank and model groups were cultured with complete medium, while the experimental group was intervened with 10 μmol·L^{-1} of each compound. Both the blank group and the model group were divided into two groups after 48 h, one of which was stimulated with 10^{-7} mol·L^{-1} of insulin for 30 min, and the other group was not stimulated. The experimental group was treated identically after the treatment was completed, the glucose content in the supernatant of the medium was measured using GOD-POD method and according to the formula (1), (2), the glucose consumption of each group was calculated.

Effects of Potentilla Bifurca Compounds on the Content of TG in 3T3-L1 Preadipocyte Differentiation

3T3-L1 preadipocytes which were divided into blank group, model group, and experimental group, were seeded (1×10^5 cells/well) in 24-well plates. Induction differentiation was performed according to the method in section 2.3. The cells were cultured at 37°C in 5% CO_2 incubator until complete fusion. 10 μmol·L^{-1} of *Potentilla bifurca* compounds were added along with the inducer to intervene, and on the 8th day after the end of induction, the content of TG in each group was determined. The operations are as follows:

1. Cells collection: The old cell culture was aspirated off on the 8th day of induction differentiation, washed twice with PBS, digested for 5-10 min, and then the cells were transferred to centrifuge tubes for centrifuging at 1000 rpm·min^{-1} for 5-10 min, and the supernatant was discarded. Cellular deposits with PBS were washed twice, centrifuged again, leaving cellular deposits. The cell lysate was added to the centrifuge tubes containing cellular deposits, and the lysed cell liquid was measured without centrifugation.
2. Determination of TG content: The samples were added according to the system as follows: the blank hole (2.5 μL distilled water+250 μL Fluid), the standard hole (2.5 μL Calibrator+250 μL Fluid), and the sample hole (2.5 μL Specimen+250 μL Fluid)

The samples were added and mixed evenly, and incubated in a microplate incubator at 37°C for 10 minutes. The absorption values of each hole were measured with a microplate card reader. The concentration of protein in the sample solution was determined with the BCA kit and protein correction was carried out.

BCA assay protein quantification: 1- Preparation of BCA working liquid: The required amount of BCA working liquid was calculated based on the standard product and the quantity of sample that was measured. The BCA working liquid was prepared according to the ratio of 50:1, shake and mix well. It can be stabilized 24 h at room temperature. 2- Dilution standard: The standard was diluted with PBS to a concentration of 0.5 mg·mL^{-1}. 0, 2, 4, 6, 8, 12, 16 and 20 μL were added to the protein standard wells of the 96-well plate, and a certain amount of PBS was added to 20 μL in each well. Also, 2 μL of sample was taken and added to the sample hole, and then PBS was added to 20 μL. 3- Add working liquid: Following the addition of the samples, 200 μL of BCA working liquid was added to each well, incubated at 37°C for 15-30 min, and the absorbance value at 562 nm was measured using a microplate reader. The X-axis represents the protein concentration of the standard wells, and the Y-axis represents the corresponding OD value. The protein concentration of the sample was calculated using a standard curve.

$$\text{TG content} = (OD_3\text{-}OD_1)/(OD_2\text{-}OD_1) \times C_1/C_2 \quad (4)$$

Where OD_1, OD_2, OD_3 were the absorbance of the group with the blank group, standard group, sample group, where C_1, C_2 were the concentration of the group with calibration product and sample protein.

Effects of Potentilla Bifurca Compounds on IL-6 Content in IR-3T3-L1 Adipocytes

Cell culture, induction of differentiation, modeling and drug delivery methods were referred to the methods in 2.6. The IL-6 content in the supernatant of IR-3T3-L1 adipocytes was measured with the IL-6 kit after the treatment was completed.

The Expression of Protein Factors in the PI3K-Akt Pathway Using Western Blot

Firstly, the total protein was extracted using RIPA lysis buffer containing 1% PMSF, and the protein concentration was determined by the BCA method. Electrophoresis is performed using polyacrylamide gel with an 8% sodium sulphate concentration after loading the same amount of

protein (60 g) into each hole. Secondly, the protein treated by electrophoresis were transferred to polyvinylidene fluoride (PVDF) membrane, which was infiltrated with 5% skim milk powder solution for 1 h. Then, the protein was incubated at 4°C with the primary antibody diluent for 8h, and then, incubated with the corresponding secondary antibody diluent for 1h. Western blotting was then performed using the hypersensitive enhanced image analysis system. The chemiluminescent substrate kit was released and can be obtained on the FluorChem E system. Finally, AlphaView software is used to quantify the corresponding bands.

Results

Separation and Preparation of Six Compounds

The results are shown in Figure 1. The resulting compounds were identified as: quercetin-3-O-β-D-glucopyranoside (QC3GP), myricetin (MC), quercetin-3-O-β-D-xylose (QC3X), quercetin-3-O-(6''-O-trans-p-coumaroyl)-β-D-glucoside (QC3G), quercetin (QC), (-)-epicatechin (EC). Compared with QC, the H of hydroxyl at position 3' of QC3GP, QC3X and QC3G were replaced by different sugar groups, and the hydroxyl of position 3', 4' was transferred to the position 4',5'. Compared with QC, a hydroxyl was added to the position 5' in MC's molecular structure, the hydroxyl in EC's B ring was transferred from the position 3', 4' to the position 4', 5', and the ketone carbonyl in EC's C ring was replaced by H, and the bond angle between the hydroxyl in the C ring and the bond between the C ring and the B ring was deflected.

