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Natural Products for the Treatment of Obesity, Metabolic Syndrome, and Type 2 Diabetes 2016

Guest Editors: Menaka C. Thounaojam, Srinivas Nammi, and Ravirajsinh Jadeja

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Editorial Board

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Editorial

Natural Products for the Treatment of Obesity, Metabolic Syndrome, and Type 2 Diabetes 2016

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The prevalence of obesity, metabolic syndrome, and type 2 diabetes is continuously on rise due to modernization of life style and changing dietary habits. Use of herbal medicines for the treatment of metabolic diseases is a viable option and list of potential candidates is ever expanding. This 2016 edition of this special issue regarding natural products for the treatment of obesity, metabolic syndrome, and type 2 diabetes contains 8 articles accepted from a total of 21 submissions consisting of 6 research articles and 2 clinical studies.

B.-S. Ko et al. reported beneficial effect of a 70% ethanol Korean mistletoe (*Viscum album* coloratum) extract (KME-E) in β-cell function and hepatic insulin sensitivity. KME-E decreased epididymal fat mass by increasing fat oxidation and exhibited greater potentiation of first-phase insulin secretion than the partial pancreatectomized rats. KME-E also increased β-cell mass by increasing β-cell proliferation and decreasing its apoptosis. In the *in vitro* studies, betulin potentiated insulin-stimulated glucose uptake via increased PPAR-γ activity and insulin signaling in 3T3-L1 adipocytes, whereas oleanolic acid enhanced glucose-stimulated insulin secretion and cell proliferation in insulinoma cells. The authors concluded that ethanolic extract of KME has more beneficial potential than its aqueous extract.

In another study, antidiabetic and hypolipidemic potential of antcin K, a triterpenoid isolated from *Antrodia camphorata*, was evaluated. It was observed that AnK-treated mice had significantly lowered blood glucose, triglyceride, total cholesterol, and leptin levels. Further, antihyperglycemic and antihypertriglyceridemic effects of AnK were comparable to metformin and fenofibrate, respectively. AnK-induced phosphorylation of AMP-activated protein kinase (phospho-AMPK) expression in the muscle and liver resulted in significantly increased skeletal muscular membrane expression of glucose transporter 4 (GLUT4) and decreased hepatic glucose-6-phosphatase (G6Pase) mRNA levels. Furthermore, AnK treatment inhibited hepatic fatty acid synthase (FAS) and sterol response element binding protein-1c (SREBP-1c) levels and enhanced peroxisome proliferator-activated receptor α (PPARα) expression.

H.-Y. Jung et al. evaluated efficacy of a polyherbal formulation (containing *Fomitopsis pinicola*, *Acanthopanax senticosus*, *Viscum album*, and *Allium tuberosum*), against high-fat diet- (HFD-) induced obesity. Treatment of HFD fed mice with this polyherbal formulation for 12 weeks reduced body and white adipose tissue (WAT) weights and occurrence of fatty liver. Additionally, the polyherbal formulation reduced serum lipids, leptin, and insulin levels along with hepatic lipids. It also suppressed lipogenic mRNA expression levels in WAT.

Y. Zhang et al. used metabolomic approach to evaluate efficacy of isoflavones rich extract of *Radix Puerariae* in HFD + streptozotocin-induced diabetes in rats. Eleven potential metabolite biomarkers related to coagulation, lipid metabolism, and amino acid metabolism were identified. In another study, the effect of *Miconia* sp. extract on mRNA expression of PPARγ and activity of α-amylase and α-glucosidase were

evaluated. The authors concluded that the ethanolic extract of *Miconia* sp. increased mRNA expression of PPARγ and inhibited α-amylase and α-glucosidase.

A meta-analysis study was conducted by X. Wei et al. to evaluate therapeutic effect of berberine in the treatment of nonalcoholic fatty liver disease (NAFLD). Authors searched Embase, PubMed, Cochrane Library, and so forth until March 2016 for randomized controlled trials using berberine to treat NAFLD. Results from six randomized controlled trials comprising 501 patients showed significant efficacy of berberine in reducing lipids, blood glucose, and HbA1c in NAFLD patients. The authors concluded that berberine has positive efficacy on blood lipids, blood glucose, liver function, insulin resistance, and fatty liver condition of NAFLD patients.

A randomized, double-blinded, double-dummy, active-controlled, and multiple-dose clinical study compared the efficacy and safety of mulberry (*Ramulus Mori*) twig alkaloid tablet and acarbose in individuals with type 2 diabetes mellitus. 24-week treatment with alkaloid extract (SZ-A) and acarbose significantly decreased HbA1c and postprandial plasma glucose levels. However, the fasting plasma glucose levels were not significantly changed in both groups. Interestingly, 1 of 23 patients in SZ-A group (4.76%) and 5 of 15 patients in acarbose group (33.33%) suffered from gastrointestinal adverse events and hence authors concluded that SZ-A tablet is a more effective and safe therapeutic option for glycemic control compared to acarbose, in patients with type 2 diabetes.

J. Tian et al. conducted a clinical retrospective trial to access the efficacy and safety of a Chinese herbal decoction in treating outpatients with type 2 diabetes mellitus (T2DM). A total of 142 diabetes outpatients were enrolled in this clinical retrospective trial. All patients received the decoction for at least 6 consecutive months. Multiple linear regression analysis showed that the change of last visited HbA1c has a significant relationship with the baseline HbA1c, duration of diabetes, and body mass index (BMI). Both fasting and postprandial glucose levels were significantly decreased compared to the baseline. The Chinese herbal decoction also improved islet cell function with decreased HOMA-IR and increased HOMA-β. Triglycerides (TG) and blood pressure (BP) were decreased significantly at months 12 and 6, respectively. During the observation period, one subject developed diabetes kidney disease (DKD) and one developed diabetic peripheral neuropathy (DPN).

Acknowledgments

Firstly we express our sincere thanks and gratitude to the Editorial Board of eCAM for their continuous help in successful publication of this annual issue. We would also like to thank contributors of this annual issue for critical assessment of each paper, their constructive criticisms, and timely response that made this special issue possible.

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Research Article

The Efficacy and Safety of Chinese Herbal Decoction in Type 2 Diabetes: A 5-Year Retrospective Study

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Background. The study was designed to assess the efficacy and safety of Chinese herbal decoction in treating outpatients with T2DM. *Methods*. All patients enrolled received decoction for at least 6 months. The primary outcome was the control rate of HbA1c and the change in HbA1c. FPG, 2hPG, HOMA-IR, and HOMA-β were also collected and evaluated. *Results*. The control rates after treatment at months 6, 12, 18, 24, 36, 48, and 60 were 45.07%, 52.78%, 47.22%, 45.83%, 50.00%, 57.14%, and 40.00%. Multiple linear regression showed the change of HbA1c has a significant relationship with the baseline HbA1c and duration of DM and BMI ($p < 0.05$). Both FPG and 2hPG levels significantly decreased compared to the baseline ($p < 0.05$). Chinese herbal decoction also improved islet cell function with decreased HOMA-IR and increased HOMA-β ($p < 0.05$). 19 and 4 subjects deactivated the antidiabetes drugs or insulin, respectively, after taking decoction. One subject developed DKD and one developed DPN, and another subject showed abnormal liver function which was irrelevant to decoction treatment. *Conclusions*. Chinese herbal decoction significantly enhanced the hypoglycemic action and had certain effect on protecting islet cell function. As a candidate diabetes therapy, it may reduce the use of antidiabetes drugs and slow the progression to diabetes complications.

1. Introduction

Diabetes mellitus (DM) has become an important public health problem worldwide [1, 2]. A recent global study indicated that the prevalence of DM was rising rapidly, particularly in developing countries [3]. With the rapid economic development, elevated standard of living, dietary shifts, lifestyle alterations, and aging, China has the largest number of diabetic patients in the world [4]. Currently the overall prevalence of diabetes was estimated to be 11.6% and the prediabetes was estimated to be 50.1% in Chinese adults [4]. It has been estimated that a total of 12.9 million people died from ischemic heart disease and stroke in the world, while diabetes is the main risk factor [5, 6]. Studies have shown that good glycemic control is critical for patients with type 2 diabetes mellitus (T2DM), since the HbA1c level is correlated with diabetic complications [7, 8]. However, according to the latest survey among 22.31 million Chinese T2DM patients, it was shocking that more than two-thirds could not effectively control their HbA1c levels [9]. Therefore, it is urging to expand the treatment.

Chinese herbal medicine has more than 2000 years of medical practical history. It is an excellent resource for discovering new innovative medications. Previous studies have reported the efficacy and safety of several Chinese herbal medicines that effectively reduced blood glucose and HbA1c levels in diabetic and prediabetic patients [10–13]. Thus, we believe that Chinese herbal medicine may play a role in treating this very common metabolic disease.

Chinese herbal decoction has been widely used in China for diabetes management. However, there is a lack of convincing clinical evidence of glycemic control treated by herbal

decoction, especially for changes in islet cell function. Additionally, the change in dosage of antidiabetes drugs or insulin after combining herbal decoction is rarely reported. Gegen Qinlian Decoction (GQD) is a formulation derived from a classic formula described in the *Treatise on Exogenous Febrile Diseases* over 1000 years ago. GQD formula contains Radix Puerariae, *Coptis chinensis*, Radix Scutellariae, and Radix Liquiritiae. Previous studies have shown that the herbs in GQD could regulate glucose metabolism and its mechanism of action was potentially linked to the improvement of gut microbiota [14]. Antidiabetic effects of GQD formula have been shown in type 2 diabetic rats [15]. We performed this long-term retrospective study to evaluate whether GQD could enhance glycemic control and islet cell function in patients with T2DM whose diabetes were poorly managed.

2. Methods

2.1. Study Design. Study subjects were recruited from outpatients of endocrine department, Guang'anmen Hospital, China Academy of Chinese Medical Sciences, between July 2009 and June 2014.

2.2. Study Population. Outpatients who met the following criteria were eligible for this study: the early type 2 diabetic status of these patients being confirmed according to the diagnosis standard issued by the WHO [16]; receiving Chinese herbal decoction on the main purpose of treating T2DM for at least 6 consecutive months; irrespective of age and sex; having HbA1c $\geq$ 7.0%, the first recorded HbA1c, fasting insulin (FINS), fasting C-peptide (FCP), and other biochemical measurements being considered as the baseline, while keeping records of HbA1c for at least six months; the fact that all the test results should be measured by the same central laboratory; and having stable diet control and programmed daily exercise during the follow-up treatment.

Patient with any of the following conditions was excluded from the study: having complications of diabetes at first treatment; having serious heart, lung, liver, kidney, brain, or other serious complications or those associated with other primary diseases; having diabetic ketoacidosis or serious infections; participating in other clinical trials; and having unstable antidiabetes drugs during medication. Patients who had poor compliance were withdrawn from the study.

The research protocol was approved by the Guang'anmen Hospital Ethics Committee. This research is a retrospective analysis of clinic cases, which focuses on the documental archives of existing clinical patients. The risk to the participants would be no larger than the minimum risk, in that the exemption of informed consent would not cause any adverse effect on the right and health of the participants; the privacy and personal information would also be protected. As we censored, the ethics committee had agreed to exempt the informed consent and approved the launching of this research.

2.3. Intervention. The individual treatment was customized according to the patients' physical conditions on the basis of the standard guide [17]; the dosage of medications remained stable during the first month. Subjects were assessed at each month, while appropriate variation was made in each session according to their measurements and symptoms. Chinese herbal medicine was supplied by Guang'anmen Hospital uniformly. The quality of these herbs and decoction preparation was in accordance with the *Pharmacopoeia of the People's Republic of China* (2005). Subjects took herbal decoction 200 mL two times daily before breakfast and dinner. Each patient should maintain stabilized standard diet and exercise during medication.

For subjects that included oral drugs or insulin therapy throughout the study, the dosages and categories of these medications should be recorded; any change in adjustment and combination related to other diseases should be also recorded at each visit.

2.4. Clinical and Biochemical Measurements. The control rate, which was defined as HbA1c level lower than 7%, and the change in HbA1c values were the primary endpoint [18]. HbA1c was measured using affinity HPLC method (Automatic HA-8160 Analyzer HA-8160, Arkray Factory, Inc., Shiga, Japan). Fingertip blood was collected and FPG was measured using a blood glucose analyzer (ACCU-CHEK Active Meter, Roche Diagnostics, Indianapolis, USA). The 2hPG level was measured after taking a standardized meal (Olympus AU640 Analyzer, Olympus Optical Co., Ltd., Shizuoka, Japan). β-cell function was evaluated from venous blood to determine plasma insulin and C-peptide level, using the homeostatic model assessment (HOMA) to quantify HOMA insulin resistance (HOMA-IR) and β-cell function (HOMA-β) [19].

Blood pressure (BP) was collected at each visit. Total cholesterol (TC), triglycerides (TG), high-density lipoprotein (HDL), and low-density lipoprotein (LDL) were measured by enzymatic methods (Olympus AU640 Analyzer, Olympus Optical Co., Ltd., Shizuoka, Japan).

All the changes in test results and symptoms of each outpatient should be recorded in detail, and we collected the records of the first visit and 6, 12, 18, 24, 36, 48, 60 months from the first visit.

2.5. Effectiveness Evaluation. A comparison of HbA1c levels before and after medication treatment was made. HbA1c < 7.0% was set as the standard normal value. Comparisons of glucose, plasma insulin, C-peptide, lipids, and BP levels were made. The changes in adjustments of combination drugs and the symptoms would also be evaluated. Subjects developed with diabetes complications during follow-up would also be collected and recorded immediately.

2.6. Safety Evaluation. Vital signs were collected at each visit. Routine blood tests, urine tests, stool tests, ECG, hepatic functions (ALT and AST), and renal function (serum creatinine) were also collected. Adverse events were recorded immediately after being reported.

2.7. Statistical Analysis. Statistical analysis was performed via SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). Count data was presented as frequency (proportion). Measurement data was presented as the mean ± SD or SE. Related factors were analyzed using multiple linear regression. Paired t-test was used to analyze the data before and after medication treatment. All statistical tests were two-sided tests. $p < 0.05$ was considered statistically significant.

3. Results

Between July 2009 and June 2014, a total of 142 subjects were enrolled, the baseline was shown in Table 1. There was one subject who developed diabetes kidney disease (DKD) after 12-month treatment and one developed diabetic peripheral neuropathy (DPN) after 22-month treatment, respectively. Among the 72 outpatients who included antidiabetes drugs, there were 3 subjects who added the dosage of drugs, while 42 kept the original, 8 reduced the dosage, and 19 deactivated the drugs during observation period. According to the records, among the 29 outpatients who included insulin, there was 1 subject who added the dosage of insulin, while 17 kept the original, 7 reduced the dosage, and 4 deactivated the insulin after treatment. There were a total of 19 patients who took Chinese herbal decoction alone and kept glycemic controlling well.

3.1. HbA1c Change. After 6-month treatment, The HbAlc decreased by 1.54 ± 0.18%. 45.07% of the subjects had normal HbA1c after treatment, based on the established criteria. At months 12, 18, 24, 36, 48, and 60, the HbA1c decreased by 1.38 ± 0.25%, 1.59 ± 0.34%, 0.86 ± 0.27%, 1.38 ± 0.58%, 2.43 ± 1.27%, and 2.09 ± 1.71%, respectively, and the control rate was 52.78%, 47.22%, 45.83%, 50.00%, 57.14%, and 40.00% (Figure 1).

We also evaluated the relationship of change in last visited HbA1c with the baseline HbA1c, duration of diabetes, BMI, and age measured by multiple linear regression analysis. It showed that the last recorded HbA1c was significantly related to HbA1c baseline ($X1$), duration of diabetes ($X2$), and BMI ($X3$) ($F = 992.032$, $p = 0.000$), and the regression equation was $Y = 0.165X1 + 0.268X2 + 0.011X3$, $R^2 = 0.965$ (Table 2).

3.2. FPG and 2hPG. A total of 142 subjects measured the FPG for 601 times and 93 subjects measured the 2hPG for 294 times during treatment. Compared to the baseline, the last recorded FPG and 2hPG decreased by 1.26 ± 0.28 and 2.34 ± 0.49 mmol/L, respectively ($p < 0.05$). FPG decreased significantly ($p < 0.05$) after intervention at 6, 12, 24, and 60 months and 2hPG decreased significantly ($p < 0.05$) after intervention at 6 and 12 months (Figures 2(a) and 2(b)).

3.3. HOMA-IR and HOMA-β. 113 subjects measured the fasting insulin for 335 times and 80 subjects measured the C-peptide for 234 times. Subjects who included the insulin were excluded from analysis of this part. The longest comparison observation was 57 months, the shortest was 6 months, and the average observation was 18.55 months. Compared to the baseline, the changes in FINS, FCP, HOMA-IR, and HOMA-β among subjects that were last recorded were −1.19 ± 1.05, 0.03 ± 0.07, −0.65 ± 0.48, and −5.29 ± 7.83, respectively. Changes in FINS, FCP, HOMA-IR, and HOMA-β over time were shown in Figures 2(c), 2(d), 2(e), and 2(f).

Stratified analysis was performed according to baseline HOMA-IR. HOMA-IR over 2.69 was regarded as insulin resistance according to Chinese characteristics [20]. There were 70 subjects of pretreatment HOMA-IR > 2.69 (61.95%) and 43 cases of pretreatment HOMA-IR ≤ 2.69 (38.05%).

It was found that the changes in FINS, FCP, HOMA-IR, and HOMA-β among subjects of pretreatment HOMA-IR > 2.69 that were last recorded were −3.99 ± 1.43, 0.41 ± 0.08, −1.87 ± 0.68, and −20.42 ± 10.60, respectively, while the changes in FINS, FCP, HOMA-IR, and HOMA-β among subjects of pretreatment HOMA-IR ≤ 2.69 that were last recorded were 3.39 ± 1.21, −0.01 ± 0.15, 1.42 ± 0.48,

Table 1: Characteristics of study subjects at baseline.

	Chinese herbal decoction (n = 142)
Age (years)	50.54 ± 12.23
Gender (M/F)	59.86%/40.14%
Height (m)	1.68 ± 0.08
Weight (kg)	72.00 ± 12.56
Duration of diabetes (month)	72.96 ± 63.45
Duration of treatment (month)	21.88 ± 18.05
Times of therapy (n)	11.90 ± 7.77
History of hypertension (n)	59 (41.55%)
History of dyslipidemia (n)	65 (45.77%)
History of fatty liver (n)	32 (22.54%)
History of hyperuricemia (n)	8 (5.63%)
Combined with insulin (n)	29 (20.42%)
Combined with metformin (n)	37 (26.06%)
Combined with repaglinide (n)	14 (9.86%)
Combined with gliclazide (n)	8 (5.63%)
Combined with glimepiride (n)	5 (3.52%)
Combined with acarbose (n)	24 (16.90%)
Combined with rosiglitazone (n)	4 (2.82%)
HbA1c (%)	8.98 ± 2.02
FPG (mmol/L)	9.96 ± 2.94
2hPG (mmol/L)	14.07 ± 4.90
CHO (mmol/L)	5.43 ± 1.19
TG (mmol/L)	2.71 ± 2.72
LDL (mmol/L)	3.17 ± 0.97
HDL (mmol/L)	1.26 ± 0.43
Systolic pressure (mmHg)	134.71 ± 20.51
Diastolic pressure (mmHg)	83.94 ± 9.68
UA (μmol/L)	315.81 ± 118.73
ALT (U)	37.42 ± 47.11
AST (U)	25.58 ± 14.75

Values are expressed as mean ± SD.

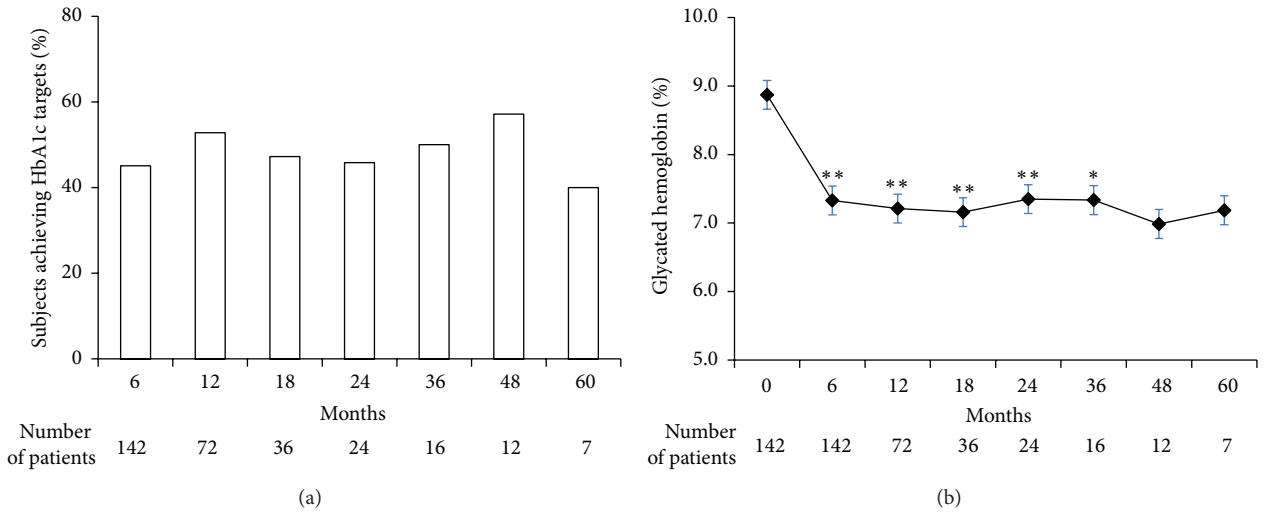

Figure 1: Subjects achieving HbA1c targets (a) and glycated hemoglobin (b) over time. $* < 0.05$ and $** < 0.01$.

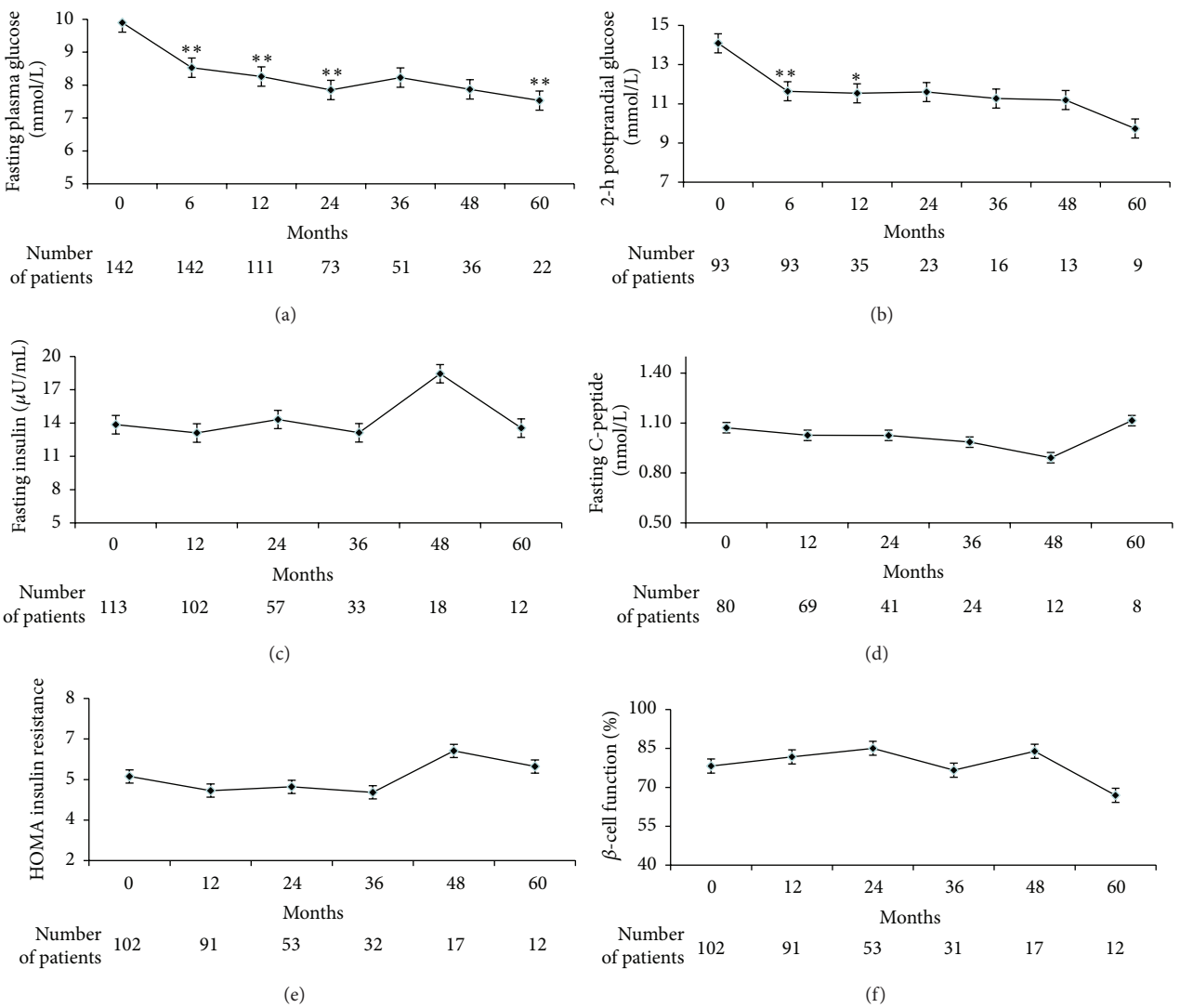

Figure 2: Fasting plasma glucose (a), 2-h postprandial glucose (b), fasting insulin (c), fasting C-peptide (d), HOMA insulin resistance (e), and β-cell function (f) over time. $* < 0.05$ and $** < 0.01$.

Table 2: Factors influencing HbA1c control of patients.

	B	SE	Standardized regression coefficients	T	p
HbA1c at baseline	0.165	0.020	0.562	8.226	0.000
Duration of diabetes	0.268	0.054	0.328	4.978	0.000
BMI	0.011	0.002	0.130	4.783	0.000
Age	0.113	1.583	0.116	0.151	0.063

and 20.20 ± 9.97, respectively. Stratified analysis also showed that FINS and HOMA-IR decreased significantly among subjects with pretreatment HOMA-IR > 2.69 and increased significantly among subjects with pretreatment HOMA-IR ≤ 2.69 ($p < 0.05$). The change in HOMA-β increased significantly among subjects with pretreatment HOMA-IR ≤ 2.69 after intervention ($p < 0.05$).

3.4. Other Biochemical Measurements. 73 subjects measured CHO for 199 times, 81 subjects measured TG for 227 times, 57 subjects measured LDL for 154 times, 60 subjects measured HDL for 154 times, and 110 subjects measured BP for 334 times. Compared with baseline, TG decreased significantly ($p < 0.05$) at 12 months and SBP and DBP decreased significantly ($p < 0.05$) at 6 months (Figure 3).

3.5. Safety. There were no serious adverse events that related to Chinese herbal decoction reported during this study. One subject had abnormal liver functions during medication, who was the carrier of hepatitis B virus (HBV). The test results were back to normal after taking hepatoprotectants. Neither hypoglycemia nor cardiovascular event was reported.

4. Discussion

Several large-scale clinical trials, such as the United Kingdom Prospective Diabetes Study (UKPDS) and Diabetes Control and Complication Trial (DCCT) [7, 21], have shown that good glycemic control is critical for patients with type 2 diabetes. In the past few decades, the incidence of DM had increased rapidly and the course of disease is not reversible. Thus, prevention of diabetes complications has become the most important part in diabetes management. Though many antidiabetes drugs have emerged in the marketplace, the circumstance is far from satisfactory. Thus, adopting alternative strategies is necessary to improve diabetes treatment, including the use of the Chinese herbal medicine.

Traditional Chinese medicine, which is an important scientific and technological resource with original and independent advantages, has been treating diabetes for thousands of years. The "whole view" and "multitargets" of TCM own unique advantages in controlling complex diseases, such as diabetes. This research is the first long-term observation for evaluating glycemic control and islet cell function treated by Chinese herbal decoction. It provides more detailed assessment of treatment features of herbal decoction and the clinical evidence for rational drug using. It is exciting that our study confirmed that TCM had better glucose controlling and performed protection of islet cell function, which is very important for slowing the disease progression. HbA1c, as the gold standard for evaluating glycemia, has been widely used in the clinic. However, the latest survey in China reported that only 30.15% of 223,114 Chinese T2DM patients had HbA1c lower than 7.0% [9]. Another Chinese study showed that the control rate of HbA1c was 41.1% [22]. It is still not optimistic in the United Kingdom; UKPDS reported about 28%~37% of diabetes patients achieved target levels, that is, HbAlc lower than 7% [23]. Another study reported that 76% of patients had HbAlc > 7.0% [24]. Our study showed that the control rate of HbA1c was between 40.00% and 57.14%. ADOPT showed that the HbA1c was within 7.1%–7.7% in the fifth-year intervention [25]. Our results showed that after five-year intervention HbAlc was 7.2%. Additionally, FPG and 2hPG decreased significantly after intervention. Thus our results preliminarily proved herbal decoction was effective on DM.

The impaired islet cell function is the important pathophysiological mechanism of diabetes, regardless of type I or II diabetes; the progressive exhaustion of islet cell function is the central link of the disease [26]. We could not reverse the diabetes pathology if we are only focusing on the hyperglycemia recovery. It is expected to delay the disease progression for performing early intervention against islet cell dysfunction. Currently the treatment focuses too much on the glycemia while it disregards islet cells as the primary target. The treatment of diabetes in the future should exert more efforts on improving insulin resistance and maintaining normal insulin secretion.

To the best of our knowledge, there is no observation on the efficacy of Chinese herbal decoction treating islet cell function before. Though our information is limited, it is exciting that herbal decoction may improve the islet cell function through assessment. UKPDS has demonstrated that T2DM is a progressive disease that the glycemic deterioration is associated with progressive loss of beta-cell function and no medication could reverse this recession [27]. According to Chinese characteristics, we performed a more rational stratified analysis to evaluate the impact of herbal decoction on islet cell function. It was found that Chinese herbal decoction could mitigate insulin resistance. As for patients without insulin resistance, herbal decoction could increase FINS and islet cell function which may delay the exhaustion of islet function. Moreover, the fifth-year islet cell function maintenance is similar to ADOPT in our study which was 70% to 80% [25]. It is worth mentioning that, in the late period of observation, the islet cell function variation trend was positive. Chinese herbal decoction might alleviate the continuous stimulation of hyperglycemia to islet cell.

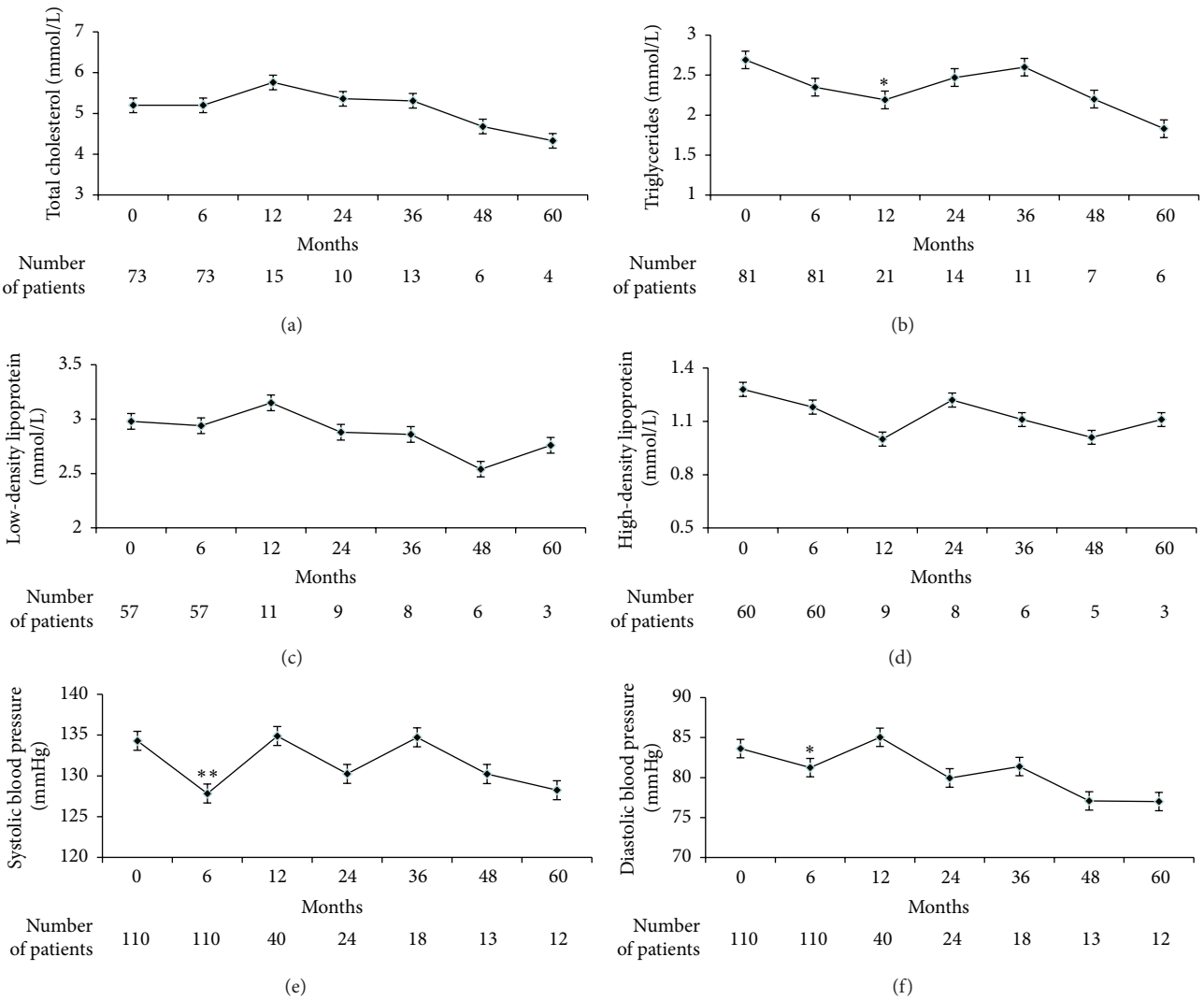

Figure 3: Total cholesterol (a), triglycerides (b), low-density lipoprotein (c), high-density lipoprotein (d), systolic blood pressure (e), and diastolic blood pressure (f) over time. $* < 0.05$ and $** < 0.01$.

The effective components may promote the proliferation and differentiation of islet cell, to postpone the exhaustion of islet cell function. This result is encouraging which indicates that traditional Chinese medicine might provide a new alternative therapy for DM.

UKPDS showed that, after six-year intervention, 12.1% of the patients experienced macrovascular events and 5.7% of them experienced microvascular events [27]. In our study the incidences of both macrovascular and microvascular events were low. Traditional Chinese medicine might have advantages in preventing complications. The study showed that Chinese herbal decoction has certain effects on lowering lipids and BP, which reflects the positive impacts of herbal decoction on multiple targets. It is notable that herbal decoction may reduce the dosage or even deactivate the oral drugs or insulin. Safety analysis showed that herbal decoction did not increase the risk of hypoglycemia, which further confirmed the safety of herbal decoction.

Since this study is retrospective, there exist some limitations unavoidably. Large-scale clinical trials or long-term experiment is required to confirm the trends of islet cell function, thus making a more complete assessment and judgment. The study preliminarily proved that Chinese herbal decoction was effective on glycemic control, whereas we could not perform the more detailed stratified analysis as subjects were lost to follow-up during the long-term observation. We also preliminarily proved herbal decoction had positive effect on delaying the disease progression, whereas subjects should measure more timely in the future trials, which is important to explore more deeply on diabetes with complications. Additionally, BMI and waist-to-hip ratio would also be collected in the future study. At present, the precise mechanism of Chinese herbal medicine for glycemia has not been fully understood yet. The suitable scope of application in the corresponding individual syndromes should be further explored and interpreted. To achieve more accurate results

and avoid bias, large-scale randomized clinical trials should be launched to estimate the efficacy of herbal decoction on T2DM.

In summary, data from this retrospective study preliminarily demonstrated that the Chinese herbal decoction is effective and safe to use in glycemic controlling and islet cell function improving for diabetes patients. Further clinical studies are needed to confirm the observed effectiveness of Chinese herbal decoction as a new treatment option in the clinical management of diabetes. By the application of TCM comprehensive system for the prevention and control, the prevention of diabetes complication would be improved significantly. The tendency of the rising rates of diabetes and its complications would be controlled, or even the inflection point would appear.

Competing Interests

The authors indicated no conflict of interests.

Authors' Contributions

Jiaxing Tian, Fengmei Lian, and Xiaolin Tong contributed to study design. Xiaotong Yu, Yashan Cui, Tianyu Zhao, and Yang Cao contributed to data collection. Jiaxing Tian, Fengmei Lian, and Xiaolin Tong contributed to data analysis and interpreted results. Jiaxing Tian and Fengmei Lian wrote the paper. Jiaxing Tian, Fengmei Lian, and Xiaolin Tong revised and reviewed the final paper.

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Research Article

Randomized, Double-Blinded, Double-Dummy, Active-Controlled, and Multiple-Dose Clinical Study Comparing the Efficacy and Safety of Mulberry Twig (Ramulus Mori, Sangzhi) Alkaloid Tablet and Acarbose in Individuals with Type 2 Diabetes Mellitus

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Aims. To evaluate the efficacy and safety of mulberry twig alkaloid (SZ-A) tablet compared with acarbose in patients with type 2 diabetes. *Methods*. This clinical trial enrolled 38 patients who were randomized into two groups (SZ-A: 23; acarbose: 15) and were treated for 24 weeks. Patients and clinical trial staffs were masked to treatment assignment throughout the study. The primary outcome measures were glycated hemoglobin (HbA1c) and 1-hour and 2-hour postprandial and fasting plasma glucose levels from baseline to the end of treatment. Analysis included all patients who completed this study. *Results*. By the end of this study, HbA1c level in SZ-A group was decreased from baseline significantly ($P < 0.001$). No significant difference was found when compared with acarbose group ($P = 0.652$). Similarly, 1-hour and 2-hour postprandial plasma glucose levels in SZ-A group were decreased from baseline statistically ($P < 0.05$), without any significant differences compared with acarbose group ($P = 0.748$ and 0.558, resp.). The fasting plasma glucose levels were not significantly changed in both groups. One of 23 patients in SZ-A group (4.76%) and 5 of 15 patients in acarbose group (33.33%) suffered from gastrointestinal adverse events. *Conclusions*. Compared with acarbose, SZ-A tablet was effective and safe in glycemic control in patients with type 2 diabetes.

1. Introduction

Diabetes is one of the largest global health emergencies of the 21st century. In most countries, diabetes and its complications are the leading causes of death. The scary number of diabetes has been increasing in most regions. In 2015, 415 million adults are estimated to currently have diabetes and 318 million adults with impaired glucose tolerance in the world [1]. Moreover, due to the cost of essential medicines, diabetes has a substantial economic impact on individuals, their families, and national health systems [1]. In addition to public health education and early diagnosis, effective treatment of diabetes is the indispensable role in halting the rise of diabetes.

Among three main types of diabetes, type 2 diabetes is the most prevalent form. Patients with early type 2 diabetes may be able to maintain normal blood glucose levels by means of a meal plan and physical exercise. As the disease progresses, oral hypoglycemic drugs are indicated. To decrease the postprandial rise in glucose levels in these patients, carbohydrate absorption can be decreased or delayed by the prandial use of acarbose, an α-glucosidase inhibitor that acts on the small intestine by blocking the digestion of complex carbohydrates

[2]. However, the most frequent gastrointestinal side effects of acarbose are flatulence and diarrhea, due to its mechanism of action [3]. In a STOP-NIDDM randomized trial, the percentage of gastrointestinal adverse events of acarbose was 13% (flatulence 9%, diarrhea 5%, abdominal pain 3%, and other 1%), which were more frequent than in those given placebo ($P < 0.0001$) [4].

Herbal medicine has been wildly used to treat type 2 diabetes in Asia for centuries. Many antidiabetic herbs are adopted extensively in clinical units with proven efficacy and safety [5–8]. Moreover, screening of α-glucosidase inhibitors from plants has been a hot research topic [9–11]. The most reported plant is mulberry (Latin name: *Morus alba* L.) [12–16], one kind of herbs, which is commonly used in Chinese medicine. Mulberry twig (Latin name: *Ramulus Mori*, Chinese name: Sang Zhi, SZ), is the dry branch of mulberry, which is widely distributed in Asia.

1-Deoxynojirimycin (1-DNJ) is a main active constituent of effective fraction of alkaloids from SZ [12]. It has been reported that 1-DNJ is an α-glucosidase inhibitor [17–19] which can delay glucose absorption and significantly reduce postprandial blood glucose levels [20]. In a previous study [21], the effective fraction of alkaloids from SZ (SZ-A) was found to have a strong α-glucosidase inhibitory activity *in vitro* and *in vivo*. Compared with acarbose, SZ-A showed stronger inhibition of sucrase (IC_{50} = 21.9 ng/mL), equal inhibition of maltase (IC_{50} = 40.4 ng/mL), and less inhibition of amylase *in vitro* [22]. Also, in the study of blood glucose of normal mice after loading sucrose, the results showed that the SZ-A in the dosage of 10 mg/kg–40 mg/kg can significantly reduce elevated blood glucose, and the blood glucose area under the curve was significantly less than acarbose group. The results of these two groups (10 mg/kg–40 mg/kg) had no significant differences. In another study of blood glucose of alloxan induced diabetic mice after loading sucrose, the results showed that the SZ-A could lower and postpone the peak of blood glucose and reduce the blood glucose area under the curve. The effects of 20 mg/kg and 40 mg/kg SZ-A treatment groups were better than acarbose groups. These results suggested that the hypoglycemic activity of SZ-A was similar to acarbose [21, 22].

In our study, we compared the efficacy and safety of SZ-A tablet with acarbose for 24 weeks. Monotherapy and polytherapy with metformin were also included. The aims of this study were to (1) evaluate the efficacy and safety of SZ-A tablet, (2) find the lowest effective dose of SZ-A tablet (as the same effectiveness of acarbose), and (3) investigate whether the polytherapy with metformin has better effects on glycemic control, compared with monotherapy. This is the first clinical study of SZ-A tablet compared with acarbose in patients with type 2 diabetes.

2. Materials and Methods

2.1. Patients and Study Design. Eligible patients in the study were 18–70 years of age, with a diagnosis of type 2 diabetes according to the 1999 World Health Organization diagnostic criteria, who were not on a regimen of antidiabetic medical treatment at least 3 months before screening, who were on a regimen of antidiabetic treatment no more than 3 months at any time in the past, who were on a stable regimen of metformin monotherapy for at least 8 weeks, and who had a glycated hemoglobin concentration (HbA1c) ≥7.0% (53 mmol/mol) and ≤10.0% (86 mmol/mol), a fasting plasma glucose level ≤13 mmol/L (234 mg/dL), and a body mass index (BMI) of 19–30 kg/m^2. Patients were excluded for a difference of fasting plasma glucose levels between 1st follow-up and 2nd follow-up >2.5 mmol/L (45 mg/dL), severe diabetes complications (e.g., diabetic ketoacidosis), allergy to or intolerance of α-glucosidase inhibitors, confounding concomitant drug use (including insulin, incretin mimetics, thiazolidinediones, antidiabetic herbal medicine, and glucocorticoids), poor blood pressure control (SBP > 160 mmHg or DBP > 100 mmHg), liver disease, kidney disease, intestinal conditions (e.g., inflammatory bowel disease), substantial alcohol consumption (>20 g/day for women or >30 g/day for men), pregnancy, and disorders such as a medical history of major pathology. Written informed consent was obtained from all patients before the trial began (to convert mmol/L to mg/dL, multiply by 18).

This was a randomized, double-blinded, double-dummy, active-controlled, and multiple-dose clinical trial evaluating the efficacy and safety of SZ-A tablet compared with acarbose in patients with type 2 diabetes. Eligible patients were randomly assign by a computer-generated, centrally administered randomization schedule via an interactive web response system (IWRS). Each patient was associated with a randomization code. Allocation concealment was achieved by packaging both SZ-A and acarbose groups with a unique identification number by the manufacturer. Patients, investigators, clinical trial staffs, and physicians were masked to treatment assignment throughout the study. Emergency unblinding service is provided. The double-dummy design was accomplished by use of the placebo formulations of SZ-A and acarbose. In order to be indistinguishable in terms of odor and taste, SZ-A placebo contains 1/20 the dosage of SZ-A, which is an ineffective dose, while acarbose placebo imitates acarbose tablet in appearance and weight.

The tablets SZ-A (each tablet 50 mg) were produced by Beijing Wuhe Boao Pharmaceutical Technology Development Co., Ltd., which was approved to produce tablets in September 2008 by CFDA (China Food and Drug Administration, number 2008L05752). An effective fraction of alkaloids of SZ-A is prepared from mulberry twig and the active ingredients are a composition of alkaloids, including N-methyl-1-deoxynojirimycin (1-DNJ), 3-epi-fagomine, fagomine, 1,4-dideoxy-1,4-imino-D-arabinitol, and 1,4-dideoxy-1,4 imino-(2-O-β-D-glucopyranosyl)-D-arabinitol. Among these alkaloids, 1-DNJ is the highest content [21]. The tablets acarbose (each tablet 50 mg) were produced by Bayer Healthcare Pharmaceutical Inc., Germany. In January 22, 2014, the Ethics Committees of Peking University First Hospital where the study was conducted approved the trial protocol. The study was registered at http://db.yaozh.com/ (CTR20140034).

To improve accuracy and screen compliance, all enrolled patients underwent a 4-week lead-in period (1st follow-up: −4 weeks), given SZ-A placebo tablet 50 mg and acarbose placebo tablet 50 mg with first bite of food, three times

daily (t.i.d.). After randomization, all patients underwent a multiple-dose procedure: for SZ-A group, its initial dose is SZ-A tablet 50 mg t.i.d. + acarbose placebo tablet 50 mg t.i.d (for 4 weeks) and maximum dose is SZ-A tablet 100 mg t.i.d. + acarbose placebo tablet 50 mg t.i.d. (till end of treatment); for acarbose group, its initial dose is acarbose tablet 50 mg t.i.d. + SZ-A placebo tablet 50 mg t.i.d. (for 4 weeks) and maximum dose is acarbose tablet 50 mg t.i.d. + SZ-A placebo tablet 100 mg t.i.d. (till end of treatment).

2.2. Study Endpoints and Assessments. Patients returned for study visits at weeks −4, 0, 4, 8, 16, and 24 (end of treatment). Patients had physical examination, routine blood tests, urine tests, and electrocardiogram (ECG) during each visit. The primary objective of the study was to demonstrate effectiveness of SZ-A tablet on HbA1c change during the period, compared with acarbose. Secondary objectives were to evaluate the changes of fasting plasma glucose level and 1-hour and 2-hour postprandial plasma glucose levels during the study.

Safety assessments included adverse events, hypoglycemia, vital signs (blood pressure), ECGs, and laboratory variables. Hypoglycemia was defined as a measured plasma glucose concentration ≤3.9 mmol/L and/or symptoms and/or signs attributable to hypoglycemia [23]. Severe hypoglycemia was defined as an episode requiring the assistance of another person to actively administer therapy.

Patients were not given any additional therapy during the trial. No dose reductions of SZ-A, acarbose, or their placebo were allowed throughout the 24-week treatment period. Participants with previous treatment of metformin were continued at the same dose as before randomization.

2.3. Statistical Analysis. We used SPSS 17.0 software (SPSS, Inc., Chicago, IL) for all statistical analysis. For normally distributed quantitative data, independent-samples t-test or one-way analysis of variance (ANOVA) was used to test the effects of treatment on the changes of HbA1c, plasma glucose levels, and other routine blood tests; paired t-test was used to test the effects of each group before and after the treatment. If the drug effect was found to be significant by ANOVA, multiple comparison of LSD was used to test the difference between the different treatments. Qualitative data were analyzed by using Chi-square (Fisher's exact test or Monte Carlo exact test) or nonparametric technique using Kruskal-Wallis test. All tests were two-tailed, and the level of significance was set at 0.05 ($P < 0.05$).