Establishment of IR Model

In order to ensure the accuracy of the experiment, the stability of the modeled cells was investigated. At 24 h and 48 h, the glucose uptake capacity in the model group was lower than that in the blank group, the data difference was significant ($p<0.05$). After insulin stimulation, the glucose uptake capacity in the blank group increased significant, the data difference was extremely significant ($p<0.01$), but the glucose uptake capacity in the model group remained unchanged, the data difference was not significant ($p>0.05$). This indicates that the cells in the model group were still insensitive to insulin at this time (Figure 2A and B). Glucose uptake capacity in the model group began to rise at 72 h of model establishment and the 3T3-L1 adi-

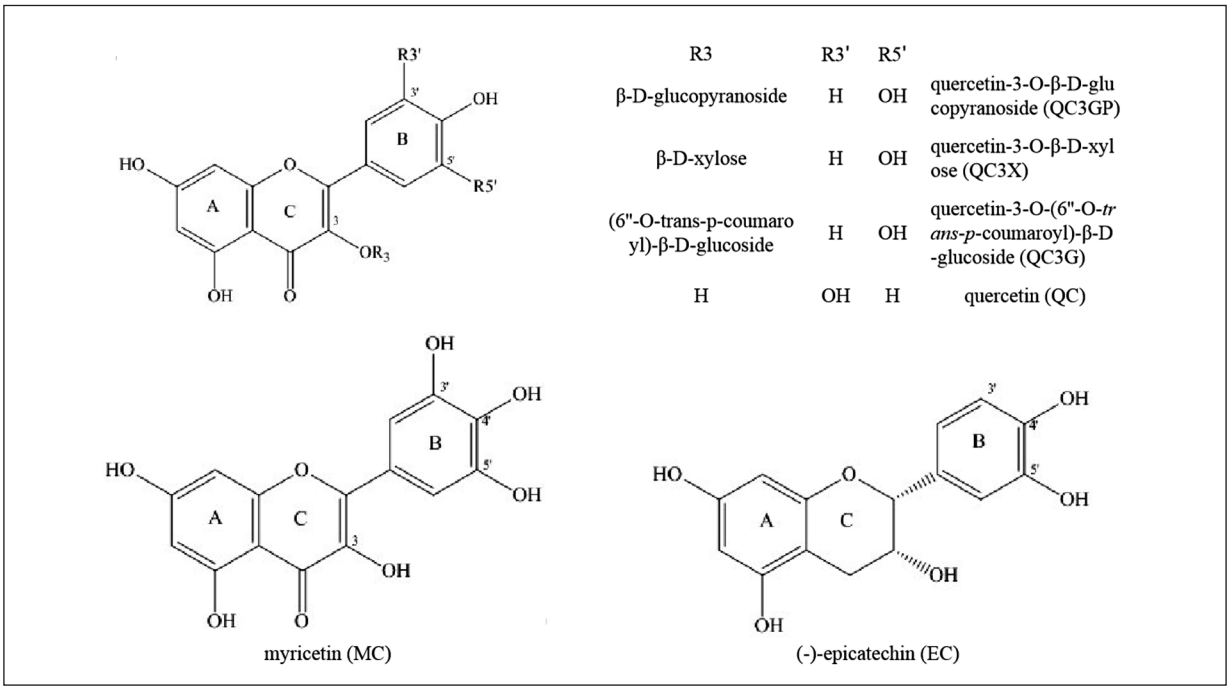

Figure 1. The molecular structure of six compounds. The six compounds belong to polyphenols, among which QC3GP, QC3X, QC3G, QC and MC belong to flavonoids.