3. Results

3.1. Patients. From June 25, 2014, to December 29, 2014, 69 patients were recruited in this study. After systemic review, we excluded 31 patients who met our exclusive criteria. Finally, 38 patients were randomly assigned to receive SZ-A ($n = 23$) or acarbose ($n = 15$). Two patients in SZ-A group were lost to follow-up because of the failure of getting in contact with them. No patient was lost to follow-up in acarbose group (Figure 1). We analyzed the records of 36 patients in total, 21 patients in SZ-A group, and 15 patients in acarbose group, respectively. Baseline demographic, clinical, and laboratory features were similar in the two groups (Table 1).

3.2. Efficacy. Twenty-one (91%) patients in SZ-A group and 15 (100%) patients in acarbose group had group-comparison and self-comparison in terms of HbA1c, fasting plasma glucose levels, postprandial plasma glucose levels, lipids, liver functions, and kidney functions both at baseline and at week 24 (Table 2).

For the self-comparison in each group, the significant changes from baseline to 24 weeks in HbA1c were −0.776% ($P < 0.001$) and −0.827% ($P < 0.05$) (SZ-A and acarbose group, resp.). Compared with acarbose, SZ-A reduced HbA1 with 95% confidence interval (CI): 0.18 (−0.64 to 1.00). The differences between two groups were not statistically significant (Figure 2(a); $P > 0.05$). As for postprandial plasma glucose levels, treatments in both groups significantly decreased 1-hour postprandial plasma glucose levels from baseline to 24 weeks (self-comparison; Table 2; $P < 0.05$), while the difference between groups was not significant (Figure 2(b); $P > 0.05$). Similarly, in each group, 2-hour postprandial plasma glucose levels were significantly decreased from baseline to 24 weeks (self-comparison; Table 2; $P < 0.05$), while the difference in group-comparison was not significant (Figure 2(c); $P > 0.05$). Both treatments did not change fasting plasma glucose levels significantly in self-comparison and group-comparison (Figure 2(d); $P > 0.05$).

There was no significant difference in lipids, liver functions, or kidney functions between the two groups from baseline to end of treatment (Table 2).

In addition, the results of 2-hour postprandial plasma glucose levels in group-comparison showed a significant reduction in patients receiving acarbose at week 8 compared with SZ-A (Figure 3; $P < 0.05$).

In comparison of monotherapy and polytherapy with metformin, there was no significant difference between four treatment groups (SZ-A + metformin, SZ-A, acarbose + metformin, and acarbose groups) in terms of HbA1c ($P = 0.945$), fasting plasma glucose ($P = 0.720$), and 1-hour and 2-hour postprandial plasma glucose levels ($P = 0.940$, $P = 0.597$, resp.; Figures 4(a)-4(b)).

3.3. Safety. Adverse events were recorded in detail in the two treatment groups in each follow-up (Table 3). The two patients in the SZ-A group withdrew from treatment because of failure to contact them. There were no serious adverse events (deaths, heart failure, liver failure, or kidney failure and other conditions) during the treatment period. All gastrointestinal adverse events were considered to be of mild intensity. The percentage of gastrointestinal disorders in SZ-A group was obviously lower than that of in acarbose group, which included increased release of gas (one (4.76%) patients in SZ-A group versus 2 (13.33%) in acarbose group) and diarrhea (none versus 3 (20%)).

According to liver and kidney function tests recorded at each visit, the level of increased alanine aminotransferase, aspartate aminotransferase, and urine acid and the level of reduced creatinine clearance rate in SZ-A group were slightly higher than that in acarbose group. Long-time clinical

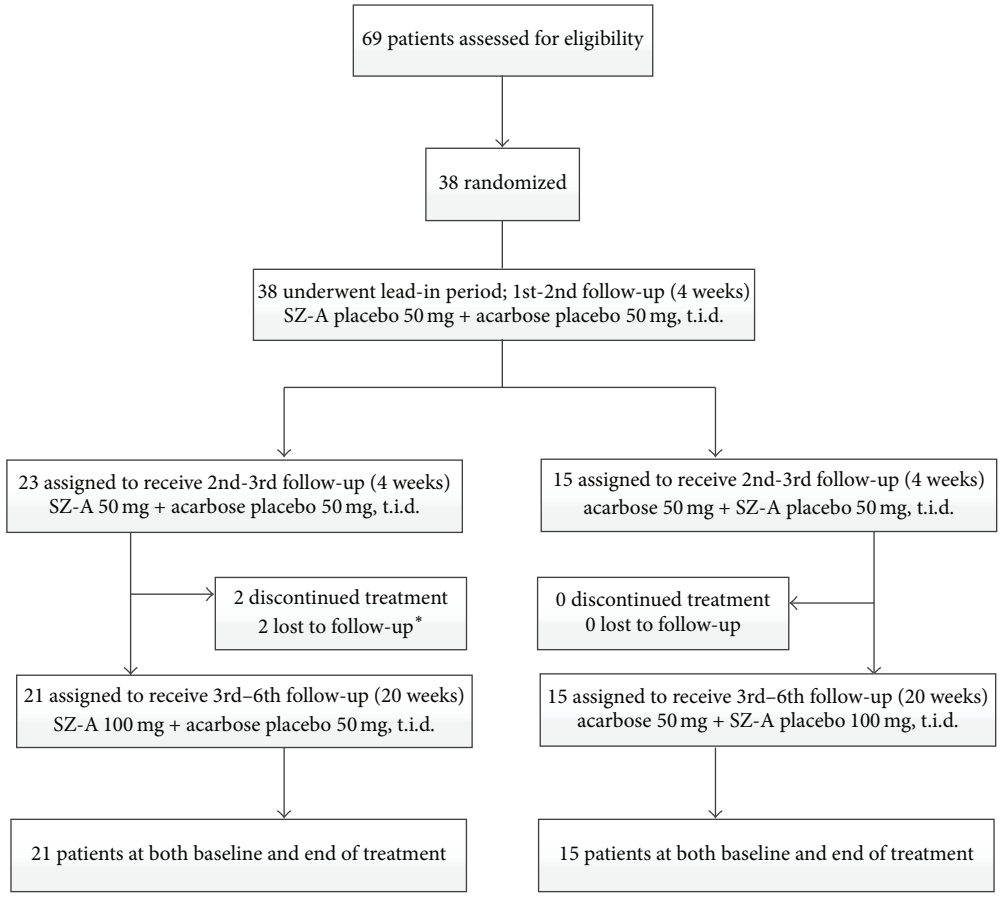

Figure 1: Trial profile. *Two patients assigned to the SZ-A group were lost to follow-up (one patient was lost at week 4 after receiving drugs; one patient was lost at week 8 after receiving drugs) because of the failure to getting in contact with them.

observation is needed and we should pay more attention to the cause-effect relationship between SZ-A tablet and mildly increased aminotransferase and urine acid or reduced creatinine clearance rate. We noticed that the abnormal creatinine clearance rate of two patients in SZ-A group and two patients in acarbose group turned to be normal after the study. We also noted that there were two (9.52%) urinary tract infection cases in the SZ-A group, both of which were considered unrelated to the treatments. Changes from baseline in ECG are shown in Table 3. No cardiovascular events were observed.

4. Discussion

Intensive glucose control is the predominant factor to prevent the development of chronic complications in type 2 diabetes [24–27]. An important component of dysglycemia is postprandial hyperglycemia [28]. Postprandial hyperglycemia remains a prominent feature in the early stage of diabetes and has been demonstrated in Chinese patients with type 2 diabetes [29]. Sustained postprandial hyperglycemia is an independent risk factor for cardiovascular complications and death [30]. There were two main categories of the treatment of postprandial hyperglycaemia: (1) therapeutic agents that act on intestinal digestion of carbohydrates and (2) therapeutic agents that stimulate or mimic the postprandial insulin response [27]. The α-glucosidase inhibitor belongs to the first category and plays a significant role in pharmacological treatments to control postprandial glycemic excursions. Acarbose was the first agent of α-glucosidase inhibitor to be made available and adopted extensively.

In this randomized, double-blinded, double-dummy, active-controlled clinical trial, the α-glucosidase inhibitor, SZ-A, met the predefined primary endpoint and led to significant reduction of HbA1c and postprandial plasma glucose in 24-week treatment period. Fasting plasma glucose levels had no significant reduction in both groups (SZ-A and acarbose group), which indicated that fasting blood glucose is poorly sensitive to α-glucosidase inhibitors. In addition, there was no statistical difference of HbA1c levels in both SZ-A and acarbose group. Similar results were presented in terms of postprandial plasma glucose levels in group-comparison. That is, compared with acarbose 50 mg t.i.d., the dose of SZ-A tablet 100 mg t.i.d. can achieve similar hypoglycemic effects.

According to a previous unpublished randomized, dose-escalation, active-controlled, multicenter phase II clinical study, SZ-A tablets 100 mg (once taken) showed the similar effects as acarbose 50 mg (once taken) on reducing postprandial blood glucose levels by inhibiting and delaying

Table 1: Baseline characteristics of trial population.

	SZ-A (n = 23)	Acarbose (n = 15)	P value
Demographics			
Age (years)	56 (9.71)	57 (6.70)	0.747[a]
Gender (male/female)	8/15	5/10	1.000[b]
Comorbidities			
Hypertension	6 (26.1%)	6 (40.0%)	0.481[b]
Hyperlipidaemia	17 (73.9%)	10 (66.7%)	0.722[b]
Concomitant drug use			
Metformin	12 (52%)	7 (47%)	1.000[b]
Antilipidemic	2 (0.09%)	0	NA
Antihypertensive	1 (0.04%)	0	NA
Metabolic factors			
Disease duration (months)	36 (6,180)	30 (9,120)	0.800[c]
Fasting glucose (mmol/L)	8.94 (1.63)	8.85 (1.56)	0.873[a]
HbA1c (%)	8.30 (0.91)	8.24 (0.95)	0.847[a]
Weight (kg)	71.63 (12.06)	69.30 (13.00)	0.576[a]
BMI (kg/m^2)	25.67 (2.744)	25.47 (2.612)	0.822[a]
Systolic blood pressure (mmHg)	131.48 (11.735)	129.67 (15.267)	0.682[a]
Diastolic blood pressure (mmHg)	81.39 (8.217)	78.67 (9.123)	0.345[a]
Smoking history (current smoker)	1 (4.3%)	4 (26.7%)	0.069[b]
Drinking history (current drinker)	2 (8.7%)	0	NA
Proteinuria			0.898[c]
0	12 (63.2%)	9 (64.3%)	—
Trace	5 (26.3%)	4 (28.6%)	—
1+	2 (10.5%)	1 (7.1%)	—
Glycosuria			
0	12 (63.2%)	7 (50.0%)	1.000[b]
Trace	2 (10.5%)	3 (21.4%)	0.365[d]
1+	0	1 (7.1%)	NA
2+	0	0	NA
3+	2 (10.5%)	3 (21.4%)	0.365[d]
4+	3 (15.8%)	0	NA
Lipids			
Total cholesterol (mmol/L)	5.17 (1.06)	4.85 (0.91)	0.350[a]
Triglycerides (mmol/L)	2.26 (1.30)	1.89 (1.12)	0.367[a]
HDL (mmol/L)	1.18 (0.37)	1.15 (0.27)	0.803[a]
LDL (mmol/L)	3.04 (0.93)	2.97 (0.69)	0.790[a]
Liver function tests			
Alanine aminotransferase (U/L)	22.39 (9.41)	26.60 (15.04)	0.294[a]
Aspartate aminotransferase (U/L)	17.91 (5.43)	20.07 (6.27)	0.269[a]
Kidney function tests			
Creatinine clearance (mL/min)	77.08 (20.66)	76.59 (14.56)	0.937[a]
Uric acid (mmol/L)	292.91 (80.62)	241.20 (90.95)	0.078[a]
ECG			
Normal	11 (57.89%)	7 (50%)	1.000[b]
ST segment depression	2 (10.53%)	0 (0%)	NA
T wave nonspecific changes	3 (15.79%)	3 (21.43%)	0.663[d]
Low voltage	1 (5.26%)	0 (0%)	NA
High voltage of left ventricle	1 (5.26%)	1 (7.14%)	1.000[d]
PR interval shortened	1 (5.26%)	0 (0%)	NA
Left ventricle hypertrophy	0 (0%)	1 (7.14%)	NA
Right atrium hypertrophy	0 (0%)	1 (7.14%)	NA
Sinus bradycardia	0 (0%)	1 (7.14%)	NA
Compliance (%)	97.66 (2.95)	97.36 (2.08)	0.734[a]

Data are mean (SD) or n (%) or median (minimum, maximum). [a]Independent-samples t-test. [b]Chi-square tests (Fisher's exact test). [c]Nonparametric tests. [d]Chi-square tests (Monte Carlo exact test).

Table 2: Changes in plasma glucose levels, lipids, liver functions, and kidney functions from baseline to week 24.

	Mean (SD) change from baseline to week 24				Mean (95% CI) changes from baseline (SZ-A versus acarbose)	*P* value
	SZ-A ($n = 21$)	*P* value	Acarbose ($n = 15$)	*P* value		
Plasma glucose levels						
Fasting glucose (mmol/L)	1.04 (2.61)	0.083	−0.02 (2.25)	0.976	−0.88 (−2.45 to 0.70)	0.266
Postprandial blood glucose 1 h (mmol/L)	4.10 (5.11)	0.003*	4.02 (4.92)	0.007*	0.46 (−2.44 to 3.36)	0.748
Postprandial blood glucose 2 h (mmol/L)	3.84 (5.05)	0.003*	3.83 (5.83)	0.029*	0.97 (−2.36 to 4.29)	0.558
HbA1c (%)	0.78 (0.85)	0.000*	0.83 (1.35)	0.033*	0.18 (−0.64 to 1.00)	0.652
Lipids						
Total cholesterol (mmol/L)	0.06 (1.21)	0.824	−0.01 (0.65)	0.969	0.30 (−0.36 to 0.96)	0.366
Triglycerides (mmol/L)	0.10 (1.17)	0.706	0.31 (0.93)	0.214	0.61 (−0.11 to 1.33)	0.094
HDL (mmol/L)	−0.04 (0.19)	0.386	−0.02 (0.15)	0.527	0.07 (−0.14 to 0.27)	0.513
LDL (mmol/L)	−0.09 (0.72)	0.586	0.06 (0.49)	0.634	0.25 (−0.21 to 0.71)	0.271
Liver functions						
Alanine aminotransferase (U/L)	−0.38 (8.39)	0.837	2.07 (14.21)	0.582	−2.53 (−9.77 to 4.70)	0.482
Aspartate aminotransferase (U/L)	0.33 (4.20)	0.720	1.27 (6.15)	0.438	−2.28 (−5.50 to 0.95)	0.160
Kidney functions						
Creatinine clearance (mL/min)	2.36 (13.94)	0.447	−2.56 (16.30)	0.553	−3.71 (−17.02 to 9.61)	0.575
Urine acid (mmol/L)	−5.55 (58.59)	0.760	−12.83 (38.79)	0.455	36.50 (−40.03 to 113.03)	0.327

* $P < 0.05$.

Table 3: Adverse events.

	SZ-A ($n = 23$)	Acarbose ($n = 15$)
Overall treatment withdrawal rate	2 (0.09%)	0
Treatment withdrawal due to adverse event	0	0
Participants with serious adverse event	0	0
Adverse event		
Gastrointestinal disorders	1 (4.76%)	5 (33.33%)
Increased release of gas	1 (4.76%)	2 (13.33%)
Diarrhea	0	3 (20%)
Abdominal pain	0	0
Dyspepsia	0	0
Flatulence	0	0
Urinary tract infection	2 (9.52%)	0
Increased alanine aminotransferase (mild)	2 (9.52%)*	0
Increased aspartate aminotransferase (mild)	1 (4.76%)**	0
Reduced creatinine clearance (mild)	1 (4.76%)	0
Increased urine acid	1 (4.76%)***	0
ECG changes from normal		
Sinus bradycardia	1 (4.76%)	0
T wave nonspecific changes	1 (4.76%)	0
ST segment ischemic changes	1 (4.76%)	0

No deaths were reported in the trial period (weeks 0–24). * One patient's alanine aminotransferase was normal on further testing until end of treatment. ** This patient's aspartate aminotransferase was normal on further testing until end of treatment. *** This patient was lost to follow-up.

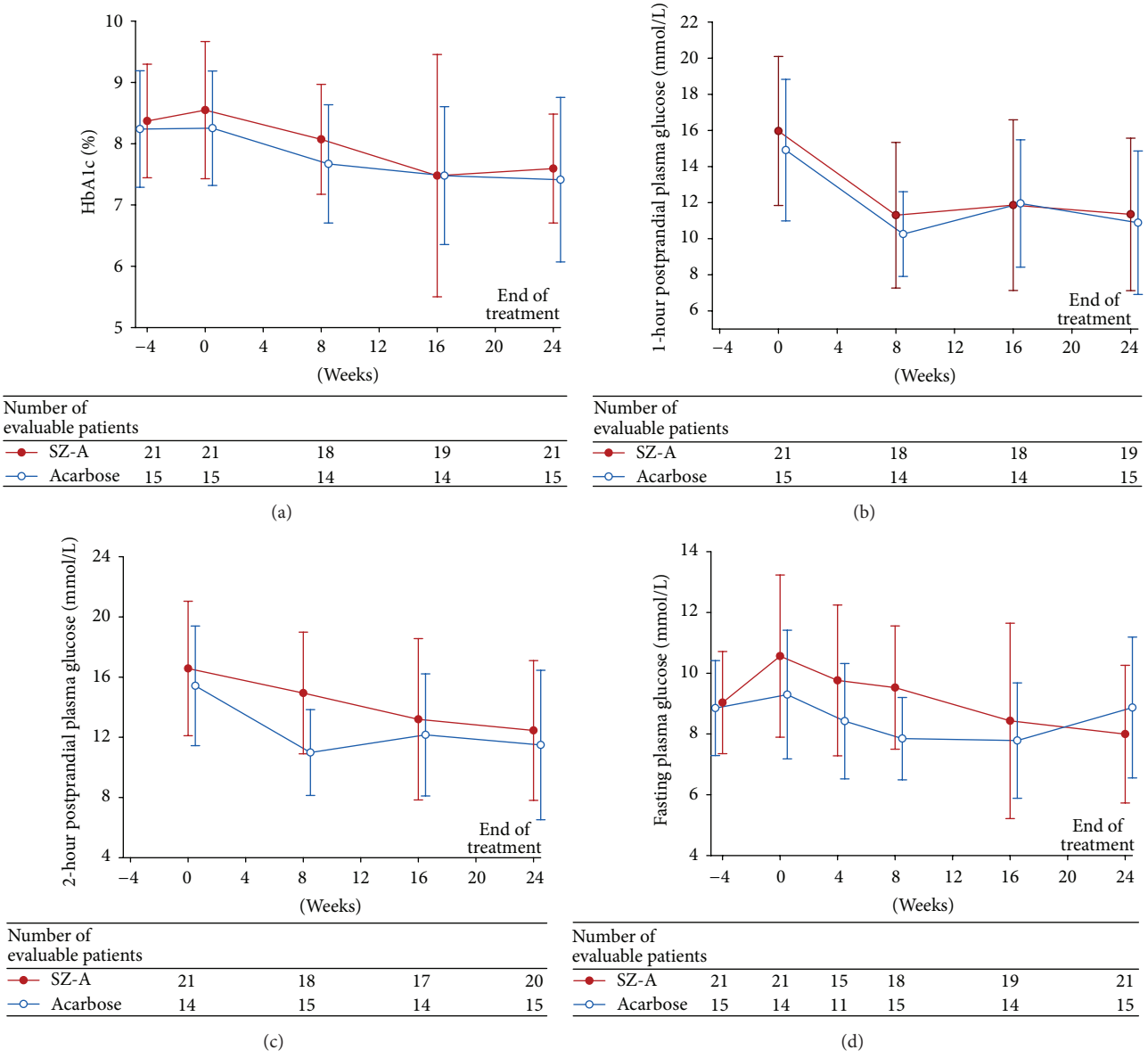

FIGURE 2: Glycated hemoglobin (HbA1c), postprandial plasma glucose, and fasting plasma glucose from baseline to week 24. (a) The difference of HbA1c between SZ-A group and acarbose group was not statistically significant ($P > 0.05$). (b) The difference of 1-hour postprandial plasma glucose levels was not significant in group-comparison ($P > 0.05$). (c) The difference of 2-hour postprandial plasma glucose levels was not significant in group-comparison ($P > 0.05$). (d) The difference of fasting plasma glucose levels was not significant in group-comparison ($P > 0.05$).

digestion and absorption of carbohydrate. In this study, from the beginning to the end of week 4, we applied SZ-A 50 mg t.i.d.; after week 4, we applied a dose-escalation of SZ-A tablet to 100 mg t.i.d., while the dose of acarbose still remained 50 mg t.i.d. As a result, compared with SZ-A group, the 2-hour postprandial plasma glucose level was significantly decreased ($P = 0.003$) in the patients in acarbose group only at week 8 but was not shown in other follow-up weeks or records. Similar to the previous clinical study, we could not acquire an ideal hypoglycemic effect on 2-hour postprandial plasma glucose level when SZ-A tablet 50 mg t.i.d. was applied. This result was reflected by SZ-A group at week 8; even the dose-escalation of SZ-A tablet had been applied for 4 weeks (from week 5 to week 8) in this study. This could be explained by the fact that drugs always need a time period to reach a stable drug concentration and reveal its best efficacy. Dose-escalation for only 4 weeks was not quite enough for SZ-A tablet. However, we continued dose-escalation to 100 mg t.i.d. of SZ-A till end of treatment, and there was no significant difference in 2-hour postprandial plasma glucose after 8 weeks. Besides, in terms of HbA1c and 1-hour postprandial plasma glucose levels, no significant difference appeared in group-comparison during the whole 24 weeks, whether the dose-escalation of SZ-A applied or not. We considered that 50 mg t.i.d. SZ-A tablet had similar hypoglycemic effects on HbA1c and 1-hour postprandial plasma glucose as the same

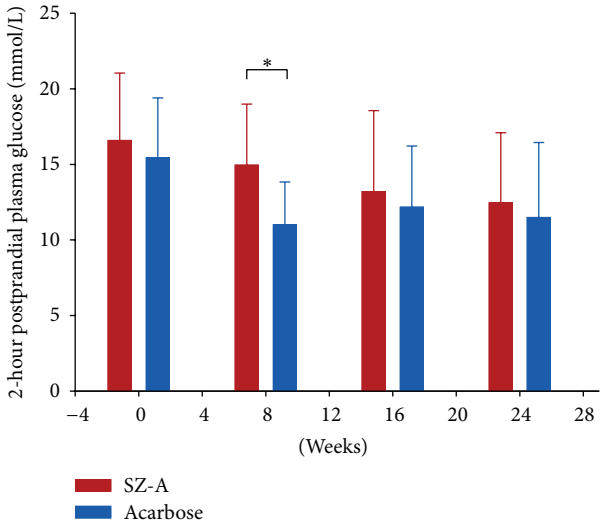

Figure 3: Two-hour postprandial plasma glucose levels in group-comparison from baseline to week 24. It showed a significant reduction in patients receiving acarbose at week 8 compared with SZ-A ($^{*}P < 0.05$).

dose of acarbose. In the consideration of the drawback of SZ-A on 2-hour postprandial plasma glucose control, 100 mg t.i.d. of SZ-A should be recommended in clinical practice for patients with type 2 diabetes mellitus as monotherapy. Further studies are still needed to investigate whether a combination of low dose of SZ-A (e.g., 50 mg t.i.d.) with other hypoglycemic treatments could acquire better efficacy of 2-hour postprandial plasma glucose control.

Combination therapy is commonly prescribed in the treatment of type 2 diabetes. The combination of acarbose and metformin is recommended in clinic because of their different and complimentary mechanisms of action, which provides effective glycemic control with additional cardiovascular benefits and minimizes adverse events [31, 32]. In this study, the combination therapy of SZ-A and metformin was also applied without any adverse events. But there was no significant difference in primary outcome measures between monotherapy and polytherapy with metformin. Further large-scale clinical study with long-time follow-ups should be conducted to assess the effects of combination of SZ-A and metformin on glycemic control and provide more clinical evidence.