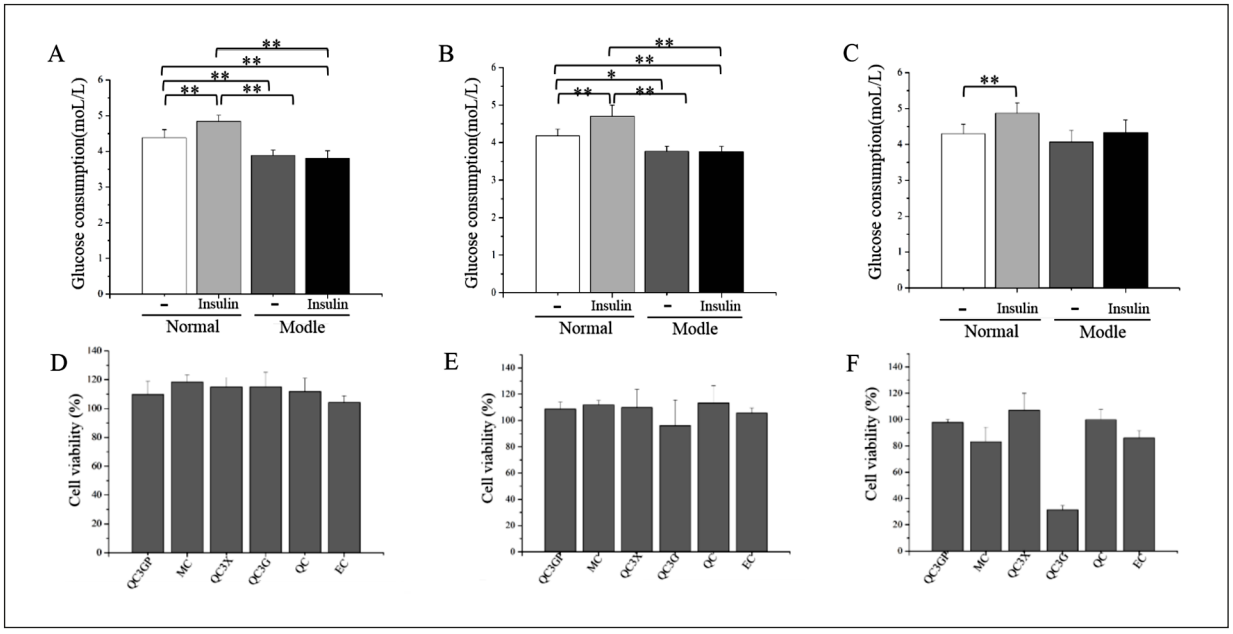

Figure 2. The stability of insulin resistance in cells was investigated by measuring glucose uptake. **A,** Study on the stability of 24 h after insulin stimulation. **B,** Study on the stability of 48 h after insulin stimulation. **C,** Study on the stability of 72 h after insulin stimulation. Cells viability of compounds were measured using CCK-8 ki. **D,** 1 μmol·L^{-1} Toxicological examination of compounds. **E,** 10 μmol·L^{-1} Toxicological examination of compounds. **F,** 100 μmol·L^{-1} Toxicological examination of compounds. Results are shown as mean $\bar{x}$ ± SD. $^{**}p < 0.01$.$^{*}p < 0.05$.

pocytes became insulin-sensitive (Figure 2C). Therefore, in the establishment of the 3T3-L1 adipocyte IR cell model, the condition was with a concentration of 10^{-6} mol·L^{-1} of insulin treated for 48 h.

Cytotoxic Experiments

When the concentration was 100 μmol·L^{-1}, the cell viability of 3T3-L1 preadipocytes was relatively low under the intervention of six compounds, and the cell activity was less than 40% after QC3G intervention (Figure 2F). When the concentration was 10 μmol·L^{-1} or less, the cell viability of 3T3-L1 preadipocytes reached more than 90% (Figure 2D and E). The findings demonstrated that the concentration is safe in the case of 10 μmol·L^{-1}, so the concentration of 10 μmol·L^{-1} is selected for the follow-up test.

The Lipid Metabolism Research

To further explore the effect of *Potentilla bifurca* compounds on T2DM, the effects of *Potentilla bifurca* compounds on obesity was studied, mainly include oil red O staining observation, glucose uptake capacity determination, TG content determination, IL-6 content determination. The results are as follows:

Effects of Lipid Droplet Accumulation

The shape of 3T3-L1 preadipocytes is typically shuttle-shaped or irregularly triangular with no lipid droplets in the cytoplasm. After the addition of the inducer, the cells gradually became larger, changing from spindle or polygonal to round or oval, and by 6-8 d of differentiation, the cells gradually differentiated into adipocytes containing small reflective lipid droplets. After staining with ORO, red "ring-like" lipid droplets were observed under the microscope, indicating that the 3T3-L1 preadipocytes differentiated into mature adipocytes after induced differentiation.

As shown in Figure 3A, there were no "ring-shaped" lipid droplets in the normal group, indicating that the cells in the normal group were undifferentiated or the degree of differentiation was relatively low, while a large number of lipid droplets appeared in the model group. Compared with the model group, the number of lipid droplets reduced after the compound's intervention. This indicates that all six compounds can reduce the accumulation of lipid droplets during the differentiation of 3T3-L1 adipocytes.

Effects of Glucose Uptake Capacity

Under normal conditions, insulin can promote glucose uptake and utilization, reduce blood sug-

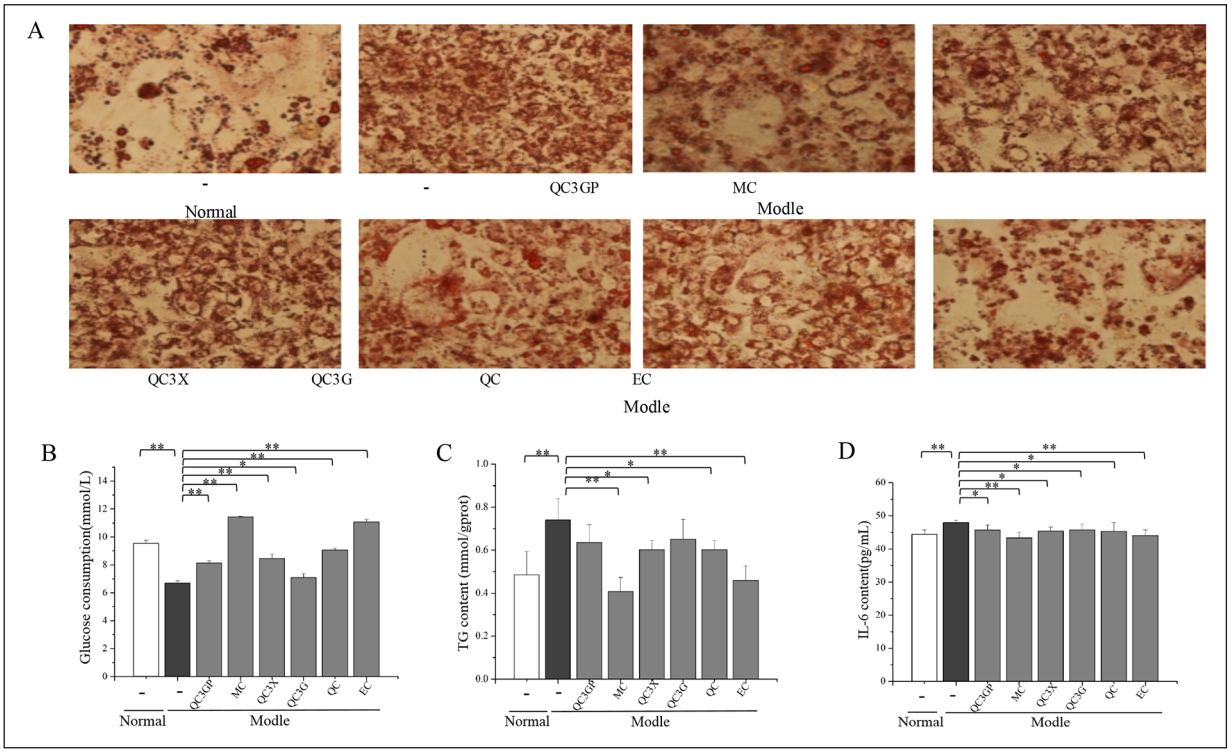

Figure 3. The lipid metabolism of insulin resistance in cells was investigated. **A,** Study was observed by oil red O staining on the the effect of 3T3-L1 adipocytes under different compounds (100×). **B,** Study on the improvement of glucose uptake in IR by Potentilla bifurca compounds. **C,** Study on the improvement of TG content in IR by Potentilla bifurca compounds. **D,** Study on the improvement of IL-6 content in IR by Potentilla bifurca compounds; Results are shown as mean $\bar{x} \pm$ SD. $^{**}p < 0.01$.$^{*}p < 0.05$.

ar. IR is insulin action not sensitive and related to the decline of the human body's action on insulin. Glucose intake is the most intuitive reflection of insulin resistance. Therefore, IR patients are usually associated with disorders of glucose metabolism. IR-3T3-L1 adipocytes were used to evaluate the improvement of IR by six compounds. After intervention with six compounds, the glucose uptake capacity of IR-3T3-L1 adipocytes was determined by glucose kit. Compared with the blank group, glucose uptake was significantly reduced in the model group, the data difference was extremely significant ($p<0.01$), indicating that the IR model was successfully modeled. After the compound's intervention, glucose uptake was increased to varying degrees. The data differences between QC3GP, MC, QC3X, QC, EC and the model group were extremely significant ($p<0.01$), and the data differences between QC3G and the model group were significant ($p<0.05$) (Figure 3B). The results showed that the *Potentilla bifurca* compounds increased the glucose uptake capacity of IR-3T3-L1 adipocytes and improved IR status, which has potential for the treatment of diabetes mellitus.

TG Content Determination

Compared with the model group, all six compounds reduced the TG content in cells after the compound intervention. The data differences in MC and EC were extremely significant ($p<0.01$), significant ($p<0.05$) in QC3X and QC, and not significant ($p>0.05$) in QC3GP and QC3G (Figure 3C).

IL-6 Content Determination

Compared with the blank group, the model group had significantly higher IL-6 levels, the data difference was extremely significant ($p<0.01$), indicating that the IR model was successfully modeled. All six compounds can reduce the IL-6 content of IR-3T3-L1 adipocytes after the intervention. The data differences in MC and EC were extremely significant ($p<0.01$), and significant ($p<0.05$) in QC3GP, QC, QC3X and QC3G (Figure 3D).

PI3K/AKT Signaling Pathway Research

The Protein Expression of p-Akt /Akt

Akt which is an extremely important protein in insulin signaling pathway is a very important factor in the PI3K/Akt pathway. Its phosphorylation is closely related to glycolipid metabolism[22]. In this study, the IR-3T3-L1 adipocyte model was used to intervene with the *Potentilla bifurca* compounds to extract cell protein, and Western blot was used to determine the expression of p-Akt/Akt protein in each group of cells. As shown in Figure 4A and C, p-Akt/Akt content in the model group was lower than that in the blank group. After the compound's intervention, the p-Akt/Akt ratio of all compounds to IR-3T3-L1 adipocytes increased to varying degrees. Compared to the model group, the data differences in the group of MC, QC3X, QC, EC were extremely significant ($p<0.01$), and in the group of QC3GP, QC3G were not significant ($p>0.05$).

The Protein Expression of IKKβ and p-NF-κBp65/NF-κBp65

The expression of IKKβ protein in the IR-3T3-L1 adipocyte condition was determined. IKKβ content of the model group was higher than that of the blank group, indicating successful modeling. After the compound intervention, compared with the model group, all six compounds can reduce the IKKβ protein expression content of IR-3T3-L1 adipocytes, the data difference was significant ($p<0.05$). The results suggested that *Potentilla bifurca* compounds may regulate related inflammatory signaling pathways by reducing IKKβ protein content in IR-3T3-L1 adipocytes (Figure 4B and D).

In this study, the content of p-NF-κBp65/NF-κBp65 in IR-3T3-L1 adipocytes was measured after the intervention of the *Potentilla bifurca* compounds. After calculating the ratio, all six compounds can reduce the content of p-NF-κBp65/NF-κBp65. Compared with the model group, the data difference in MC was extremely significant ($p<0.01$). The differences between the group of QC3GP, QC, QC3X, QC3G, EC and the model group were significant ($p<0.05$), indicating that *Potentilla bifurca* compounds may regulate the inflammatory response pathway by lowering p-NF-κBp65/NF-κBp65 levels (Figure 4B and E).