α-Glucosidases are membrane-bound enzymes that digest disaccharides such as amylase, maltose, and sucrose in the small intestine [33]. Gastrointestinal side effects are one of the limitations of α-glucosidase inhibitor in clinical application. Our study also demonstrated this kind of side effect in both SZ-A and acarbose groups. However, the percentage of gastrointestinal side effects in patients receiving SZ-A was lower than that in acarbose group. The reason for this result should be carefully discussed. Acarbose, an aminooligosaccharide isolated from the fermentation broth of *Actinoplanes sp.*, inhibits brush-border α-glucosidases in humans [34, 35]. Acarbose can give rise to major adverse effects such as abdominal distention, flatulence, meteorism, and possibly diarrhea [4]. Such adverse effects are attributed to the impaired digestion of starch by strong inhibition of intestinal α-amylase. When undigested starch increases, it could be hydrolyzed by the bacteria residing in the colon and be used for fermentation, releasing gas and low-molecular-weight substances [36]. Then, this abnormal fermentation results in several undesirable side effects as mentioned above [36]. Compared with acarbose, plant-derived α-glucosidase inhibitor, mulberry leaf extract (MLE) has lower α-amylase inhibitory activity. The *in vitro* inhibitory activity of MLE on intestinal α-glucosidase was potent and that on intestinal α-amylase was very weak compared with acarbose [37]. Similar results were also reported in previous *in vitro* experiment of SZ-A [21]. Thus, SZ-A tablet is a strong α-glucosidase inhibitor but has less α-amylase inhibitory activity. SZ-A could be effective for postprandial hyperglycemia with minimal side effects. To some extent, it will improve the patient's compliance.

Limitations of our study should be considered when interpreting the results. Firstly, sample size was not large. Comparative study about effects of SZ-A and acarbose on glycemic control in patients with type 2 diabetes has not yet been published. Thus, the estimate of sample size was based on the feasibility of conducting a clinical trial. Albeit similar to other proof-of-concept studies [5, 38], our sample size was smaller than some later stage studies [39]. Despite this, every patient was strictly matched for features of type 2 diabetes and inclusion criteria in our study. Secondly, observation time was only 24 weeks. Due to the previous phase II clinical study, it was shown that the gastrointestinal side effects of SZ-A mainly occurred within 4 weeks after receiving tablets. With the extension of received time, the better tolerance for SZ-A was observed. For other adverse events, we think that our study should be with an extension (such as 28 weeks mentioned in a previous study [39], or maybe even longer observation period [40]). Thirdly, we did not analyze the BMI changes in our study. We collected the baseline BMI data (weight and height) of eligible patients when screening for the first time, but we missed some data in follow-ups. Further investigations with larger sample size and longer observation time are still needed to clarify the safety and efficacy of SZ-A tablet.

In conclusion, the efficacy and safety of SZ-A (mulberry twig alkaloid) tablet in reducing postprandial plasma glucose levels and HbA1c render it an attractive therapy for individuals with type 2 diabetes.

Competing Interests

No potential competing interests relevant to this paper were reported.

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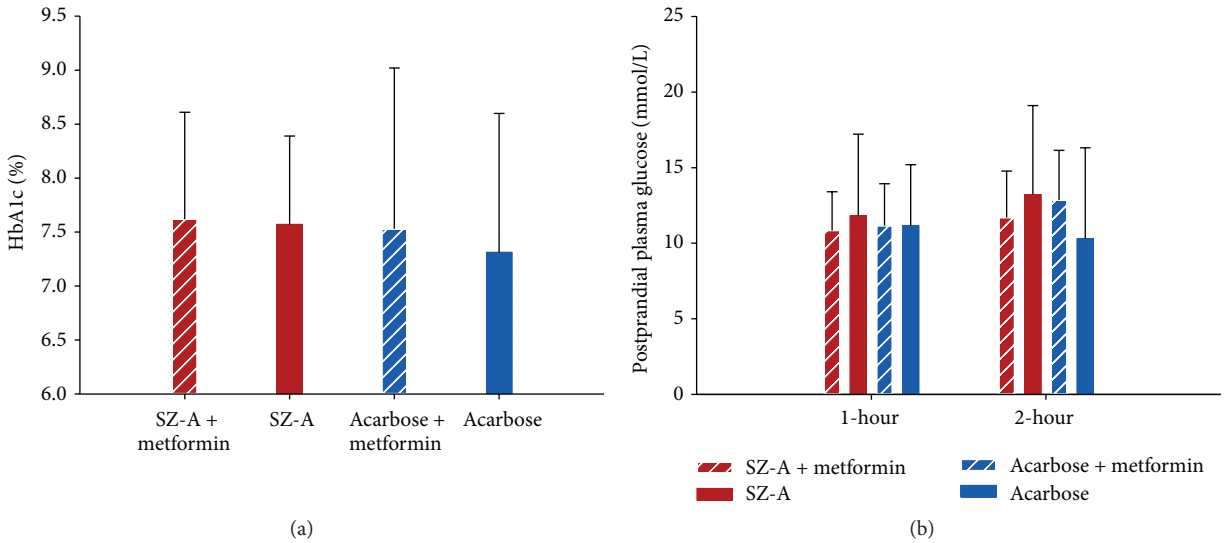

Figure 4: Comparisons of HbA1c and postprandial plasma glucose levels between monotherapy and polytherapy at week 24. (a) The difference of HbA1c among SZ-A + metformin, SZ-A, acarbose + metformin, and acarbose groups was not significant at week 24. (b) The differences of 1-hour and 2-hour postprandial plasma glucose levels between four groups were not significant at week 24.

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Research Article

Miconia sp. Increases mRNA Levels of PPAR Gamma and Inhibits Alpha Amylase and Alpha Glucosidase

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Diabetes mellitus is a public health problem worldwide. For this reason, ethanolic extract of *Miconia* sp. from Oaxaca, Mexico, was selected in search of an alternative against this disease. The effect of *Miconia* sp. on mRNA expression of PPARγ on cell line 3T3-L1, its effect on alpha amylase and alpha glucosidase, lipid accumulation during adipogenesis, and cell viability on VERO cells were evaluated. The mRNA levels of PPARγ increased on 1.393 ± 0.008 folds, lipid accumulation was increased by 29.55% with *Miconia* sp. extract and 34.57% with rosiglitazone, and α-amylase and α-glycosidase were inhibited with IC_{50} values from 28.23 ± 2.15 μg/mL and 1.95 ± 0.15 μg/mL, respectively; the IC_{50} on antiproliferative activity on VERO cells was 314.54 ± 45.40 μg/mL. In case of α-amylase and α-glycosidase assays, IC_{50} (inhibitory concentration 50) refers to necessary extract amounts to inhibit 50% of enzymatic activity. On the other hand, on antiproliferative activity, IC_{50} (inhibitory concentration 50) refers to necessary extract amounts to inhibit 50% of cell proliferation. It was concluded that the compounds present in *Miconia* sp. ethanolic extract increase mRNA expression of PPARγ, inhibit α-amylase and α-glucosidase, and increase lipid accumulation. It constitutes an alternative as adjuvant in diabetes mellitus treatment; therefore, we recommend continuing identifying the compounds responsible for its promising in vivo antidiabetic activity.

1. Introduction

Diabetes mellitus is a chronic metabolic disease considered a serious global public health problem. In 2010, approximately 285 million people suffered from this disease and this amount is expected to double up within the next 20 years [1]. Diabetes mellitus type 2 (DM2) is the most common form of diabetes. It is a complex metabolic alteration characterized by an insulin combination resistance (IR, low sensitivity of one or multiple tissues to insulin) and insulin secretion alteration [2].

The search for new drugs that act against peroxisome proliferator-activated receptor gamma (PPARγ) is very important because ligands of these transcription factors exhibit multiple biological responses such as decreasing the IR and avoiding high levels of plasm glucose. It has been shown that the adipogenesis process is under the control of a complex cascade of transcriptional regulatory factors in which PPARγ and other transcriptional factors of C/EBP family play a fundamental role [3, 4].

Enzymes α-amylase and α-glucosidase found in saliva and the brush border of the small intestine, respectively, act on hydrolysis oligosaccharides and disaccharides to produce easy absorption monosaccharides such as glucose. For the above mentioned, delaying absorption of glucose through inhibition of enzymatic hydrolysis of carbohydrates, carried

out by alpha amylase and alpha glucosidase, could be another form of combating DM [4].

About 80% of the population worldwide use medicinal plants to treat various diseases. These constitute a major source of drugs; about 25% of prescribed drugs worldwide originate from plant species [5].

A species of the genre *Miconia* in Mexican traditional medicine is used in south Mexico as an alternative for diabetes mellitus treatment. However, such fact has not been scientifically confirmed yet. *Miconia* is a genus of about 1000 species distributed in tropical America and belongs to the Melastomataceae family, which has about 4300 species distributed in 166 genera. Very few studies on biological activity have been performed on this genre, but it has been shown that extracts and compounds isolated from species of the genus *Miconia* have antibiotic, antitumor, analgesic, and antimalarial activities. A phytochemical analysis of methanolic extract of *Miconia cabucu* reveals the presence of glycosylated flavonoids (quercetin, myricetin, and kaempferol all with different glycosides), a tannin, and rare bioflavonoid. The phytochemical research of *M. rubiginosa* extract led to the identification of several glycosylated forms such as quercetin, gallic acid, and epicatechin. Similarly, quercetin, myricetin, and catechin derivatives *M. stenostachya* were found [6].

2. Materials and Methods

2.1. Preparation of the Extract. *Miconia* sp. aerial part was collected in Oaxaca, Mexico. The plant was dried in shade at room temperature and extraction with ethanol was performed by the Soxhlet method (PYREX) for 48 h; solvent was removed under reduced pressure using a rotary evaporator (Yamato RE-200). Extract yield was determined and then stored at room temperature until it was used.

2.2. Cell Culture Preparation and Adipocyte Differentiation. Murine cell line 3T3-L1 preadipocytes were used. DMEM medium supplemented with 10% fetal bovine serum (FBS) was used for propagation; it was incubated at 37°C at 5% CO_2 atmosphere, until reaching a confluence of 90%. The 100% confluency was used for adipocyte differentiation. It could be DMEM with 10% FBS, 1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, and 10 μg/mL insulin (all brands from Santa Cruz Biotechnology). After 48 hours of incubation (day 2), the differentiation medium was removed and replaced every 48 hours until maturation of adipocytes (days 9–11), with maturation medium of adipocytes (DMEM with 10% FBS and insulin 10 μg/mL called MM). The maturation was confirmed by microscope observation of the typical morphology of an adipocyte [7–10].

2.3. Expression of PPARγ in Adipocytes. The study was performed in the cell line 3T3-L1 differentiated, following the methodology described above, using microplate 6-well culture with an inoculum of 100,000 cells per well in a final volume of 2 mL with the corresponding culture medium. At day 10, adipocyte differentiation was observed and replaced in the medium (MM). The following treatments were added: Group 1: DMEM with 0.1% ethanol (expression control group), Group 2: MM supplemented with 1 μM of rosiglitazone (positive control), Group 3: MM supplemented with *Miconia* sp. extract (40 μg/mL), and Group 4: MM only to observe the effect of the medium. After 24 hours of treatment application of the total RNA extraction, the retrotranscription (cDNA) and PCR were performed in real time to determine the effect on mRNA expression [8–10].

The total RNA isolation of treated adipocytes was performed with an extraction kit SV Total RNA Isolation System # Z3100, Promega (following the manufacturer's instructions) and stored at −80°C until use. Total RNA integrity, purity, and quantification analysis was made in NanoDrop 8000 Spectrophotometer with Thermo Scientific and an electrophoresis gel (1.5% agarose).

CDNA synthesis was done with the ImProm-II™ Reverse Transcription System kit Promega # A3800 with 500 ng of RNA isolated. The cDNA was stored at −20°C, until use.

Analysis of mRNA expression PPARγ was performed by qRT-PCR using LightCycler 480 II Roche equipment, and 50 ng of cDNA, 0.5 μM of the primers, 12.5 μL of Maxima SYBR Green qPCR Master Mix (2x) # K0251 Thermo Scientific, and the proper amount of nuclease-free water to have a final volume of 25 μL. The 36B4 was used [Thomson] as reference gene. The sequence of the primers used is PPARγ forward 5′-CTGGCCTCCCTGATGAATAAAG-3′, reverse 5′-AGGCTCCATAAAGTCACCAAAG-3′, 36B4 forward 5′-ACTGGTCTAGGACCCGAGAAG-3′, and reverse 5′-TCAATGGTGCCTCTGGAGATT-3′. The relative mRNA expression was calculated based on the $2^{-\Delta\Delta Ct}$ method.

2.4. Lipid Accumulation in Adipocytes. Lipid accumulation was evaluated on the cell line 3T3-L1 using Oil Red O, with modifications to the method described by other authors. The method described above was used for cell differentiation. Microculture plates were used having 24 wells with an inoculum of 30,000 cells per well leading to a final volume of 500 μL with culture medium. At 48 h after confluence (day 0) the differentiation medium (Dm) was applied to stimulate adipogenesis, supplemented with the substances to be evaluated: treatment 1 [Dm (control reference)], treatment 2 [Dm supplemented with 40 μg/mL extract *Miconia* sp.], and treatment 3 [Dm supplemented with 1 μM rosiglitazone (positive control)]. Subsequently, the microplate was incubated for 48 h with the established conditions. After that, Dm was replaced by MM (day 2) and incubated for 8 d. The culture medium was removed, and formalin 10% was used for 1 h to fix monolayer cells. After monolayer cells had been washed twice with distilled water, the dye Oil Red O (0.5% in 60% isopropanol) was applied for 15 min at room temperature. The cells were washed three times with distilled water; the dye inside the cells was removed with 100% isopropanol and read the optical density at 540 nm [9–12].

2.5. Inhibitory Activity of α-Amylase. The enzyme inhibition was evaluated by the method of dinitrosalicylic acid 3,5-(DNS) with some modifications. The *Miconia* sp. extract was applied at different concentrations (25, 50, 75, 100, and 125 μg/mL) as a vehicle using phosphate buffer with

20 mM sodium 6.7 mM sodium chloride, at pH 6.9. α-Amylase enzyme (Sigma-Aldrich # A9857-250KU) was used at 0.5 U/mL concentration with the same buffer as a vehicle. Starch 0.5% in phosphate buffer was used as a substrate. Acarbose at different concentrations (312.5, 625, 1250, 2500, and 5000 μg/mL) was used as a positive control. Additionally, DNS solution was used at 96 mM. In 1.5 mL conical tubes, 50 μL of substances was placed to be evaluated: treatment 1: *Miconia* sp. extracts, treatment 2: buffer phosphate with 2% ethanol (negative control), and treatment 3: acarbose (positive control). Briefly, to the above treatments 50 μL of the α-amylase was added and incubated at 37°C for 10 min. After that, to initiate the reaction, 50 μL of the substrate was added immediately and incubated at 37°C for 15 min. Then, the reaction was stopped by adding 50 μL of DNS and heated in a water bath at 95°C for 5 min; the tubes were left to cool at room temperature, and the optical density was measured at 540 nm. The absorbance rates were used to calculate the percent inhibition of each treatment using the following equation:

$$\%\ \text{Inhibition} = \frac{(\text{Abs control} - \text{Abs treatment})}{\text{Abs control}} \times 100. \quad (1)$$

In this equation, "Abs treatment" is the product's light absorption of the enzyme-substrate reaction in the presence of the *Miconia* sp. or negative control as appropriate. "Abs control" is the reaction product's light absorption of enzyme-substrate in the presence of phosphate buffer as a treatment. The percentage inhibition using the statistical package (SPSS v.20) allows us to calculate the inhibitory concentration: 50 (IC_{50}) is the amount required to inhibit 50% of the enzyme (Sigma-Aldrich # A8980-1G) [5, 13–15].

2.6. α-Glucosidase Inhibitory Activity. Inhibition of the enzyme was evaluated by the pNPG method (p-nitrophenyl-α-D-glucopyranoside) with some modifications. *Miconia* sp. extract at different concentrations (1, 2, 3, 4, and 5 μg/mL) was used as a vehicle a buffer of sodium phosphate mentioned above. Acarbose at different concentrations (62.5, 125, 250, 500, and 1000 μg/mL) is positive control, using buffer sodium phosphate as a vehicle. α-Glucosidase enzyme (Sigma-Aldrich # G5003-100UN) at a concentration of 0.2 U/mL using phosphate buffer as a vehicle. Additionally, a preparation of pNPG 2 mM (Sigma-Aldrich # N1377-1G) was used as a substrate in the same vehicle. After that, in a 96-well microplate, 25 μL of the substances was mixed with the enzyme to the assay: treatment 1 [*Miconia* sp. extracts], treatment 2 [phosphate buffer with 1% ethanol (negative control)], and treatment 3 [acarbose (positive control)]. Besides the above treatments, 25 μL of the enzyme suspension was added and incubated at 37°C for 15 min; immediately after that, 50 μL of pNPG was added and incubated at 37°C for 10 min; then, the reaction was stopped by adding 50 μL sodium carbonate (0.2 M).

Finally, the optical density at 405 nm was measured. The absorbance rates were used to calculate the percent inhibition of each treatment using the following equation:

$$\%\ \text{Inhibition} = \frac{(\text{Abs control} - \text{Abs treatment})}{\text{Abs control}} \times 100. \quad (2)$$

In this equation, "Abs treatment" is the product's light absorption of the enzyme-substrate reaction in the presence of the *Miconia* sp., acarbose or negative control as appropriate. "Abs control" is the product's light absorption of the enzyme-substrate reaction in the presence of phosphate buffer as a treatment. Percent inhibitions using a statistical package (SPSS v.20) were used to determine the inhibitory concentration 50 (IC_{50}) that is the amount of treatment necessary to inhibit the enzyme 50% [4, 13–15].

2.7. Cell Proliferation Assay. Cell proliferation with MTT colorimetric method (3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide) was evaluated. The VERO cell (monkey kidney epithelial) lines are widely used in research to evaluate the effect of chemicals and toxins in mammals [16]. 1×10^5 cells/well were seeded in a 96-well microplate and adjusted to a final volume of 200 μL with culture medium (DMEM supplemented with 10% FBS) and incubated at 37°C in an atmosphere of 5% CO_2. After 24 h and until reaching a layer of 80% confluence, the culture medium was removed and cells were washed with phosphates (PBS), a buffered saline solution; further substances were added and evaluated: treatment 1 [extracts of *Miconia* sp. (62.5, 125, 250, 500, and 1000 μg/mL)], treatment 2 [DMEM with 1% ethanol (negative control)], and treatment 3 [cells with culture medium (reference of proliferation)]. After that, the microplate was incubated for 24 h at the above conditions. Then, 10 μL MTT (5 mg/mL) were added to each well and incubated again for 4 h; the culture medium was removed and cells were washed with PBS. Immediately afterwards, 200 μL dimethyl sulfoxide (DMSO) was added; then the optical density was measured at 570 nm. By the following formula the percentage of cell proliferation was determined:

$$\%\ \text{cell proliferation} = \frac{\text{Abs treatment}}{\text{Abs control}} \times 100. \quad (3)$$

In this equation, "Abs treatment" is the product's light absorption of the treated cells and "Abs control" is the product's light absorption of cells used as proliferation reference. The IC_{50} is calculated with the percentages of cell proliferation and with the support of SPSS v.20 [17, 18].

3. Results

In this study, different biological activities of ethanolic extract of *Miconia* sp. were evaluated in their effect on expression of mRNA PPARγ, lipid accumulation during adipogenesis, the activity of α-amylase and α-glucosidase, and the effect on proliferation of VERO cell line.

In determining the expression level of mRNA of PPARγ in mature mouse adipocytes, Group 1 was used as an expression control, which was normalized to a value of 1. Therefore, the

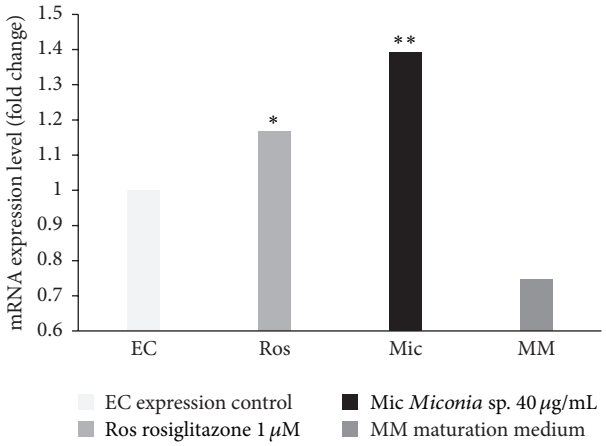

Figure 1: Effect of different compounds on the expression of mRNA PPARγ. On day 10 the treatments were applied; Group 1: DMEM with 0.1% ethanol (EC), Group 2: MM and rosiglitazone (Ros) as a positive control, Group 3: MM and *Miconia* (Mic), and Group 4: MM. On day 11 of adipocyte differentiation the total RNA was isolated. $^{*}P \leq 0.05$: there is a significant difference with the control of expression; $^{**}P \leq 0.05$: there is a significant difference with the control of expression and rosiglitazone ($n = 3$).

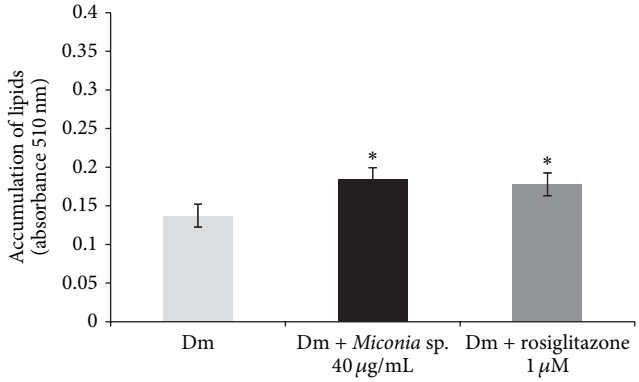

Figure 2: Effect of different substances in lipid accumulation during adipogenesis in 3T3-L1 cells. The treatments were applied at day 0 of differentiation and withdrawn at day 2. On day 10 when adipocytes reached maturity, the staining was performed. $^{*}P \leq 0.05$ significant difference between treatments and Dm ($n = 3$).

Table 1: Enzymatic inhibition from acarbose and *Miconia* sp. extract.

Treatment	α-Amylase ($IC_{50} \pm$ DE μg/mL)	α-Glucosidase ($IC_{50} \pm$ DE μg/mL)
Miconia*	28.23 ± 2.15	1.95 ± 0.15
Acarbose (positive control)	993.84 ± 157.13	331.00 ± 72.08
Phosphate buffer with 1% ethanol (negative control)	NI	NI

Data are expressed as mean ± the SD ($n = 3$). NI: no inhibition. $^{*}P \leq 0.05$ significant difference with acarbose.

values above 1 indicate an overexpression or upregulation. In Group 2 the value in the expression levels was 1.166 ± 0.007 fold change; at the same time, in Group 3 a value was 1.393 ± 0.008 and finally Group 4 showed a value of 0.746 ± 0.034 fold change (Figure 1).

Miconia sp. 40 μg/mL produced upregulation in the expression of mRNA of PPARγ with a value greater than that of the drug rosiglitazone, which increases the expression of PPARγ as expected. The use of maturation medium with both extracts as rosiglitazone did not increase by itself the expression levels of the gene PPARγ.

In the lipid accumulation test during adipogenesis with different treatments, Group 1 (Dm) showed absorbance of 0.137; this value represents the accumulated lipids during adipogenesis induced differentiation medium already established. At the same time, Group 2 (Dm + *Miconia* sp. 40 μg/mL) showed absorbance of 0.184; Group 3 (Dm + rosiglitazone 1 μM) showed an absorbance value of 0.177.

Taking the absorbance value from Group 1 as 100% lipid accumulation, when extract *Miconia* sp. was added to the differentiation medium, lipid accumulation was increased to 34.57% compared to Group 1. Like the *Miconia* sp. extract, adding the drug rosiglitazone to differentiation medium, it causes an increase of 29.55% compared to Group 1. The increased absorbance of the extract and the drug is significant compared with Dm; *Miconia* sp. presented a very similar result to rosiglitazone, difference between them not of great significance (Figure 2).

In the α-amylase inhibition assay, different treatments to the mixture of enzyme-substrate reaction were added, obtaining the following results: for treatment 1, *Miconia* sp. presented IC_{50} of 28.23 ± 2.15 μg/mL. At the same time, for treatment 2, phosphate buffer and 2% ethanol (negative control) showed no inhibition by the vehicle used; for treatment 3: acarbose presented IC_{50} of 993.84 ± 15.13 μg/mL (positive control).