Discussion

The flavonoid skeleton is composed of (C6-C3-C6) and is the plant secondary metabolite

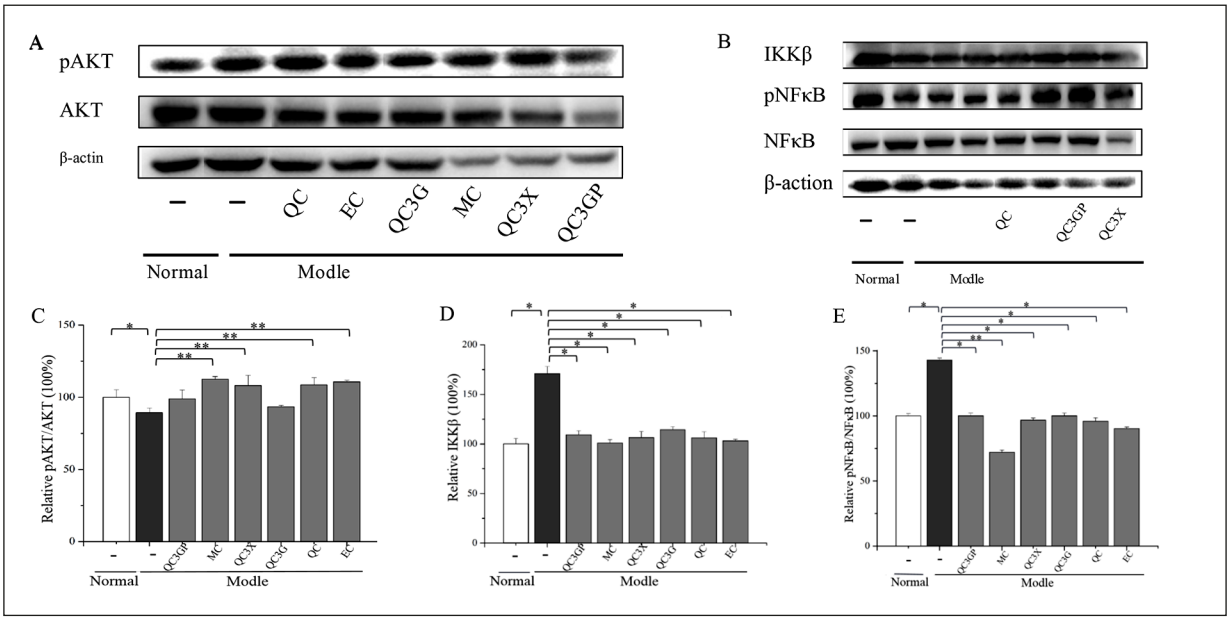

Figure 4. The expression of major proteins in PI3K/AKT signal pathway. **A, B,** Results of related protein expression, the data were determined by Western blotting. **C,** Study on the improvement of pAKT/AKT in IR by *Potentilla bifurca* compounds. **D,** Study on the improvement of IKKβ in IR by *Potentilla bifurca* compounds. **E,** Study on the improvement of p-NF-κBp65/NF-κBp65 in IR by *Potentilla bifurca* compounds. Results are shown as mean $\bar{x} \pm$ SD. $^{**}p < 0.01$.$^{*}p < 0.05$.

with a strong therapeutic effect on diabetes. It can be sub-classified into six distinct types consisting of flavonols, flavones, flavan-3-ols, anthocyanidins, flavanones, and isoflavones[23]. Glycosylation is one of the major modifications of flavone and is mainly composed of two types of glycosylation: O-glycosylation and C-glycosylation, which play vital roles in many physiological characteristics and functions of plants[24]. Studies have shown that quercetin glycosides, flavone derivatives, and catechins have been addressed to exploit antioxidant and pharmacological properties[25]. In addition, there is evidence that the absorption of glycosides is much higher than that of the corresponding aglycones[26].

In a previous study, the model of IR induced by the revulsant was investigated to determine the optimal time of induction and the cytotoxicity of each compound was explored. The compounds were most cytotoxic at a cell concentration of 100 μmol·L^{-1}, and on this basis, we determined the following experiments with a 10 μmol·L^{-1} concentration. The most significant feature of IR is the increase in blood glucose levels. The six compounds showed significant results in promoting blood glucose consumption, the most obvious of which were myricetin, epicatechin, and quercetin. The quercetin glycoside derivatives were not obvious compared with them. This has been apparent to confirm the hypoglycemic effect of *Potentilla bifurca*. To make the experimental data more reliable, the WB method was used to detect protein expression in the PI3K/AKT signaling pathway. The phosphatidylinositol3-kinase (PI3K)/protein kinase B (Akt) signaling pathway is a crucial pathway in insulin signaling[27-29]. Firstly, in the PI3K/AKT insulin signaling pathway, insulin stimulation alters PI3K signaling in the case of IR. Insulin binds to its receptor and induces insulin receptor substance (IRS) phosphorylation at the tyrosine site, thereby activating PI3K, which subsequently activates protein kinase AKT, ultimately activating glucose transporter-4 (GLUT-4), which plays an important role in mobilizing glucose throughout the body[30]. To investigate the effect of *Potentilla bifurca* on the PI3K/AKT signaling pathway, AKT protein downstream of the pathway was determined. Akt, also known as protein kinase B (PKB), is a central node in cell signaling downstream of growth factors, cytokines, and other cellular stimuli. At the same time, Akt activation underlies the pathophysiological properties of a variety of complex diseases, including T2DM[31]. Combined with the observations made in the present study (Figure 4C), the intensity of increase in pAKT/AKT expression by the six compounds was MC > EC> QC > QC3X > QC3GP > QC3G.

Much evidence supports the view that low-grade chronic inflammation exists in obese people[32]. Obese people are generated locally in the adipose tissue and enter into the bloodstream with the release of numerous pro-inflammatory cytokines[33]. Some research showed that insulin resistance in mice lacking pro-inflammatory factors was improved despite high fat diets[34]. The prevalence of T2DM increases along with obesity[35]. Therefore, obesity-induced insulin resistance can be ameliorated through the reduction of inflammation.

In addition to studying the improvement of diabetes by the six compounds, the experiment also explored lipid metabolism pathways associated with inflammation. The IKKβ/NF-κB signaling pathway in the hypothalamus induced by an HFD was causative for the development of obesity[36]. First of all, six compounds were investigated for their ability to improve the appearance of 3T3-L1 adipocytes. It was found that all six compounds could reduce the formation of lipid droplets in adipocytes, but the difference in effect between the compounds was not significant. Six compounds of *Potentilla bifurca* can also reduce the TG content in 3T3-L1 adipocytes compared with the model group, among which the data differences in MC and EC were extremely significantly (p<0.01), QC and QC3X were significantly (p<0.05), and QC3GP was not significantly (p>0.05). Secondly, this research focused further on the protein expression and inflammatory factor content in the IKKβ/NF-κB pathway. The IKK complex is an activator of the NF-κB pathway and thus regulates inflammation, while IKKβ is one of the most important proteins. Glucolipotoxicity activates inflammatory signals such as nuclear factor-κB (NF-κB), interleukin-1β (IL-1β), IL-6, interferon-γ (IFN-γ) and tumor necrosis factor-alpha (TNF-α) in experimental conditions, impairing insulin secretion. Combined with the observation in the present study (Figure 4D), all six compounds were able to reduce the content of IKKβ, among which the MC, QC and EC effects were the most obvious, and the effect of the four glycosides QC was slightly weaker. In addition, this paper also studies the expression of NF-κB protein in the downstream pathway of IKKβ/NFκB. In the unstimulated state, NF-κB remains in the cytoplasm in an inactive form and

is bound to the inhibitory binding protein IκBα[37]. A broad range of immune stimuli can activate the upstream kinase IKKβ, leading to IκBα phosphorylation (at Ser32 and Ser36) and degradation, and subsequently to the release of NF-κB[37]. The expression of downstream pathways under the action IKKβ/NFκB six compounds was explained by measuring the ratio of p-NF-κBp65/NF-κBp65. The p-NF-κBp65/NF-κBp65 of MC reduction was the most significant, followed by EC, QC, and subsequent glycosidic bonds. The activated NF-κB stimulates the products of many inflammatory regulators, including IL-6 and TNF-α, which eventually lead to inflammation. NF-κB is transported to the nucleus, binds to genomic DNA in the nucleus and regulates the expression of inflammatory cytokines, initiates or aggregates inflammatory reactions, and ultimately leads to the occurrence of IR[38]. The IL-6 kit was used to determine the effect of compounds on the inflammatory response of IR-3T3-L1 adipocytes, as shown in Figure 3C. Compared with the model group, MC and EC can significantly reduce the IL-6 content in cells, the data differences were extremely significant ($p<0.01$), and the differences in QC, QC3X, QC3GP, and QC3G were significant ($p<0.05$).

Among the six compounds, MC has the most significant effect. Compared with QC, MC has a hydroxyl group at the 5'position, which improves the ability to bind to other compounds and significantly enhances its lipid-lowering and hypoglycemic activities. Compared with QC, the B ring connected to the C ring and the hydroxyl bond connected to the C ring both rotate, which makes the lipid-lowering and hypoglycemic activity of EC enhanced compared to QC, QC3X, QC3GP, and QC3G are all glycoside derivatives of QC. Compared with QC, the activities of QC3X, QC3GP, and QC3G are reduced. Among them, QC3X is the strongest, followed by QC3GP, and finally QC3G. This shows that the factors affecting QC activity are not only related to the added glycosidic bonds but also related to the complexity of the added glycosidic bonds.

Although this study is of great positive significance for the study of the treatment of type 2 diabetes mellitus, the current research is only at the *in vitro* level, and the experimental data has certain limitations, so it is planned to be used in animal experiments in the next step, in order to further verify the value of *Potentilla bifurca* compounds in the treatment of type 2 diabetes.

Conclusions

The improvement effect of *Potentilla bifurca* compounds on high-fat diet-induced IR in 3T3-L1 adipocytes was studied and the possible mechanisms were explored. At the same time, the differences in the structures of flavonoids and their effects on the metabolism of glucose and lipids were discussed. The results showed that IR could be improved by *Potentilla bifurca* compounds through activating the IRS/PI3K/AKT signal pathway to accelerate glucose consumption and inhibiting the IKKβ/NFκB signal pathway to improve inflammatory pathways. Based on quercetin, the difference between the changes in the structure of the five compounds and the lowering of blood sugar and lipids was analyzed. The study suggested that *Potentilla bifurca* has the potential to be developed as a hypoglycemic agent in regulating hyperglycemia and IR in T2DM.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Funding

This work was supported by the Natural Science Foundation of Qinghai Province (2020-ZJ-918), the Innovative Team of Comprehensive Utilization and Development of Medicinal Plant Resources (2020-2023) and from the Key Laboratory of Qinghai Province on Plant and Animal Resources in Tibetan Plateau (2020-ZJ-Y40).