According to the results and despite being a complete extract, the extract of *Miconia* sp. reveals a greater capacity than acarbose to inhibit 50% of enzyme activity; this difference between the two treatments is statistically significant (Table 1).

In the inhibition assay of α-glucosidase, the following results were obtained: for treatment 1, *Miconia* sp. extract provided IC_{50} of 1.95 ± 0.15 μg/mL. At the same time, for treatment 2, phosphate buffer with 1% ethanol (negative control) did not show any inhibitory effect on the enzyme; for treatment 3, acarbose (positive control) showed IC_{50} of 331.00 ± 72.08 μg/mL (Table 1).

Miconia sp. extract also has a greater inhibition than acarbose, and acarbose is used as a reference compound in the inhibition of these enzymes. The differences in the treatments are statistically significant.

In determining the proliferation on the VERO cell line, IC_{50} of 314.54 ± 45.40 mg/mL considered toxic was obtained for *Miconia* sp. extract.

4. Discussion

PPARγ is a nuclear receptor that acts as a transcription factor; it improves insulin sensitivity by the cells and enhances

glucose utilization [19]. A diverse set of natural and synthetic molecules is classified as ligands and can induce activation and that expression of PPARγ. These ligands include nutrients, endogenous ligands, and drugs [20]; one of those drugs is thiazolidinediones (TZDs), such as rosiglitazone. *Miconia* sp. showed an increased mRNA expression of PPARγ, even more than rosiglitazone. Moreover, *Miconia* sp. was able to increase lipid accumulation during adipogenesis in 3T3 cell line L-1 similar to positive control rosiglitazone. It is known that PPARγ is the master regulator of adipocyte differentiation, and during adipogenesis PPARγ is induced [21]. PPARγ activation in adipocytes ensures proper balance and secretion of adipokines, such as leptin and adiponectin; they are mediators in insulin action in peripheral tissues, which causes insulin sensitivity throughout the body [22]. The products mentioned above that stimulate the production of PPARγ are candidates to induce the proper functioning of insulin and recognition, considering them as potential antidiabetic agents.

The results suggest that the presence of secondary metabolites could be involved in upregulation of PPARγ gen. Quercetin, catechin, and kaempferol have been reported in some species of *Miconia* sp. and these compounds could act as ligands of PPARγ [6, 21] just as rosiglitazone does. Some TZDs as rosiglitazone have been associated with a significant increase of cardiovascular diseases. For this reason, FDA restricted their prescription in the United States [23].

Avoiding the increase of postprandial glucose is important to keep the glycemic levels low in diabetic patients. The inhibition of α-amylase and α-glucosidase present in the gastrointestinal tract could keep the levels of glycemia low. Drugs inhibit these enzymes, such as acarbose, miglitol, voglibose, nojirimycin, and 1-deoxynojirimycin, which allow the slow absorption of carbohydrates [23]. The ethanolic extract of *Miconia* sp. inhibits α-amylase by 50% to a less concentration than the acarbose drug. Additionally, the α-glucosidase is inhibited by *Miconia* sp. at low concentrations, lower than the drug. Therefore, it is believed that in the presence of molecules with antihyperglycemic effect in *in vitro* model, these compounds could be an alternative to existing treatments or adjunctive to them, which may have undesired side effects.

Miconia sp. showed cytotoxicity at a greater concentration than necessary to increase expression of PPARγ, increase lipid accumulation, and inhibit α-glucosidase and α-amylase. There are reports that the species of the genus *Miconia*, *M. stenostachya*, *M. cabucu*, *M. albicans*, and *M. rubiginosa,* lack cytotoxicity or mutagenicity at lower concentrations of 100 μg/mL [6].

5. Conclusions

The ethanolic extract of *Miconia* sp. showed increase of the level of mRNA expression of PPARγ at a significantly higher level than rosiglitazone (drug). Also, *Miconia* sp. showed inhibition of the enzymes α-glucosidase and α-amylase with IC_{50} lower than acarbose (drug) and furthermore increase the capacity of lipid accumulation during adipogenesis, similar to the drug rosiglitazone. At the same time, *Miconia* sp. showed cytotoxicity on VERO cells with a concentration higher than that presenting biological activity. For this reason, the compounds present in the ethanolic extract of *Miconia* sp. can be an alternative for the treatment of diabetes mellitus or like an adjunctive, with the recommendation of continuing with in vivo tests and elucidation of bioactive compounds.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

The Therapeutic Effect of Berberine in the Treatment of Nonalcoholic Fatty Liver Disease: A Meta-Analysis

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Aim. To assess the efficacy of berberine in the treatment of nonalcoholic fatty liver disease through meta-analysis. *Method*. We searched Embase, Pubmed, Cochrane Library, and so forth, until March 2016 for randomized controlled trials using berberine to treat NAFLD. *Result*. Six randomized controlled trials involving 501 patients were included in this study. The results showed that the efficacy of reducing TC, LDL, ALT, 2hPG, and HbA1c in NAFLD patients of the berberine group were significantly higher than that of control group. The subgroup analyses on TG, AST, and FBG indicated that treatment combined with berberine decreased TG level in NAFLD patients significantly. Compared with other drugs, berberine alone decreased TG level in NAFLD patients significantly. We also conducted a descriptive analysis on insulin resistance and radiography results that berberine can improve NAFLD patients' insulin resistance and fatty liver. *Conclusion*. According to analysis result, berberine has positive efficacy on blood lipids, blood glucose, liver function, insulin resistance, and fatty liver condition of NAFLD patients. However, due to the limitation of number and quality of trials included, more clinical randomized controlled trials with high quality are needed for further verification of the efficacy of berberine on NAFLD patients.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is a kind of metabolic stress-induced liver injury which is closely related to insulin resistance and genetic susceptibility. The spectrum of this liver disease includes nonalcoholic simple fatty liver (NAFL), nonalcoholic steatohepatitis (NASH), relevant liver cirrhosis, and hepatocellular carcinoma [1]. Around the world, the morbidity of NAFLD is 6.3%–33% [2–4], while the morbidity in overweight people and type 2 diabetic patient is, respectively, up to 58% and 74%. One longitudinal analysis of clinical data indicated that about 1/3 of NAFL patients could develop into NASH, and once they developed into NASH, the risks of liver cirrhosis, hepatocellular carcinoma, and liver failure may increase markedly [5]. Finally, these risks may lead to liver disease related disability or death of patients.

The pathogenesis of NAFLD has not been completely clarified yet. Nowadays, the possible pathogenesis involves two-hit hypothesis, insulin resistance, leptin resistance, oxidative stress, endoplasmic reticulum stress, and alteration of intestinal flora. The main therapies of this disease in modern medicine are change of life style intervention and drug therapy. In recent years, researches aimed at new drugs to treat NAFLD have not achieved new breakthrough, and the most used drugs are metformin and thiazolidinediones (in order to improve insulin resistance) as well as statins (in order to reduce blood fat). Therefore, to research and develop an effective drug which is effective in NAFLD is very significant to present medical situation.

Berberine, also called berberine hydrochloride, is the main active ingredient of traditional Chinese medicines Coptis Root and Cortex Phellodendri. Berberine is a common kind of isoquinoline alkaloid whose molecular formula is $C_{20}H_{18}NO_4$. For the past few years, extensive researches found that berberine could regulate blood glucose level, reduce blood lipid, provide an effect of antiarrhythmia and antiplatelet aggregation, enhance body immunologic function, and so on [6–9]. The latest researches demonstrated that berberine was a quite good effective drug to treat NAFLD. The

pharmacokinetics research shows that liver is the organ which contains the highest concentration of berberine metabolites, and the concentration in liver is about 70 times as large as that in plasma [10]. In addition, the half-life of berberine in liver tissue is longer than that in other tissues, and these results may explain that liver is the main target organ of berberine [11]. In the study of Li et al. [12], berberine can promote the excretion of cholesterol from liver to bile and, as a result, blood lipid can be reduced. Furthermore, berberine can regulate lipid metabolism and improve hepatic steatosis through increasing the expression of low density lipoprotein receptor (LDLR) [13]. In the study of Pérez-Rubio et al. [14], berberine can enhance patients' insulin sensitivity through PPAR-γ pathway and promote tyrosine phosphorylation in insulin receptor substrate (IRS); thus insulin resistance can be improved. Meanwhile, berberine can increase the amount of glucose transporter 4 (Glut-4) which can accelerate the ingestion of glucose and, as a result, blood glucose can be reduced [15].

Though there are several clinical trials to verify the preferable efficacy and toxic and side effects of berberine on treating NAFLD, the effectiveness of berberine has not been proved precisely due to small sample size. This study aimed to evaluate the therapeutic effect of berberine in treating NAFLD using meta-analysis in order to provide evidence for clinical decision.

2. Methods

2.1. Data Sources and Searches. The article selection process is shown in Figure 1. Electronic searches up to March 2016 were conducted in Embase, Pubmed, Cochrane CENTRAL Register of Controlled Trials, and Cochrane Database of Systematic Reviews. With regard to Chinese databases, SinoMed, Chinese journal full-text database (CNKI), VIP database, and Wanfang digital periodical full-text database were searched. The references lists of articles identified in the electronic search were hand-searched for other relevant articles.

Search terms were NAFLD, nonalcoholic fatty liver disease, NASH, nonalcoholic steatohepatitis, fatty liver, fatty liver disease, randomized controlled trial, controlled clinical trial, berberine, BBR, and huang lian su.

2.2. Study Selection. Inclusion criteria were English and Chinese articles with participants who are aged older than 18 years, who are of any sex or ethnic origin with NAFLD, who conformed to the diagnostic criteria of NAFLD (like China, the United States, Japan, Italy, and other guidelines).

Exclusion criteria were nonhuman studies, drug-induced, total parenteral nutrition-induced, and viral or genetic causes of liver injury, nonrandomized controlled trials, studies enrolling fewer than 10 subjects, case reports, reviews, and treatment time of the studies less than 2 weeks.

2.3. Intervention Measures. Berberine group (experiment group) was versus placebo group, lifestyle intervention (ISI) group, or other medicines' group (control group). Berberine group combined with other medicine group (experiment group) versus corresponding medicine group (control group).

2.4. Outcome Indicators. Total cholesterol (TC), triglyceride (TG), low density lipoprotein (LDL-C), high density lipoprotein (HDL-C), alanine transaminase (ALT), aspartate transaminase (AST), gamma-glutamyl transferase (GGT), fasting plasma glucose (FPG), 2 h postprandial plasma glucose (2hPG), glycated hemoglobin (HbA1c), insulin resistance (IR), and hepatic pathology.

2.5. Data Extraction, Management, and Analysis. Two authors independently extracted data from all included articles (Lili Yang and Xiaoyun Wei), and any disagreement was discussed and documented. Again, a third author settled disagreements that could not be solved by discussion.

Authors of studies were contacted for clarification when necessary. The quality of randomized controlled trials (RCTs) was assessed by the Cochrane Risk of Bias Tool, attributing 1 point to each item.

Review Manager (RevMan 5.3) was used to estimate pooled mean difference (MD) for continuous outcomes and odds ratio (OR) for binary outcome measures. 95% confidence interval (95% CI) will be used as effective size for the combined analysis. Heterogeneities were estimated using the I^2 statistics. When $I^2 < 50\%$ and $P > 0.10$, the results were considered homogeneous and the fixed-effect model was used; when $50\% \leq I^2 < 75\%$, the results were considered heterogeneous and the random-effect model was used. When $I^2 \geq 75\%$, sensitivity analysis or subgroup analysis was conducted to identify the causes of the heterogeneity, and if I^2 remained 75% or greater, we only provided descriptive results without pooling estimates. Statistical significance was set at $P < 0.05$. A funnel plot was used to evaluate publication bias.

3. Result

3.1. Study Description and Quality Assessment. Initially, we totally searched out 1812 records and then retained 776 records after removing duplicate records. Excluding animal experiments, case reports, reviews, and articles with incongruent intervention measure and research orientation, we achieved 6 records: 1 in English [21] and 5 in Chinese [16–20]. Among these records, the study of Yan et al. 2015 [21] includes two control groups: ISI group and pioglitazone group, so we divided this study into Yan et al. 1.2015 and Yan et al. 2.2015, two parts, before analysis. The characteristics of studies included are clarified in Table 1, the publication year ranges from 2011 to 2015, and the case load ranges from 44 to 155 (median is 84). Total case load is 501 and, among them, 231 patients are in treatment group and 270 patients are in control group.

For all the studies included, we developed an analysis of patients' baseline information. The result shows that baselines of patients' data are not different between two groups, and all

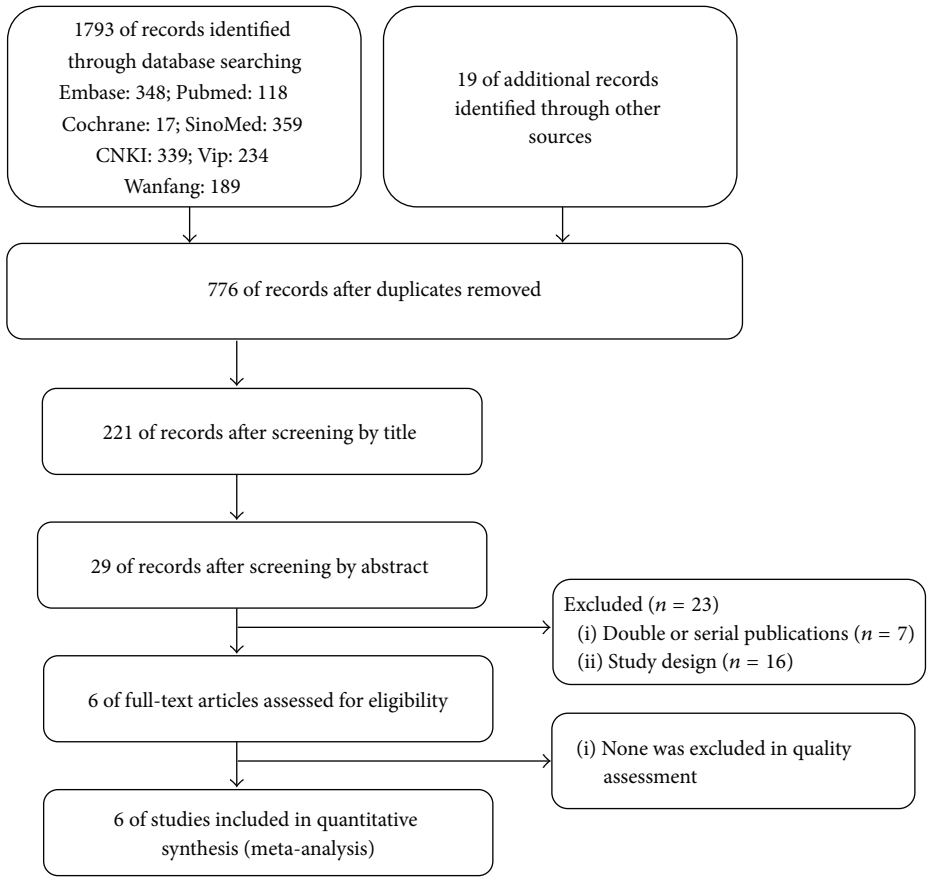

Figure 1: Identification of included studies.

Table 1: Characteristics of the 6 included studies.

Study	Sample size, n	Intervention of experimental group	Intervention of control group	Dose of berberine	Duration	Outcomes
Bai et al. 2011 [16]	78	BBR	ISI	0.5 g, tid	3 months	1, 2, 3, 5, 6, 7, 8, 11
Cao et al. 2012 [17]	78	BBR + metformin	Metformin	0.5 g, tid	16 weeks	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11
Li 2015 [18]	96	BBR	Acarbose	0.3 g, tid	3 months	1, 2, 3, 4, 5, 6, 7, 8, 9, 10
Ning et al. 2013 [19]	44	BBR + metformin	Metformin	0.5 g, tid	16 weeks	1, 2, 8, 10
Xie et al. 2011 [20]	60	BBR	Xuezhikang	0.3 g, tid	12 weeks	1, 2, 3, 4, 5, 6
Yan et al. 2015 [21]	155	BBR	ISI or pioglitazone	0.5 g, tid	16 weeks	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11

1: TC; 2: TG; 3: LDL; 4: HDL; 5: ALT; 6: AST; 7: GGT; 8: FBG; 9: 2hPG; 10: HbA1c; 11: HOMA-IR.

of the studies meet the inclusion criteria with good compatibility. The quality assessment of studies included is performed in Table 2. In the aspect of random sequence generation, 1 study applied random number table to group [18]; 1 study applied random allocation sequence produced by computer [21]; 4 studies mentioned "random" but without details of method. In the aspect of blinding, 1 study mentioned blinding but without details of method; 5 studies did not report situation of blinding and all the studies included did not report the plan of allocation and concealment. Four studies had no report about the withdrawals but provided complete outcome data, while the other 2 studies, respectively, had 1 [17] and 29 [21] patients withdrawal with the cause of loss to follow-up.

3.2. The Effect of Berberine on Blood Lipids in NAFLD Patients. Six trials reported the data of TC and TG. These trials involved 501 patients, with 231 patients and 270 patients in treatment groups and control groups, respectively. There was heterogeneity ($I^2 = 52\%$) among these trials for TC. We conducted a random-effects model for TC, the result indicated that the reduction of the levels of TC in NAFLD patients who received berberine was more obviously than ones who received other drugs or lifestyle intervention (TC

Table 2: Risk of bias assessment.

Type of bias	Bai et al. 2011 [16]	Cao et al. 2012 [17]	Li 2015 [18]	Ning et al. 2013 [19]	Xie et al. 2011 [20]	Yan et al. 2015 [21]
Random sequence generation (selection bias)	?	?	Low	?	?	Low
Allocation concealment (selection bias)	?	?	High	?	?	?
Blinding of participants and personnel (performance bias)	?	?	?	?	?	?
Blinding of outcome assessment (detection bias)	?	Low	Low	Low	Low	Low
Incomplete outcome data (attrition bias)	Low	?	Low	Low	Low	High
Selective reporting (reporting bias)	Low	Low	Low	?	Low	Low
Other biases	Low	Low	Low	Low	Low	Low

mmol/L: MD = −0.52; 95% CI −0.95 to −0.09; $P < 0.0001$). There was high statistical heterogeneity for TG ($I^2 = 81\%$, $P < 0.0001$). Therefore, we performed subgroup analysis according to berberine alone groups and berberine combination groups, including berberine combined with lifestyle intervention or other drugs. A random-effects model analysis indicated that the combination with berberine significantly reduced the levels of TG in NAFLD patients (TG mmol/L: MD = −0.68; 95% CI −0.95 to −0.40; $P < 0.0001$). There was high heterogeneity ($I^2 = 79\%$) among trials when comparing berberine alone to other drugs. Sensitivity analysis suggested that the study carried out by Xie et al. 2011 [20] made a great contribution to this high heterogeneity. When this study was excluded, the statistical between-studies heterogeneity (I^2) was 0%, $P = 0.97$. Further analysis manifested that Xuezhikang, the hypolipidemic medicine commonly used in clinic in [20], may be the main factor for the heterogeneity. So this study was excluded; as a result we found that berberine alone could significantly reduce TG than other drugs in the NAFLD patients (TG mmol/L: MD = −0.35; 95% CI −0.56 to −0.14; $P < 0.0001$) (Figure 2).

Five trials reported the data of LDL. These trials involved 449 patients, with 201 patients and 248 patients in treatment groups and control groups, respectively. The result showed statistically significant heterogeneity among studies with I^2 of 73%, $P = 0.002$. The result of a random-effects model analysis showed that berberine significantly reduced the level of LDL in NAFLD patients when compared with other drugs or lifestyle intervention (LDL mmol/L: MD = −0.45; 95% CI −0.67 to −0.23; $P < 0.0001$) (Figure 2).

Four trials reported the data of HDL. These trials involved 389 patients, with 171 patients and 218 patients in treatment groups and control groups, respectively. There was a substantial heterogeneity between trials with I^2 of 88% ($P < 0.00001$). Though sensitivity analysis and subgroup analysis were performed, the high heterogeneity remained. Therefore, we only did descriptive analysis. Two of these trials [17, 18] reported that berberine increased the level of HDL significantly in NAFLD patients. And the other two trials [20, 21] reported that berberine was likely to increase the level of HDL in NAFLD patients (Figure 2).

3.3. The Effect of Berberine on Liver Function in NAFLD Patients. Five trials reported the data of ALT and AST. These trials involved 457 patients, with 209 patients and 248 patients in treatment groups and control groups, respectively. There was a heterogeneity for ALT with I^2 of 73%. A random-effects model demonstrated that the reduction of ALT in NAFLD patients treated by berberine was more significant than that of other drugs or lifestyle intervention (ALT U/L: MD = −7.37; 95% CI −12.31 to −2.42). There was high statistical heterogeneity for AST ($I^2 = 76\%$, $P < 0.0001$). The subgroup analysis was conducted according to berberine-alone groups and berberine combination groups. It revealed that the berberine-alone groups were more likely to reduce AST in NAFLD patients than other drugs or lifestyle interventions, which were analyzed by a random-effects model (AST U/L: MD = −2.06; 95% CI −5.86 to 1.74; $P = 0.15$). There was substantial heterogeneity among berberine combination trials with I^2 of 82%, $P < 0.003$. So we just performed descriptive analysis where berberine decreased the levels of AST in two trials; and, in one trial, berberine groups substantially reduced the levels of AST from baseline, but the reduction was insignificant compared with control groups (Figure 3).

Four trials reported the data of GGT. These trials involved 397 patients, with 179 patients and 218 patients in treatment groups and control groups, respectively. There was high unexplained heterogeneity of GGT among four trials with I^2 of 80%, $P = 0.0005$. Berberine decreased GGT in three trials [16–18]; and one trail showed that berberine had a tendency of decreasing GGT in NAFLD patients [21] (Figure 3).

3.4. The Effect of Berberine on Blood Glucose in NAFLD Patients. Five trials reported the data of FBG. These trials involved 441 patients, with 201 patients and 240 patients in treatment groups and control groups, respectively. High heterogeneity was present in these five studies with I^2 of 80%, $P = 0.0001$. We preformed the subgroup analysis in the light of berberine-alone groups and berberine combination groups. The analysis indicated that berberine-alone groups were more inclined to reduce the level of FBG in NAFLD

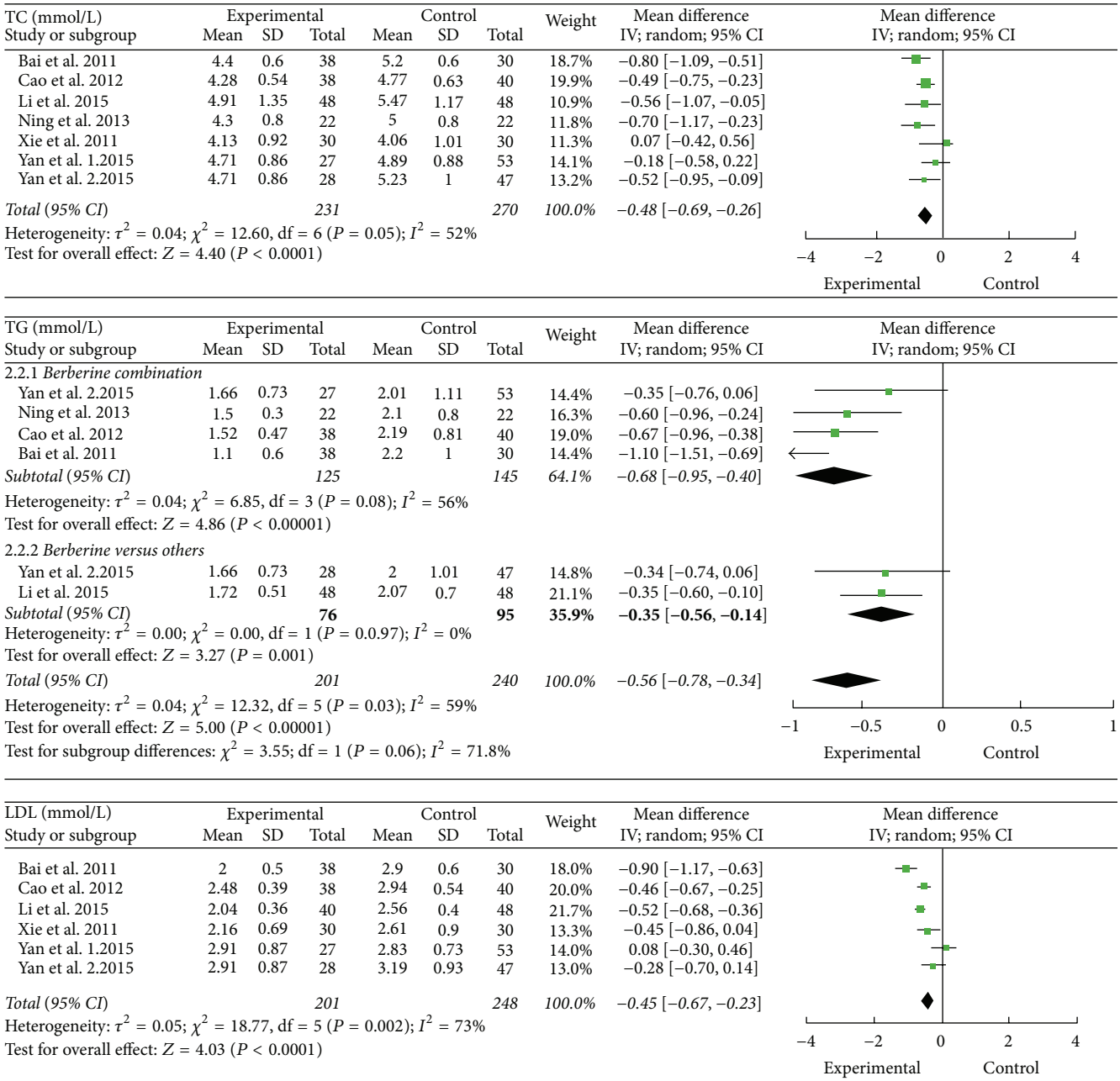

Figure 2: The effect of berberine on blood lipid in NAFLD patients.

patients than other drugs, which were analyzed by a random-effects model (FBG mmol/L: MD = −0.26; 95% CI −0.58 to −0.05; $P = 0.10$). The results of berberine combination for NAFLD patients showed high heterogeneity with I^2 of 79%, $P = 0.003$. Therefore, description analysis was used and it suggested that the treatment of berberine combination had the tendency of reducing the levels of FBG (Figure 4).

Four trials reported the data of HbA1c. These trials involved 373 patients, with 163 patients and 210 patients in treatment groups and control groups, respectively. There was heterogeneity among these four trials (I^2 of 68%, $P = 0.01$). A random-effects model analysis was used, and the result showed that berberine reduced HbA1c in NAFLD patients significantly compared with other drugs or lifestyle interventions (HbA1c (%): MD = −0.35; 95% CI −0.61 to −0.09; $P = 0.01$) (Figure 4).

Three trials reported the data of 2hPG. These studies involved 329 patients, with 141 patients and 188 patients in treatment groups and control groups, respectively. There was no significant heterogeneity with I^2 of 0%, $P = 0.89$. A fixed-effects model analysis showed that berberine significantly reduced 2hPG in NAFLD patients compared with other drugs or lifestyle interventions (2hPG mmol/L: MD = −0.43; 95% CI −0.70 to −0.17; $P = 0.001$) (Figure 4).

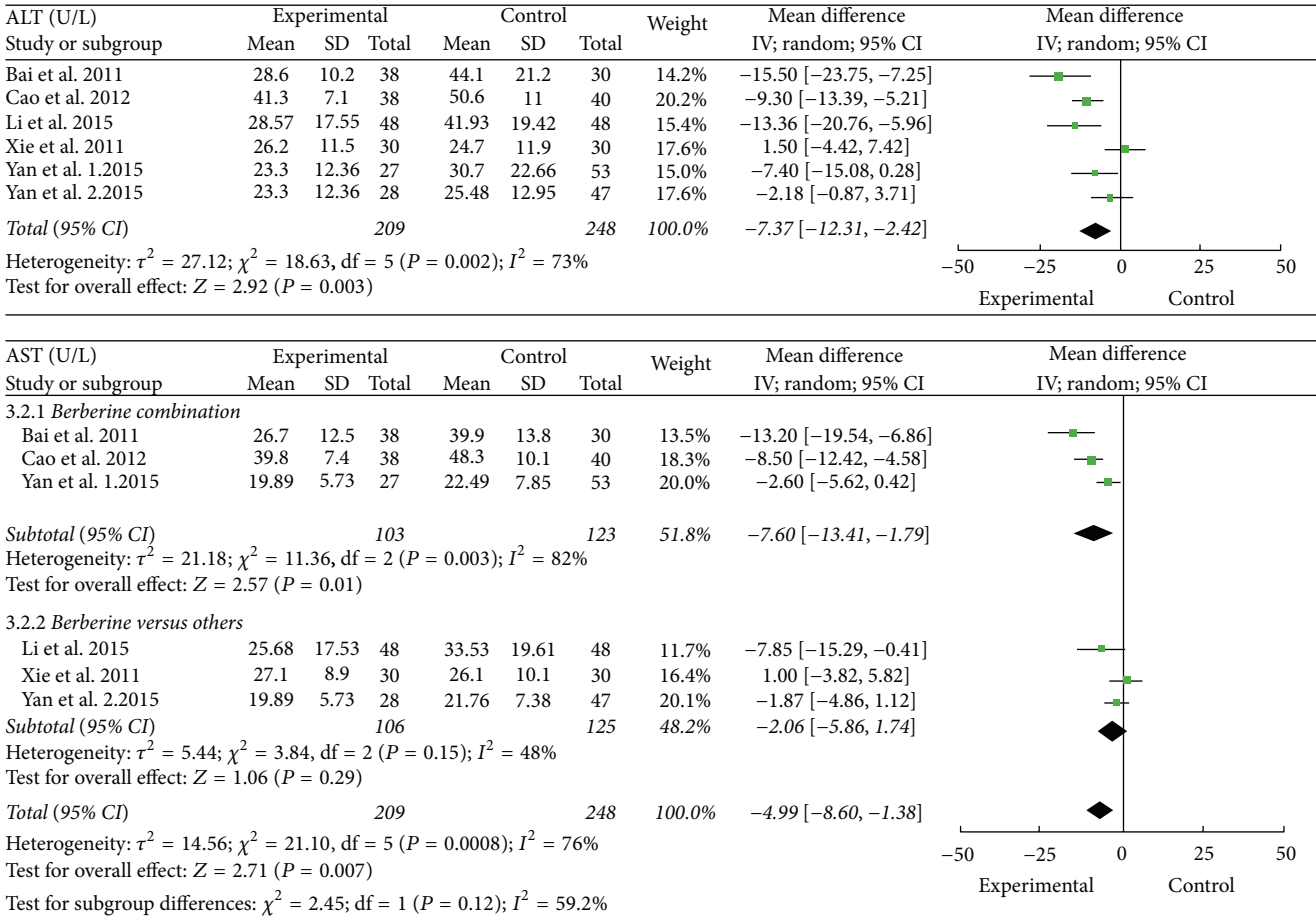

Figure 3: The effect of berberine on liver function in NAFLD patients.

3.5. The Effect of Berberine on Insulin Resistance in NAFLD Patients. Three trials reported the data of homeostatic model of assessment-insulin resistance (HOMA-IR) using the formula HOMA-IR = FPG × FINS/22.5. These trials involved 301 patients, with 131 patients and 170 patients in treatment groups and control groups, respectively. Among them, one trial providing the data of HOMA-IR with the mean differences and 95% confidence interval cannot undertake the data transformation, and there was high statistical heterogeneity for two other trials with I^2 of 92%, $P = 0.0004$. Therefore, here we just did descriptive analysis for this indicator.

In the study of Bai et al. 2011 [16] and Yan et al. 2015 [21], compared with lifestyle intervention, berberine could significantly lower HOMA-IR; the study of Cao et al. 2012 [17] found that, compared with single intervention of metformin, metformin combination with berberine could significantly decrease HOMA-IR of patients with NAFLD; Yan et al. 2015 [21] also found that the combination of berberine and pioglitazone had the trend of improving insulin resistance; however, the difference had no statistical significance.

3.6. The Effect of Berberine on Fatty Liver in Patients with NAFLD. A total of three trials studied the effect of berberine on fatty liver in patients with NAFLD, involving a total of 259 patients, and the treatment group consisted of 107 patients and the control group consisted of 152 patients. There were 2 trials [19, 20] that used the liver ultrasonic examination to evaluate the degree of fatty liver, and 1 trial [21] evaluated the degree of fatty liver by a proton magnetic resonance spectroscopy (H MRS). Due to different evaluation methods, the data cannot be merged, and we just did descriptive analysis.

In the study of Yan et al. 2015 [21], it was found that, compared with lifestyle intervention group, berberine could significantly lower liver lipid content in patients with NAFLD; in the study of Xie et al. 2011 [20], it was found that the obvious effect rate was 70% after treatment with berberine via liver type-B ultrasonic examination, and there was no statistically significant difference compared with control group; in the study of Ning et al. 2013 [19] with liver type-B ultrasonic examination hemodynamic evaluation of fatty liver found that, compared with control group, berberine could significantly improve the condition of fatty liver in patients with NAFLD.

4. Discussion

Currently, the incidence of NAFLD shows an increased and low aging tendency along with the improvement of people's living standard, and, in patients with type 2 diabetes and

FGB (mmol/L) Study or subgroup	Experimental Mean	SD	Total	Control Mean	SD	Total	Weight	Mean difference IV; random; 95% CI
4.1.1 *Berberine combination*								
Bai et al. 2011	5.1	0.6	38	6.9	2.4	30	9.4%	−1.80 [−2.68, −0.92]
Cao et al. 2012	6.7	0.9	38	7.2	1	40	17.4%	−0.50 [−0.92, −0.08]
Ning et al. 2013	7.1	0.4	22	8.1	0.7	22	19.1%	−1.00 [−1.34, −0.66]
Yan et al. 1.2015	5.92	1.1	27	6.09	1.05	53	15.7%	−0.17 [−0.67, 0.33]
Subtotal (95% CI)			*125*			*145*	*61.7%*	*−7.90 [−1.32, −0.26]*
Heterogeneity: $\tau^2 = 0.22$; $\chi^2 = 14.06$, df = 3 ($P = 0.003$); $I^2 = 79\%$								
Test for overall effect: $Z = 2.92$ ($P = 0.003$)								
4.1.2 *Berberine versus others*								
Li et al. 2015	5.51	0.31	48	5.87	0.38	48	22.4%	−0.36 [−0.50, −0.22]
Yan et al. 2.2015	5.92	1.1	28	5.92	0.97	47	15.9%	−0.00 [−0.49, 0.49]
Subtotal (95% CI)			*76*			*95*	*38.3%*	*−0.26 [−0.58, 0.05]*
Heterogeneity: $\tau^2 = 0.03$; $\chi^2 = 1.90$, df = 1 ($P = 0.17$); $I^2 = 47\%$								
Test for overall effect: $Z = 1.62$ ($P = 0.10$)								
Total (95% CI)			*201*			*240*	*100.0%*	*−0.56 [−0.91, −0.20]*
Heterogeneity: $\tau^2 = 0.14$; $\chi^2 = 25.32$, df = 5 ($P = 0.0001$); $I^2 = 80\%$								
Test for overall effect: $Z = 3.10$ ($P = 0.002$)								
Test for subgroup differences: $\chi^2 = 2.82$; df = 1 ($P = 0.09$); $I^2 = 64.6\%$								

Mean difference IV; random; 95% CI

−4 −2 0 2 4

Experimental Control

HbA1c (%) Study or subgroup	Experimental Mean	SD	Total	Control Mean	SD	Total	Weight	Mean difference IV; random; 95% CI
Cao et al. 2012	7.4	0.9	38	7.8	0.7	40	20.0%	−0.40 [−0.76, −0.04]
Li et al. 2015	5.61	1.18	48	6.12	1.17	48	15.8%	−0.51 [−0.98, −0.04]
Ning et al. 2013	7.8	0.8	22	8.9	1.2	22	11.9%	−1.10 [−1.70, −0.50]
Yan et al. 1.2015	5.93	0.42	27	6.06	0.65	53	25.5%	−0.13 [−0.37, 0.11]
Yan et al. 2.2015	5.93	0.42	28	6.03	0.46	47	26.9%	−0.10 [−0.30, 0.10]
Total (95% CI)			*163*			*210*	*100.0%*	*−0.35 [−0.61, −0.09]*
Heterogeneity: $\tau^2 = 0.06$; $\chi^2 = 12.48$, df = 4 ($P = 0.01$); $I^2 = 68\%$								
Test for overall effect: $Z = 2.63$ ($P = 0.009$)								

Mean difference IV; random; 95% CI

−4 −2 0 2 4

Experimental Control

2hPG (mmol/L) Study or subgroup	Experimental Mean	SD	Total	Control Mean	SD	Total	Weight	Mean difference IV; fixed; 95% CI
Cao et al. 2012	8.1	1.2	38	8.7	1.4	40	20.7%	−0.60 [−1.18, −0.02]
Li et al. 2015	8.11	0.75	48	8.48	0.81	48	70.9%	−0.37 [−0.68, −0.06]
Yan et al. 1.2015	8.69	2.55	27	9.15	2.98	53	4.4%	−0.46 [−1.71, 0.79]
Yan et al. 2.2015	8.69	2.55	28	9.37	3.24	47	4.0%	−0.68 [−2.00, 0.64]
Total (95% CI)			*141*			*188*	*100.0%*	*−0.43 [−0.70, −0.17]*
Heterogeneity: $\chi^2 = 0.61$, df = 3 ($P = 0.89$); $I^2 = 0\%$								
Test for overall effect: $Z = 3.23$ ($P = 0.001$)								

Mean difference IV; fixed; 95% CI

−10 −5 0 5 10

Experimental Control

Figure 4: The effect of berberine on blood glucose in NAFLD patients.

obesity, the incidence of NAFLD is much higher. Studies found that, compared with normal people, patients with NAFLD had a higher mortality rate, and the risk for the development of cardiovascular disease and metabolic syndrome related cancer increased [22]. Nowadays, researches of NAFLD receive more attention, and NAFLD has become one of the world's important public health problems in the 21st century.

It is still important that improving the way of life and exercise dominate the treatment for NAFLD; however, patients often cannot adhere to it because the effect needs a long term to be seen, so adherence to this therapy is poor. Other treatments for NAFLD include drug therapy (lipid-lowering drugs, insulin sensitizing agent, liver-protection medicine, antioxidant, etc.) and surgery, but their efficacies are not precise.

With meta-analysis through 6 studies including 501 patients, this review suggests that berberine has a positive effect on many aspects in patients with NAFLD including improving blood lipids (TC, TG, and LDL), liver function (ALT, AST), and blood glucose (FBG, 2hPG, and HbA1c). Compared with control group, berberine can decrease the TC, LDL, ALT, 2hPG, and HbA1c level in patients with NAFLD, and the difference is statistically significant. The subgroup analysis of studies on TG, AST, and FBG indicates that the combination of berberine treatment can significantly reduce TG levels in patients with NAFLD, and results of researches on combination of berberine treatment tend to the fact that berberine has the effect of reducing AST and FBG, but, at present, the results have considerable heterogeneity. Compared with other drugs, treatment with berberine alone reduced TG levels in patients with NAFLD, and the effect of

berberine alone has a tendency to decrease the AST and FBG in patients with NAFLD. Berberine also has an improvement effect on NAFLD patients' insulin resistance and fatty liver condition.

Since the number of included studies was fewer than 10, this review did not assess risk of publication bias. The research time of these articles included in this study was shorter, in which the longest one was 16 weeks, while NAFLD is chronic disease, and it might take longer time to manage the improvement of liver pathological index and biochemical index. Therefore, it cannot well figure out the therapeutic effect of berberine on NAFLD, which might be shown better if more studies with longer term were included. In addition, articles included in this study did not provide the methods of blind and allocation concealment, and the random allocation concealments of most studies were not described in detail. In terms of evaluation indexes, not widely using imaging evaluation including CT and MRI, besides the small sample sizes of most articles included, can be the factors causing bias; therefore we should be cautious about the results of the meta-analysis.

In conclusion, based on current evidence, berberine can significantly improve blood lipids and liver function in patients with NAFLD and has good advantage in reducing blood glucose in patients with NAFLD, which might be a new choice for the treatment of NAFLD. Due to the limit of the number and quality of the trials included, the conclusions need to be further validated by more strictly designed multicentered RCTs of high quality and large scale.

Competing Interests

The authors declare that there are no competing interests involved in this paper.

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Research Article

Antcin K, a Triterpenoid Compound from *Antrodia camphorata*, Displays Antidiabetic and Antihyperlipidemic Effects via Glucose Transporter 4 and AMP-Activated Protein Kinase Phosphorylation in Muscles

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The purpose of this study was to screen firstly the potential effects of antcin K (AnK), the main constituent of the fruiting body of *Antrodia camphorata*, *in vitro* and further evaluate the activities and mechanisms in high-fat-diet- (HFD-) induced mice. Following 8-week HFD-induction, mice were treated with AnK, fenofibrate (Feno), metformin (Metf), or vehicle for 4 weeks afterward. In C2C12 myotube cells, the membrane GLUT4 and phospho-Akt expressions were higher in insulin and AnK-treated groups than in the control group. It was observed that AnK-treated mice significantly lowered blood glucose, triglyceride, total cholesterol, and leptin levels in AnK-treated groups. Of interest, AnK at 40 mg/kg/day dosage displayed both antihyperglycemic effect comparable to Metf (300 mg/kg/day) and antihypertriglyceridemic effect comparable to Feno (250 mg/kg/day). The combination of significantly increased skeletal muscular membrane expression levels of glucose transporter 4 (GLUT4) but decreased hepatic glucose-6-phosphatase (G6 Pase) mRNA levels by AnK thus contributed to a decrease in blood glucose levels. Furthermore, AnK enhanced phosphorylation of AMP-activated protein kinase (phospho-AMPK) expressions in the muscle and liver. Moreover, AnK treatment exhibited inhibition of hepatic fatty acid synthase (FAS) but enhancement of fatty acid oxidation peroxisome proliferator-activated receptor α (PPARα) expression coincident with reduced sterol response element binding protein-1c (SREBP-1c) mRNA levels in the liver may contribute to decreased plasma triglycerides, hepatic steatosis, and total cholesterol levels. The present findings indicate that AnK displays an advantageous therapeutic potential for the management of type 2 diabetes and hyperlipidemia.

1. Introduction

Diabetes mellitus hardly occurs in isolation but is most often part of an array of metabolic abnormalities that includes insulin resistance, hyperinsulinemia, and hypertriglyceridemia. The population of type 2 diabetes prevalence by 2025 will reach approximately 300 million [1]. Pathogenesis of type 2 diabetes has been proposed to display more than 90% of all diabetes mellitus patients [2]. Type 2 diabetes mellitus has revealed mechanisms of insulin resistance that target either impairs in β-cell function or insulin insensitive action at adipose tissue, skeletal muscle, or liver tissues.

Antrodia camphorata (Polyporaceae, Aphyllophorales) is edible as a folk remedy in the treatment of a variety of diseases in Taiwan. It is rare and expensive because it grows only on the inner heartwood wall of the endemic evergreen *Cinnamomum kanehirai*. The mycelia, filtrate of broth, and fruiting body of *A. camphorata* exhibit numerous physiological

functions [3]. The fruiting body of *A. camphorata* consisted of terpenoids, such as antcins (A, B, and C), zhankuic acids (A, B, C, D, and E), 15α-acetyl-dehydrosulphurenic acid, dehydroeburicoic acid and dehydrosulphurenic acid, antcin E and F, methyl antcinate G and methyl antcinate H, and eburicoic acid. The solid culture of fruiting body and the filtrate in submerged culture have been shown to have hepatoprotective effects and antioxidant activities [4, 5]. Previous study had demonstrated that, in terms of *in vivo* metabolism, 13 terpenoids in *A. camphorata* were determined by using LC/MS/MS in rats plasma after oral administration, and plasma concentrations of ergostanoids were much higher than lanostanoids, and the ergostanoids underwent reduction and hydroxylation reactions *in vivo* [6]. Their mean residence time (MRT) ranged from 3 to 6 hr, and the lanostanoids were not active to metabolic reactions and were slowly eliminated with an MRT of 9–16 hr [6]. Antcin K (3α,4β,7β-trihydroxy-4α-methylergosta-8,24(28)-dien-11-on-26-oic acid, 2; AnK) (Figure 1), an active triterpenoid from the fruiting bodies of basswood cultivated *A. cinnamomea*, could induce apoptotic cell death in human liver cancer Hep3B cells [7]. Antcin K isolated from ethanol extracts of wild fruiting body has shown concentration-dependent (1–25 Mm) anti-inflammatory effects (by modulation of leukocyte activity and inhibition of ROS) induced by fMLP and TPA in human neutrophils [8, 9]. Our recent studies demonstrated that ergostatrien-3β-ol and dehydroeburicoic acid from *A. camphorata* exhibited an excellent antihyperglycemic and antihyperlipidemic activity [10, 11]. Nevertheless, the effects of antcin K, the main constituent of the fruiting body of *A. camphorata*, on diabetes and dyslipidemia are still unknown *in vitro* and in diet-induced diabetic rodents.

The glucose transporter 4 (GLUT4) has been regarded as a vital determinant of blood glucose homeostasis [12]. The elevated glucose levels, after huge caloric ingestions, are rapidly returned to normal. Insulin stimulates or contraction causes glucose uptake via eliciting translocation of GLUT4 from intracellular sites to the membrane [13, 14]. Levels of insulin-induced GLUT4 translocation in skeletal muscle of type 2 diabetic patients are markedly decreased [15]. Therefore, the improvement of GLUT4 levels or induced translocation may accelerate drug development. Peripheral glucose uptake into membrane of skeletal muscle could be promoted by two pathways including insulin-dependent mechanisms leading to Akt/PKB activation and contraction-regulated stimulation [16, 17] or hypoxia-regulated AMPK activation [17, 18]. AMPK play a dominant role in glucose and lipid metabolism. Since dysregulation of glucose and lipid catabolism in type 2 diabetes, AMPK activators would be promising therapies [19].

Metformin is used in the clinics as an antidiabetic drug in the management of type 2 diabetes [19] and it activates AMPK in both hepatocyte and skeletal muscle [19, 20].

Peroxisome proliferator-activated receptor α (PPARα) plays a key role in regulation of lipid metabolism [21] and reduces circulating triglyceride (TG) concentrations via regulated numerous genes associated with lipogenic and fatty acids oxidation [22]. Fenofibrate is one of PPARα agonists and has been used in the treatment of hypertriglyceridemia [23, 24].

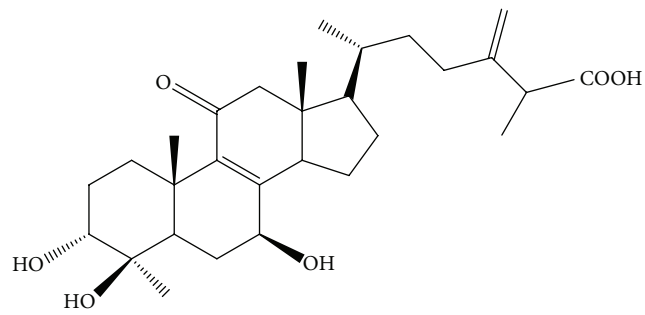


Figure 1: Chemical structure of antcin K (AnK).

The high-fat diet- (HFD-) fed C57BL/6J mouse could induce early type 2 diabetes and markedly increased adipose weights and produced resistance to insulin and increases in blood glucose, total cholesterol (TC), and TG levels [25–27]. Thus, this model was chosen to investigate both mechanistic activities and as a tool for developing novel therapeutic interventions [25]. Phosphorylation of Thr^{172} of α subunits is essential for AMPK activity [28]. This study was to screen firstly the potential effects of AnK *in vitro* and further to investigate the hypothesis that AnK could display the beneficial metabolic effects including antidiabetic and hypolipidemic effects by modulation of GLUT4 protein expression and activation of AMPK as compared with clinical drugs such as Metf and Feno; moreover, the targeted gene expressions were determined including PPARα and fatty acid synthase (FAS) from the peripheral tissues of HFD-fed mice by the AnK treatment.