Ethical Approval

Not required.

Informed Consent

Not required.

ORCID ID

Yang Zeng: https://orcid.org/0000-0003-0370-4719.

References

1) Kumar S, Kumar V, Prakash Om. Enzymes inhibition and antidiabetic effect of isolated constituents from Dillenia indica. Biomed Res Int 2013; 2013: 382063.
2) Wang X, Wang S, Sun L, Qin G. Prevalence of diabetes mellitus in 2019 novel coronavirus: A me-

ta-analysis. Diabetes Res Clin Pract 2020; 164: 108200.

3) Zaharudin N, Staerk D, Dragsted LO. Inhibition of α-glucosidase activity by selected edible seaweeds and fucoxanthin. Food Chem 2019; 270: 481-486.
4) Wang Z, Sun L, Fang Z, Nisar T, Zou L, Li D, Guo Y. Lycium ruthenicum Murray anthocyanins effectively inhibit α-glucosidase activity and alleviate insulin resistance. Food Biosci 2021; 41: 100949.
5) Kwon EY, Lee J, Kim YJ, Do A, Choi JY, Cho SJ, Jung UJ, Lee MK, Park YB, Choi MS. Seabuckthorn Leaves Extract and Flavonoid Glycosides Extract from Seabuckthorn Leaves Ameliorates Adiposity, Hepatic Steatosis, Insulin Resistance, and Inflammation in Diet-Induced Obesity. Nutrients 2017; 9: 569.
6) Kang YE, Kim JM, Joung KH, Lee JH, You BR, Choi MJ, Ryu MJ, Ko YB, Lee MA, Lee J, Ku BJ, Shong M, Lee KH, Kim HJ. The Roles of Adipokines, Proinflammatory Cytokines, and Adipose Tissue Macrophages in Obesity-Associated Insulin Resistance in Modest Obesity and Early Metabolic Dysfunction. PLoS One 2017; 11: 0154003.
7) Shen S, Chang W, Chang C. Fraction from wax apple Syzygium samarangense (Blume) Merrill and Perry] fruit extract ameliorates insulin resistance via modulating insulin signaling and inflammation pathway in tumor necrosis factor α-treated FL83B mouse hepatocytes. Int J Mol Sci 2012; 13: 8562-8577.
8) Wang H, Li Q, Yu N, Chen W, Zha X, Wu D, Pan L, Duan J, Luo J. Dendrobium huoshanense polysaccharide regulates hepatic glucose homeostasis and pancreatic β-cell function in type 2 diabetic mice. Carbohydr Polym 2019; 211: 39-48.
9) Wu JJ, Shi SS, Wang HJ, Wang SC. Mechanisms underlying the effect of polysaccharides in the treatment of type 2 diabetes: A review. Carbohydr Polym 2016; 144: 474-494.
10) Wu H, Eduardo E, Valentina T, Tanweer KM, Robert C, Louise MH, Marcus S, Lisa M, Matteo S, Mercè PF, Gemma X, Josep M, David T, Rémy B, Wifredo R, Rosie P, José Manuel FR, Fredrik B. Metformin alters the gut microbiome of individuals with treatment-naive type 2 diabetes, contributing to the therapeutic effects of the drug. Nat Med 2017; 23: 850-858.
11) Moradi-Marjaneh R, Paseban M, Sahebkar A. Natural products with SGLT2 inhibitory activity: Possibilities of application for the treatment of diabetes. Phytother Res 2019; 33: 2518-2530.
12) Teoh Seong Lin, Das Srijit. Phytochemicals and their effective role in the treatment of diabetes mellitus: a short review. Phytochem Rev 2018; 17: 1111-1128.
13) Khaksary MM, Mehdi SM, Reza R, Reza TM, Kaveh K. Quercetin improved histological structure and upregulated adiponectin and adiponectin receptors in the placenta of rats with gestational diabetes mellitus. Placenta 2021; 106: 49-57.
14) Zhuang M, Qiu H, Li P, Hu L, Wang Y, Rao L. Islet protection and amelioration of type 2 diabetes mellitus by treatment with quercetin from the flowers of Edgeworthia gardneri. Drug Des Devel Ther 2018; 12: 955-966.
15) Liu C, Ma J, Sun J, Cheng C, Feng Z, Jiang J, Yang W. Flavonoid-Rich Extract of Paulownia fortunei Flowers Attenuates Diet-Induced Hyperlipidemia, Hepatic Steatosis and Insulin Resistance in Obesity Mice by AMPK Pathway. Nutrients 2017; 9: 959.
16) Chen CC, Kuo CH, Leu YL, Wang SH. Corylin reduces obesity and insulin resistance and promotes adipose tissue browning through SIRT-1 and β3-AR activation. Pharmacol Res 2021; 164: 105291.
17) Samatadze TE, Zoshchuk SA, Khomik AS, Amosova AV, Svistunova NY, Suslina SN, Hazieva FM, Yurkevich OY, Muravenko OV. Molecular cytogenetic characterization, leaf anatomy and ultrastructure of the medicinal plant Potentilla alba L. Genet Resour Crop Evol 2018; 65: 1637-1647.
18) Li Y, Chen S, Sun J, Yu Y, Li M. Interleukin-38 inhibits adipogenesis and inflammatory cytokine production in 3T3-L1 preadipocytes. Cell Biol Int 2020; 44: 2357-2362.
19) Bais S, Patel NJ. In vitro anti diabetic and anti obesity effect of J. communis extract on 3T3L1 mouse adipocytes: A possible role of MAPK/ERK activation. Obes Med 2020; 18: 100219.
20) Kim MS, Kim JK, Kim HJu, Moon SR, Shin BC, Park KW, Yang HO, Kim SM, Park R. Hibiscus extract inhibits the lipid droplet accumulation and adipogenic transcription factors expression of 3T3-L1 preadipocytes. J Altern Complement Med 2003; 9: 499-504.
21) Yan F, Dai G, Zheng X. Mulberry anthocyanin extract ameliorates insulin resistance by regulating PI3K/AKT pathway in HepG2 cells and db/db mice. J Nutr Biochem 2016; 36: 68-80.
22) Dai S, Hong Y, Xu J, Lin Y, Si Q, Gu X. Ginsenoside Rb2 promotes glucose metabolism and attenuates fat accumulation via AKT-dependent mechanisms. Biomed Pharmacother 2018; 100: 93-100.
23) Hussain T, Tan B, Murtaza G, Liu G, Rahu N, Kalhoro MS, Kalhoro DH, Adebowale T, Mazhar MU, Rehman Z, Martínez Y, Khan SA, Yin YL. Flavonoids and type 2 diabetes: Evidence of efficacy in clinical and animal studies and delivery strategies to enhance their therapeutic efficacy. Pharmacol Res 2020; 152: 104629.
24) Gao J, Ma B, Lu Y, Zhang Y, Tong Y, Guo S, Gao W, Huang L. Investigating the Catalytic Activity of Glycosyltransferase on Quercetin from Tripterygium wilfordii. ACS Omega 2020; 5: 1414-1421.
25) Oleszek M, Pecio Ł, Kozachok S, Lachowska-Filipiuk Z, Oszust K, Frąc M. Phytochemicals of Apple Pomace as Prospect Bio-Fungicide Agents against Mycotoxigenic Fungal Species-In Vitro Experiments. Toxins (Basel) 2019; 11: 361.