2. Materials and Methods

2.1. Chemicals. Antibodies of GLUT4 (number sc-79838) were obtained from Santa Cruz Biotech (Santa Cruz, CA, USA); phospho-AMPK (Thr^{172}), PPARα (number ab8934), and PPARγ (number ab45036) were purchased from Abcam Inc. (Cambridge, MA, USA); FAS (number 3180), phospho-Akt (Ser473) (number 4060), total-AMPK (Thr^{172}), and β-actin (number 4970) were from Cell Signaling Technology (Danvers, MA, USA). Secondary antibody anti-rabbit was from Jackson ImmunoRes. Lab., Inc. (West Grove, PA, USA).

2.2. Determination of the Active Compound. The fruiting body of *A. camphorata* was purchased from the Balay Biotechnology Corporation, Hsinchu City, Taiwan. A voucher specimen (CMPC393) was deposited at and identified by China Medical University. The fruiting bodies of AC (3.0 kg) were extracted three times with methanol and followed by chromatography using 50% ethyl acetate and 50% hexane. The procedure was as in a previously described report [29]. The purity of AnK is above 99%. Analytical instrument is the HPLC, SHIMADZU LC 20-A; the HPLC Column, TOSOH TSKgel DS-80Ts, and analytical condition, 100% MeOH.

2.3. Cell Culture. C2C12 skeletal myoblasts (ATCC, CRL-1772) were employed and performed as a previous report [11].

2.4. Detection of Expression Levels of Membrane GLUT4 and Phosphorylation of Akt (Ser473) In Vitro. The procedure was performed as a previous description [11, 30, 31]. Differentiated C2C12 cells were serum-starved in DMEM/BSA prior to incubation either with test compounds (AnK at 1, 5, 10, and 25 μg/mL) or with vehicle for 30 min or with 100 nM insulin for 25 min, as previously described [32]. The homogenates were centrifuged and the pellet was resuspended and performed within membrane; protein concentration was analyzed via BCA assay (Pierce), and equal amounts of protein were then diluted four times in SDS sample buffer and subjected to SDS PAGE and were detected by Western blotting with antibodies specific for Akt, phospho-Akt Ser473, and GLUT4; and the analysis of density blotting was as in a previous report [11].

2.5. Animals and Treatments. The part of animal studies was performed under the guidelines of the Institutional Animal Care and Use Committee (12 March 2015). The C57BL/6J mice (male) aged 4 weeks (total amount = 63) were obtained from the National Laboratory Animal Breeding Center. All rodents were haphazardly partitioned to control (CON) group (control diet) (Diet 12450B, Research Diets, Inc.; low-fat diet) ($n = 9$) and high-fat diet (HFD) (Diet 12451, Research Diets, Inc.) group [10, 33, 34]. The low-fat diet was composed of protein 20%, carbohydrate 70%, and fat 10%, whereas high-fat diet was composed of protein 20%, carbohydrate 35%, and fat 45% (of total energy, % kcal). The CON mice were on the control diet, and the HFD mice were on 45% HFD for 12 weeks [33]. The control diet or HFD is comprised of 10% fat or 45% fat, respectively. After HFD-induction for 8 weeks, the HFD-fed group (total amounts: 54 mice) was again divided into 6 groups ($n = 9$, per group) as follows: treatment with AnK (including AnK1: 10, AnK2: 20, and AnK3: 40 mg/kg/day bw), or fenofibrate (Feno: 0.25 g/kg/day bw, Sigma Chemical Co.), or metformin (0.3 g/kg/day bw), or vehicle with oral gavage one time every day for 28 days, and the CON and high-fat control (HF) groups were given only vehicle [10, 33]. After administration of AnK, Feno, or Metf for 4 weeks, the mice (12 h fasting) were sacrificed and peripheral tissues were weighed. Parts of tissues were immediately stored at −80°C for targeted genes analysis. Blood glucose analysis and biochemical parameters (including TG, TC, and FFA), adipocytokine (including insulin, adiponectin, and leptin) levels, and metabolic parameters including body weight, weight gain, and food intake were performed as previous procedures [10, 11, 33].

2.6. Assessment of Blood Glucose and Biochemical Parameters. Blood sample was obtained from the retro-orbital sinus of 12 h fasting mice. Blood glucose level (by the glucose oxidase method); plasma TG, TC, and free fatty acids level (using commercial assay kits); and insulin, leptin, and adiponectin level (by enzyme-linked immunosorbent assay (ELISA) kits) were measured as previous reports [11, 33, 35, 36].

2.7. Histopathology Examination. Parts of visceral adipose and liver specimen were measured and pictures were taken as previous reports [11, 33, 36].

2.8. Analysis of Liver Lipids. This procedure was performed as in previous reports [37].

2.9. Relative Quantization of mRNA Indicating Gene Levels and Western Blotting. These procedures of relative quantization of mRNA (the primers are described in Table 1) and immunoblots in the measurement of skeletal muscular GLUT4, phospho-AMPK (Thr^{172})/total-AMPK (Thr^{172}), or phospho-Akt (Ser473)/total-Akt (Ser473) proteins from the muscle and liver of mice were performed as previous procedures elsewhere [10, 11, 33, 35, 36]. PPARα and FAS proteins were performed from the liver tissue and PPARγ and FAS proteins from the adipose tissue of mice. Skeletal muscle from mice was subjected to GLUT4 expression level analysis. Total membrane fraction was measured; and the expression levels of GLUT4, phospho-AMPK, and total-AMPK were determined by Western blotting as in described reports [10, 11, 33, 35, 36].

2.10. Statistics. Results present means and standard error. Comparisons among groups were using ANOVA and coupled with Dunnett's tests. P values less than 0.05 were regarded as statistically significant differences.

3. Results

3.1. Membrane GLUT4 and Akt Phosphorylation Expression In Vitro. The membrane GLUT4 expressions were higher in the insulin- and AnK-treated (5, 10, and 25 μg/mL) groups than in the CON group. The phospho-Akt (Ser473)/total-Akt expressions were higher in the insulin- and AnK-treated (10 and 25 μg/mL) groups than in CON group (Figures 2(a) and 2(b)).

3.2. Metabolic Parameters. At the beginning, the average body weights of all mice were 20.05 ± 0.13 g. At the end, body weight and body weight gain were markedly enhanced in HFD-induced mice (Table 2). AnK2-, AnK3-, or Feno-treated mice had decreased body weight, while AnK1-, AnK2-, AnK3-, Feno-, or Metf- treated groups had decreased body weight gain. The HF mice consume less food intake than CON mice (Table 2). No difference was found in food intake between AnK-, Feno-, or Metf-treated groups and HF group. Feeding a HFD displayed increases in absolute epididymal, mesenteric, retroperitoneal white adipose tissue (WAT) and visceral fat weights (Table 2). The AnK1-, AnK2-, AnK3-, Feno-, or Metf-treated groups reduced epididymal, retroperitoneal WAT, mesenteric WAT, and visceral fat weights. Feno-treated mice showed a decrease in brown adipose tissue (BAT) weights, but increased weights of the liver (Table 2).

3.3. Fasting Blood Glucose Levels, Biochemical Parameters, Adipocytokine Levels, and Liver Lipids. It is evident that hyperglycemia has been observed after 12 weeks of HFD

Table 1: Primers used in this study.

Gene	Accession number	Forward primer and reverse primer	PCR product (bp)	Annealing temperature (°C)
		Liver		
G6 Pase	NM_008061.3	F: GAACAACTAAAGCCTCTGAAAC R: TTGCTCGATACATAAAACACTC	350	50
SREBP1c	NM_011480	F: GGCTGTTGTCTACCATAAGC R: AGGAAGAAACGTGTCAAGAA	219	48
DGAT2	NM_026384.3	F: AGTGGCAATGCTATCATCATCGT R: AAGGAATAAGTGGGAACCAGATCA	149	50
apo C-III	NM_023114.3	F: CAGTTTTATCCCTAGAAGCA R: TCTCACGACTCAATAGCTG	349	47
SREBP2	AF289715.2	F: ATATCATTGAAAAGCGCTAC R: ATTTTCAAGTCCACATCACT	256	48
PPARα	NM_011144	F: ACCTCTGTTCATGTCAGACC R: ATAACCACAGACCAACCAAG	352	49
aP2	NM_024406	F: TCACCTGGAAGACAGCTCCT R: TGCCTGCCACTTTCCTTGT	142	52
GAPDH	NM_008084.3	F: TGTGTCCGTCGTGGATCTGA R: CCTGCTTCACCACCTTCTTGA	99	55

Table 2: Effects of antcin K (AnK) on tissue weight, food intake, and liver lipid.

Dose (mg/kg/day)	CON	HF	HF + AnK1 10	HF + AnK2 20	HF + AnK3 40	HF + Feno 250	HF + Metf 300
			Absolute tissue weight (g)				
EWAT	0.531 ± 0.052	1.264 ± 0.147$^{\#\#\#}$	0.867 ± 0.065**	0.841 ± 0.062**	0.809 ± 0.058***	0.603 ± 0.041***	0.813 ± 0.064***
MWAT	0.278 ± 0.031	0.439 ± 0.025$^{\#\#\#}$	0.349 ± 0.020*	0.340 ± 0.013*	0.332 ± 0.025*	0.247 ± 0.025***	0.270 ± 0.018***
RWAT	0.166 ± 0.021	0.483 ± 0.064$^{\#\#\#}$	0.323 ± 0.039*	0.339 ± 0.031*	0.306 ± 0.040*	0.181 ± 0.020***	0.298 ± 0.027**
Visceral fat	0.697 ± 0.056	1.747 ± 0.208$^{\#\#\#}$	1.190 ± 0.093**	1.180 ± 0.106**	1.154 ± 0.096***	0.784 ± 0.052***	1.111 ± 0.077***
Skeletal muscle	0.308 ± 0.014	0.412 ± 0.045	0.395 ± 0.036	0.364 ± 0.022	0.364 ± 0.028	0.428 ± 0.026	0.380 ± 0.025
BAT	0.158 ± 0.004	0.224 ± 0.022$^{\#}$	0.178 ± 0.007	0.172 ± 0.010	0.175 ± 0.008	0.157 ± 0.013*	0.220 ± 0.025
Liver (g)	1.003 ± 0.024	0.987 ± 0.029	0.946 ± 0.030	0.888 ± 0.019	0.883 ± 0.018	1.700 ± 0.070***	0.908 ± 0.031
Spleen (g)	0.099 ± 0.006	0.094 ± 0.004	0.090 ± 0.003	0.085 ± 0.003	0.104 ± 0.007	0.084 ± 0.005	0.093 ± 0.006
Final body weight (g)	27.21 ± 0.47	30.43 ± 1.02$^{\#}$	28.30 ± 0.61	27.55 ± 0.72*	27.48 ± 0.46*	27.55 ± 0.84*	27.86 ± 0.72
Weight gain (g)	1.61 ± 0.15	3.42 ± 0.24$^{\#}$	1.39 ± 0.81*	0.70 ± 0.86**	0.58 ± 0.35**	0.57 ± 0.55***	0.92 ± 0.08**
Food intake (g/day/mouse)	2.34 ± 0.04	1.99 ± 0.04$^{\#\#\#}$	1.95 ± 0.05	1.92 ± 0.07	1.98 ± 0.04	1.99 ± 0.06	1.89 ± 0.04
			Liver lipids				
Total lipid (mg/g)	53.7 ± 2.7	95.9 ± 6.4$^{\#\#\#}$	73.1 ± 4.7**	66.0 ± 4.8**	64.5 ± 5.2**	64.9 ± 5.1**	65.3 ± 4.9**
Triacylglycerol (μmol/g)	40.6 ± 3.9	79.3 ± 6.3$^{\#\#\#}$	56.3 ± 4.2**	45.7 ± 3.9***	45.2 ± 4.6***	47.3 ± 4.6***	45.4 ± 4.2***

Antcin K (AnK; AnK1, AnK2, and AnK3, 10, 20, and 40 mg/kg body wt); fenofibrate (Feno, 250 mg/kg body wt); metformin (Metf, 300 mg/kg body wt); BAT, brown adipose tissue; skeletal muscle included quadriceps muscle, which contains four parts, rectus femoris, vastus intermedius, vastus lateralis, and vastus medialis. All values are means ± SE ($n = 9$). $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$ compared with the control (CON) group; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with the high-fat plus vehicle (distilled water) (HF) group. Epididymal white adipose tissue (epididymal WAT; EWAT), retroperitoneal WAT (RWAT), and mesenteric WAT (MWAT). Visceral fat represented epididymal WAT plus retroperitoneal WAT.

treatment ($P < 0.001$). Treatment with AnK1, AnK2, AnK3, Feno, and Metf markedly lowered glucose levels in blood (Figure 3(a)). HFD increased the levels of circulating TG, total cholesterol (TC), and free fatty acid (Figures 3(b) and 3(c) and Table 2). The AnK1-, AnK2-, AnK3-, Feno-, or Metf-treated mice had decreased TG, TC, and FFA levels. Plasma insulin and leptin concentrations were higher, but adiponectin levels were lower in the HF group than in the CON group. The AnK1-, AnK2-, AnK3-, Feno-, and Metf-treated mice had effectively reduced plasma leptin, insulin, and FFA concentrations but markedly enhanced adiponectin levels (Figures 3(d), 3(e), 3(f), and 3(g)). HFD enhanced

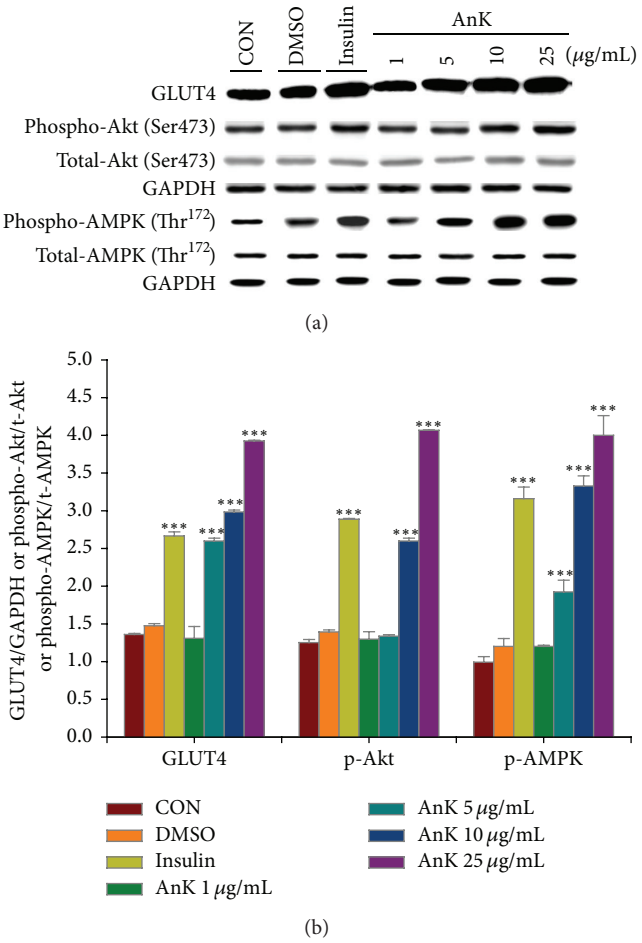


Figure 2: Effect of antcin K (AnK) on GLUT4, phospho-Akt/total-Akt, and phospho-AMPK/total-AMPK *in vitro*. C2C12 myoblasts cells were treated with AnK compounds as described in the experimental procedures and equal amounts of lysates were resolved by SDS PAGE and blotted for GLUT4, Akt, phospho-Akt (Ser473), AMPK, and phospho-AMPK (Thr^{172}). (a) Representative blots for AnK in C2C12 myoblasts cells; (b) quantification of the GLUT4 protein contents and the ratio of phospho-Akt to total-Akt and phospho-AMPK to total-AMPK. All values are means ± SE. $^{***}P < 0.001$ compared with the control group.

the levels of liver total lipids and triacylglycerol, and AnK1-, AnK2-, AnK3-, Feno-, or Metf-treated mice had decreased hepatic total lipid and triacylglycerol levels (Table 2).

3.4. Histopathology Examination. HFD caused adipocytes hypertrophy (the following data were calculated average areas: the CON mice, 6044.4 ± 359.1 μm^2; the HF group, 10142.9 ± 428.1) and following treatment with AnK1 (6548.6 ± 214.7 μm^2), AnK2 (6483.8 ± 319.8 μm^2), AnK3 (5670.8 ± 281.6 μm^2), Feno (6304.2 ± 316.9 μm^2), or Metf (5873.7±345.1 μm^2) displayed less hypertrophy (Figure 4(a)). On the basis of a previous study [38], the designation of histological hepatocellular ballooning findings is comprised of grade 0, none; grade 1, few cells; grade 2, many cells. As shown in Figure 4(b), HFD induced the ballooning of hepatocyte (mean score, 1.9 ± 0.1) as compared with the CON group (0) in liver tissue. Administration of AnK1 (0.7 ± 0.2), AnK2 (0.5 ± 0.2), AnK3 (0.4 ± 0.2), Feno (0.5 ± 0.1), or Metf (0.7 ± 0.2) decreased the ballooning as compared with the HF group.

3.5. Hepatic Targeted Gene mRNA Levels. HFD elicits increases in G6 Pase, acyl-coenzyme A: diacylglycerol acyltransferase 2 (DGAT 2), SREBP1c, aP2, apolipoprotein CIII (apo CIII), and SREBP2 mRNA levels. The AnK1-, AnK2-, AnK3-, Feno-, or Metf-treated mice had decreased mRNA levels of G6 Pase, DGAT2, SREBP1c, aP2, apo CIII, and SREBP2 mRNA levels but increased PPARα mRNA levels (Figure 5).

3.6. Targeted Protein Expression Levels in Different Tissues. HFD induced decreases in protein expression levels of skeletal muscular membrane GLUT4 ($P < 0.001$). AnK1-, AnK2-, AnK3-, Metf-, or Feno-treated groups enhanced membrane GLUT4 expressions. HFD-induced mice had decreased expression levels of phospho-AMPK/total-AMPK or phospho-Akt/total-Akt in both muscle and the liver, which were markedly enhanced in the AnK1-, AnK2-, AnK3-, Metf-, or Feno-treated mice (Figure 6). HFD-fed mice had decreased liver PPARα expressions, but increased in FAS levels. Treatment with AnK1, AnK2, AnK3, Feno, or Metf increased PPARα but decreased FAS expression levels in the liver (Figure 6). The adipose PPARγ and FAS expressions were increased in the HF group. Treatment with AnK1, AnK2, AnK3, Feno, or Metf decreased PPARγ and FAS expression levels in adipose tissue (Figure 7).

4. Discussion

Skeletal muscle and adipose tissue play unique roles in the regulation of insulin-dependent glucose homeostasis [39]. Skeletal muscle is proposed to be the primary site of whole-body insulin-mediated glucose uptake [15, 40, 41]. Adipose tissue accounts for a small fraction of glucose disposal after a meal, with the majority of glucose uptake by muscles [41, 42]. Therefore, this study was firstly designed to screen GLUT4 protein expression in *in vitro* myotubes. And we knew that if *in vitro* study of the compound displays effectiveness, it cannot be assumed to have the same effect *in vivo*, since it entered physical body and underwent biotransformation including absorption, distribution, metabolism, and excretion. Thus, this study was focused on performance of targeted gene protein expressions in different tissues of AnK-treated HFD-fed mice. This study firstly observed that AnK treatment at 5, 10, and 25 μg/mL *in vitro* significantly increased membrane expression levels of GLUT4 in C2C12 myoblast cells. We further undertake to assess whether AnK exhibit antidiabetic and antihyperlipidemic activity employing the HFD animal model since insulin resistance plays the majority of all diabetes cases and to compare with the antidiabetic drug, metformin, and the hypolipidemic drug, fenofibrate, which has also been shown to display good glycemic control [43]. Here we observed that HFD-induction was in line with the

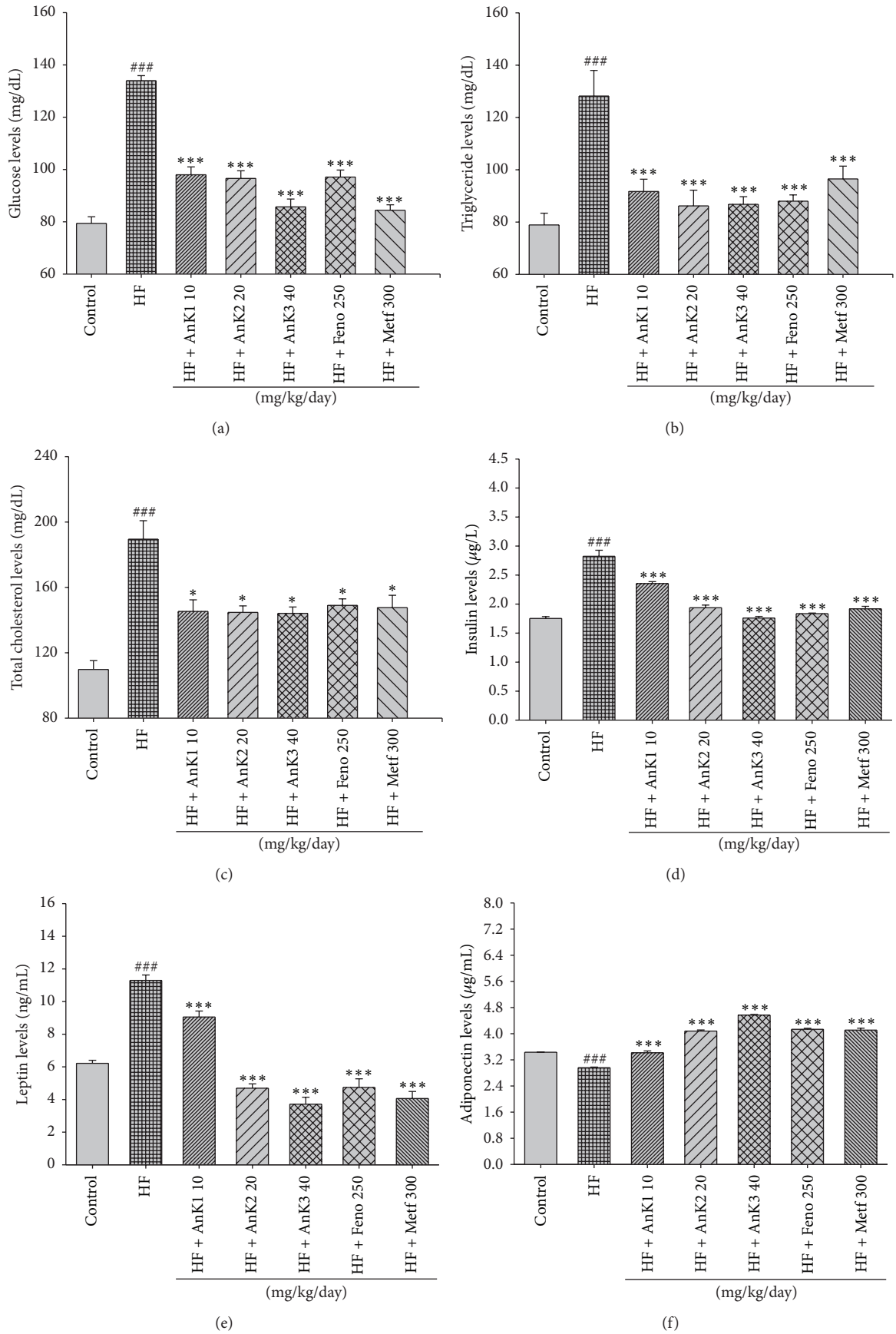


Figure 3: Continued.