26) Cezara ZT, Augustin CM, Tomasz C, Attila B, Ioan T. Sugar matters: sugar moieties as reactivity-tuning factors in quercetin O-glycosides. Food Funct 2020; 11: 5293-5307.

27) Akilavalli N, Mayilvanan C, Balasubramanian K. Ferulic acid exerts its antidiabetic effect by modulating insulin-signalling molecules in the liver of high-fat diet and fructose-induced type-2 diabetic adult male rat. Appl Physiol Nutr Metab 2015; 40: 769-781.

28) Cao C, Zhang B, Li C, Huang Q, Fu X. Structure and in vitro hypoglycemic activity of a homogenous polysaccharide purified from Sargassum pallidum. Food Funct 2019; 10: 2828-2838.

29) Kim C, Lee J, Kim MB, Hwang JK. Hypoglycemic effect of whole grain diet in C57BL/KsJ- db/db mice by activating PI3K/Akt and AMPK pathways. Food Sci Biotechnol 2019; 28: 895-905.

30) Zhou M, Ren P, Li S, Kang Q, Zhang Y, Liu W, Shang J, Gong Y, Liu H. Danhong Injection Attenuates High-Fat-Induced Atherosclerosis and Macrophage Lipid Accumulation by Regulating the PI3K/AKT Insulin Pathway. J Cardiovasc Pharmacol 2019; 74: 152-161.

31) Manning BD, Cantley LC. AKT/PKB Signaling: Navigating Downstream. Cell 2007; 129: 1261-1274.

32) Klop B, Elte J, Cabezas M. Dyslipidemia in Obesity: Mechanisms and Potential Targets. Nutrients 2013; 5: 1218-1240.

33) Dludla PV, Nkambule BB, Jack B, Mkandla Z, Mutize T, Silvestri S, Orlando P, Tiano L, Louw J, Sithandiwe E. Mazibuko-Mbeje. Inflammation and Oxidative Stress in an Obese State and the Protective Effects of Gallic Acid. Nutrients 2018; 11: 23.

34) Stuart PW, Deborah H, Reid H, Jacob L, Sarah S, Kris V, Israel C, Rudolph L, Anthony WF. CCR2 modulates inflammatory and metabolic effects of high-fat feeding. J C I 2006; 116: 115-124.

35) Huang F, Chen J, Wang J, Zhu P, Lin W. Palmitic Acid Induces MicroRNA-221 Expression to Decrease Glucose Uptake in HepG2 Cells via the PI3K/AKT/GLUT4 Pathway. Biomed Res Int 2019; 2019: 8171989.

36) Zhang X, Zhang G, Zhang H, Karin M, Bai H, Cai D. Hypothalamic IKKβ/NF-κB and ER Stress Link Overnutrition to Energy Imbalance and Obesity. Cell 2008; 135: 61-73.

37) Jonas B, Goutham KG, Dominik P, Rebecca O, Christiane EK, Karen L, Sigrid S, Carsten C, Lynda MW, Alexander T. Central inhibition of IKKβ/NF-κB signaling attenuates high-fat diet-induced obesity and glucose intolerance. Diabetes 2015; 64: 2015-2027.

38) Yang L, Wang Z, Jiang L, Sun W, Fan Q, Liu T. Total Flavonoids Extracted from Oxytropis falcata Bunge Improve Insulin Resistance through Regulation on the IKKβ/NF-κB Inflammatory Pathway. Evid Based Complementary Altern Med 2017; 2017: 2405124.