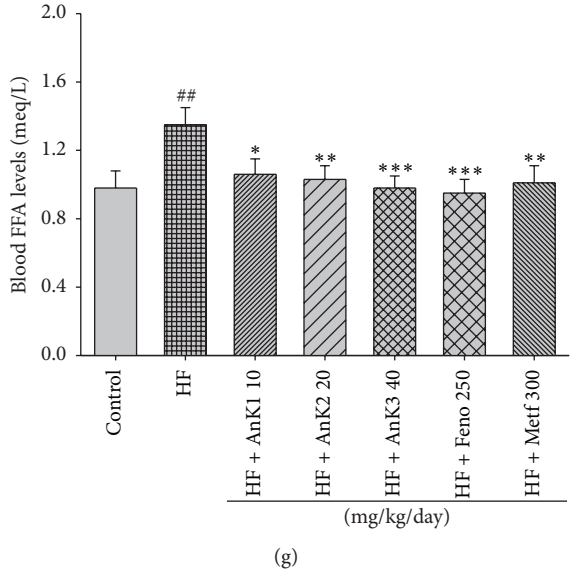


Figure 3: Effects of antcin K (AnK) on (a) blood glucose levels, (b) blood triglycerides levels, (c) blood total cholesterol levels, (d) insulin levels, (e) leptin levels, (f) adiponectin levels, and (g) blood FFA levels at week 12. Mice were fed with 45% high-fat diet (HF) or low-fat diet (CON) for 12 weeks. After 8 weeks of induction, the HF mice were treated with vehicle, or antcin K, or fenofibrate (Feno), or metformin (Metf) accompanied with HF diet for 4 weeks. All values are means ± SE ($n = 9$). $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ compared with the control (CON) group; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with the high-fat diet plus vehicle (distilled water) (HF) group by ANOVA. AnK (AnK1, AnK2, or AnK3, 10, 20, or 40 mg/kg body wt); fenofibrate (Feno, 250 mg/kg body wt); metformin (Metf, 300 mg/kg body wt). FFA, plasm free fatty acid; visceral fat represented epididymal WAT plus retroperitoneal WAT.

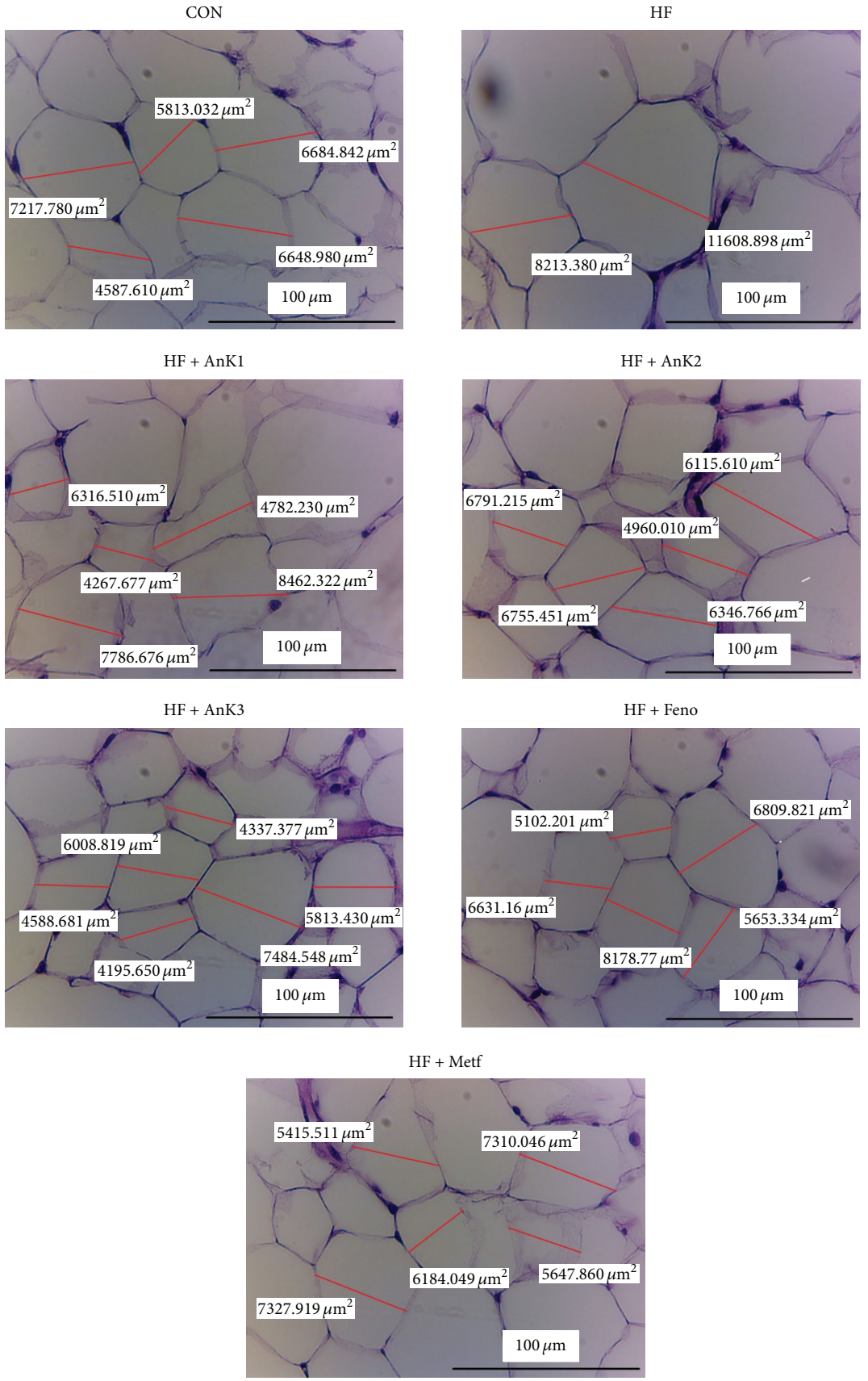
previous observation displaying increases in blood glucose, triglyceride, total cholesterol, insulin, and leptin levels [26]. After the treatment, AnK exhibited both antidiabetic and antihyperlipidemic effects in HFD-fed mice. AnK-treated mice show the glucose-lowering effect by 26.8%–36.0%. Of interest, the glucose-lowering effect of AnK at 40 mg/kg (with less than one-seventh of Meft dosage) was comparable to that of metformin. Our results demonstrated that AnK display good antidiabetic activities; moreover, AnK treatment decreased blood insulin levels and finally improved HFD- induced insulin resistance. These favorable antidiabetic effects of AnK were owing to enhancement of insulin sensitivity in peripheral tissues, particularly increased membrane GLUT4 expressions in skeletal muscle and enhanced activation of AMPK in muscle and the liver.

In addition, all of the AnK-treated groups decreased circulating triglyceride concentrations by 28.5%–32.8% comparable to that of Feno, which is a PPARα agonist with triglyceride-lowering effect [43]. The overall effects in HFD-fed mice imply that AnK had therapeutic potential for the management of type 2 diabetes accompanied with hyperlipidemia.

The first aim of this study was undertaken to assess muscular membrane GLUT4 expressions following treating HFD-fed mice with AnK. Skeletal muscle plays the major site of whole-body insulin-mediated glucose uptake [15]. The membrane GLUT4 expressions measured the translocation of insulin responsive glucose transporter GLUT4 to the plasma membrane [19]. In this study, treatment with AnK, Feno, or Metf significantly increased membrane expression levels GLUT4 by 1.52–2.20-, 1.98-, or 1.86- fold as compared with the HF group, respectively, implying that the increased membrane GLUT4 contents are enhanced to cause glucose uptake, resulting in a decrease in blood glucose levels.

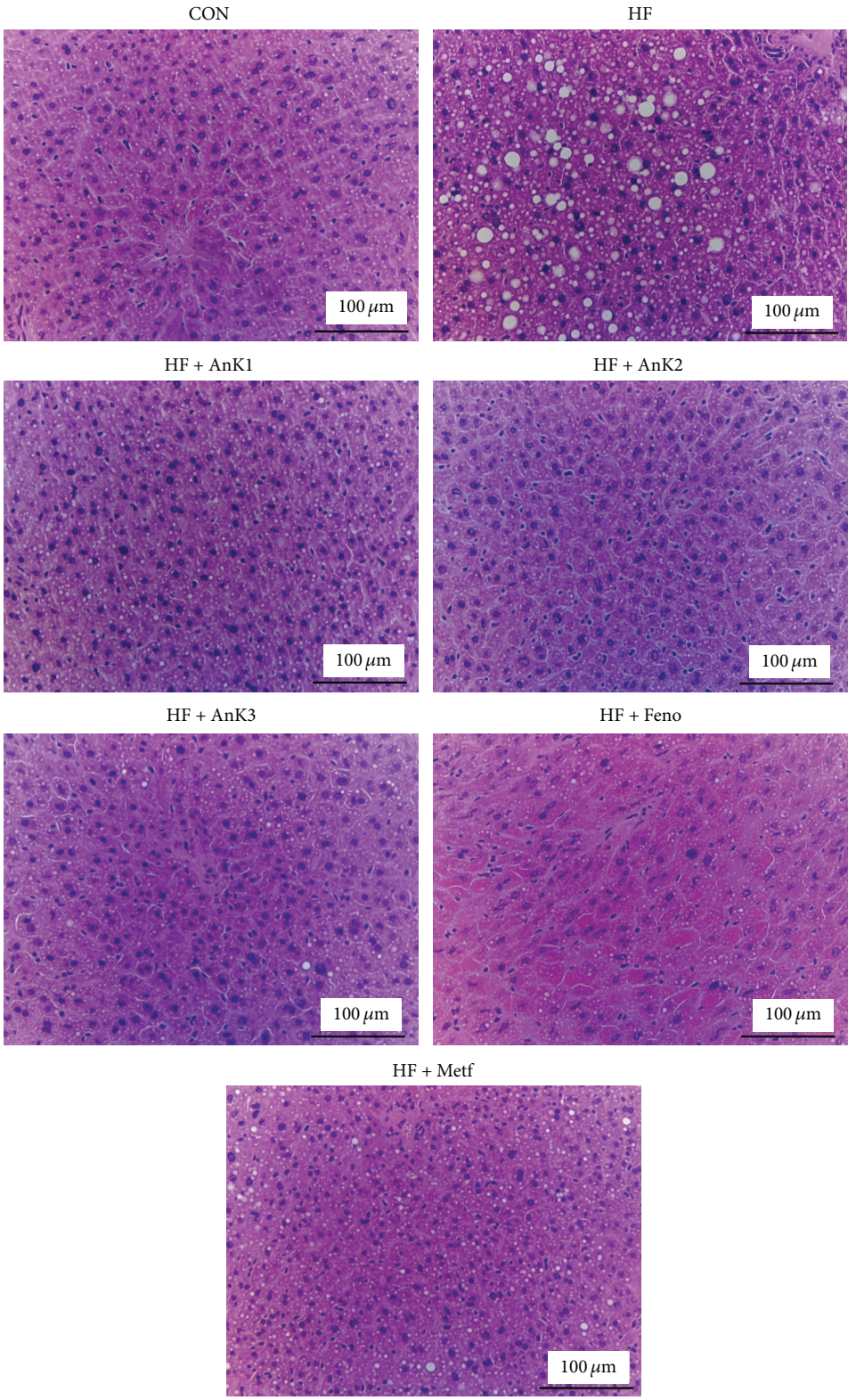
Evidence suggests that the C2C12 myotube is a useful model for analyzing GLUT4 translocation in skeletal muscle [44]. Akt (PKB) stimulates glucose uptake by modulating glucose transporter 4 (GLUT4) [45]. The promoted glucose uptake into skeletal muscle included two pathways: insulin-dependent mechanisms lead to activation of Akt and contraction-mediated stimulation of AMPK [12, 17]. In this *in vitro* experiment, our results showed that AnK (between 1 and 25 μg/mL) was not toxic to C2C12 myotubes by employing the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay (data not shown), and AnK significantly enhanced membrane GLUT4 proteins and phospho-AMPK/total-AMPK expressions at 5, 10, and 25 μg/mL and enhanced phospho-Akt/total-Akt expressions at 10 and 25 μg/mL, and we assume that AnK in myotube cells at 10 and 25 μg/mL could stimulate glucose transport activity partly by insulin pathway and partly by AMPK activation.

The second aim of this study was to evaluate the phospho-AMPK protein expression in AnK-treated HFD-fed mice, since AMPK plays the core role of glucose and lipid metabolism. These data showed that AnK treatment increased the expressions of phospho-AMPK/total-AMPK in the muscle and liver. Metformin may enhance skeletal muscular AMPK activity [17, 46]. Chronic activation of AMPK may induce GLUT4 deployment to the plasma



(a)

Figure 4: Continued.



(b)

Figure 4: Histology of (a) epididymal white adipose tissue and (b) liver tissue of mice in the control (CON), high-fat diet plus vehicle (distilled water) (HF), HF + AnK1, HF + AnK2, HF + AnK3, HF + fenofibrate (Feno), or HF + metformin (Metf) groups by hematoxylin and eosin-staining. Magnification: 10 (ocular) × 20 (object lens). Antcin K (AnK1, AnK2, or AnK3, 10, 20, or 40 mg/kg body weight, resp.); Feno, fenofibrate (250 mg/kg body weight). Metf, metformin (300 mg/kg body weight).

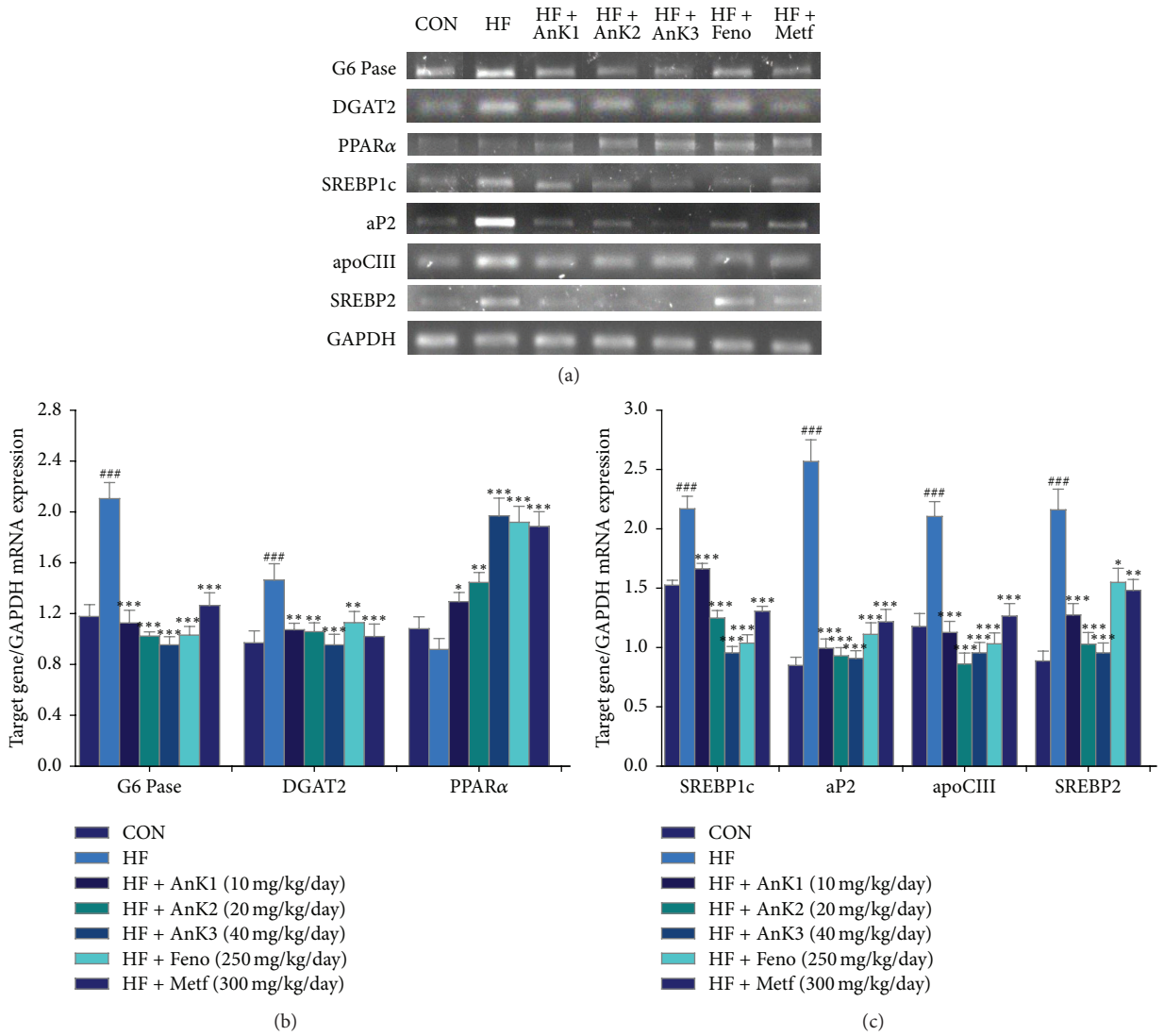


Figure 5: Semiquantitative RT-PCR analysis on G6 Pase, DGAT2, PPARα, SREBP1c, aP2, apoCIII, and SREBP2 mRNA levels in liver tissue of the mice by oral gavage antcin K (AnK1, AnK2, or AnK3, 10, 20, or 40 mg/kg body weight, resp.); Feno, fenofibrate (250 mg/kg body weight); Metf, metformin (300 mg/kg body weight): (a) representative image; (b, c) quantification of the ratio of target gene to GAPDH mRNA expression. Total RNA (1 μg) isolated from tissue was reverse-transcribed by MMLV-RT; 10 μL of RT products was used as templates for PCR. The expression levels of G6 Pase, DGAT2, PPARα, SREBP1c, aP2, apoCIII, and SREBP2 mRNA were measured and quantified by image analysis. Values were normalized to GAPDH mRNA expression. All values are means ± SE ($n = 9$). $^{###}P < 0.001$ compared with the control (CON) group; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with the high-fat-diet plus vehicle (distilled water) (HF) group.

membrane, leading to insulin-independent glucose uptake [17, 46, 47]. In skeletal muscle, AnK was found to increase AMPK phosphorylation comparable to that of metformin, suggesting that AnK activates AMPK or Akt phosphorylation to increase GLUT4 translocation in muscles, which leads to a decrease in systemic insulin resistance.

G6 Pase plays a key role in gluconeogenesis [48]. The hepatic expression of mRNA level of G6 Pase is reduced in AnK-treated mice. Collectively, our results imply that AnK display glucose-lowering effects via enhanced muscular GLUT4 proteins to increase glucose uptake and decreased hepatic G6 Pase mRNA levels to suppress hepatic glucose production.

The third aim of this study was to clarify the hypolipidemic effects and mechanisms of AnK. Evidences have shown that PPARα are abundantly expressed in the liver tissue and promoted fatty acids oxidation [49]. PPARα agonists have been proposed as a breakthrough in the management of dyslipidemia to reduce blood triglyceride levels [43, 49]. In this study, AnK displayed antihypertriglyceridemic effects. PPARα ligands could reduce the expression of the apo CIII gene [50], thus resulting in hypotriglyceridemic effect. DGAT2 play a role in the final step of triglyceride synthesis [51]. SREBP-1c, a key lipogenic transcription factor, stimulates lipogenic enzyme expression and contributes to fatty acids synthesis and TG accumulation [52]. Mice with

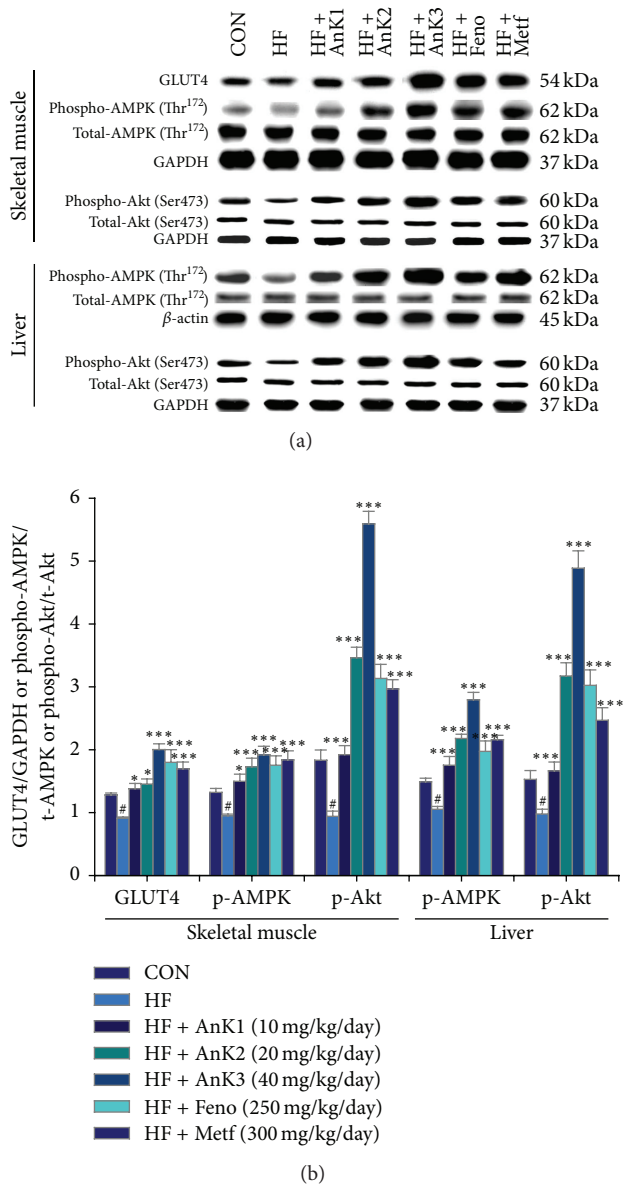


Figure 6: GLUT4 protein contents in skeletal muscle or phospho-Akt/t-Akt or phospho-AMPK (Thr^{172})/t-AMPK in liver and skeletal muscle of the mice by oral gavage antcin K (AnK): (a) representative image; (b) quantification of the GLUT4 expression levels, the ratio of phospho-AMPK to total-AMPK, or phospho-Akt/t-Akt expression levels (mean ± SE, $n = 9$). Protein was separated by 12% SDS PAGE detected by Western blot. $^{\#}P < 0.05$ compared with the control (CON) group; $^{*}P < 0.05$ and $^{***}P < 0.001$ compared with the high-fat-diet plus vehicle (distilled water) (HF) group. Antcin K (AnK1, AnK2, or AnK3, 10, 20, or 40 mg/kg body weight, resp.); Feno, fenofibrate (250 mg/kg body weight); Metf, metformin (300 mg/kg body weight).

aP2 deficiency are protected from the development of dyslipidemia, hyperglycemia, insulin resistance, and fatty liver disease in both genetic and dietary obesity [53]. Ablation of aP2 and mall show enhanced liver accumulation of longer-chain fatty acids, thus resulting in decreased SREBP1c expressions and its several downstream lipogenic enzymes [53]. We found that hepatic mRNA levels of aP2 and lipogenic SREBP1c are suppressed by AnK, thus also contributing to protecting from HFD-induced insulin resistance and hepatic steatosis. FAS is a critical focus in fatty acid synthesis [54]. SREBP2 play a core role in the regulation of cholesterol synthesis [55]. AnK lowered plasma TC concentrations coincident with reduced SREBP2 mRNA levels, implying AnK exerting TC-lowering effect may be primarily due to a decrease of cholesterol synthesis. Taken together, AnK-treated mice had increased hepatic expression of PPARα protein to enhance fatty acids oxidation but decreased FAS protein to inhibit fatty acids synthesis coincident with suppressed SREBP1c, aP2, DGAT2, and apo CIII mRNAs, thus contributing to the hepatic triglyceride output and leading to decreased plasma triglycerides, hepatic steatosis, and total cholesterol levels.

In adipose tissue, PPARγ stimulated adipogenesis and lipogenesis [56]. PPARγ is abundantly expressed in adipocytes and its expression is markedly induced during adipocyte differentiation [57]. Here we report that treatment with AnK, Feno, or Metf decreased adipose expression of PPARγ and FAS protein; as a result, adipogenesis and fatty acids synthesis and lipid accumulation are reduced in adipose tissue. Moreover, blood TG is fluctuating between the liver and adipose tissue. Lipid could usually be stored in the adipose tissue and the liver is the major organ of lipid metabolism, presuming AnK could remove fat from adipose tissue to peripheral tissues not only by increasing lipid catabolism including inhibition of fatty acid synthesis (FAS) and enhancement of fatty acid oxidation (PPARα) in the liver, but also by inhibition of adipocyte adipogenesis (PPARγ) and FAS in adipose tissue, thus leading to reduced TG levels in the liver, blood, and adipose tissue. Therefore, in histology analysis, AnK treatment resulted in a decrease in lipid accumulation in adipose tissue and liver and finally reflected hepatic lipid drops almost invisible and a reduction in adipocyte size.

Adiponectin level was found to decrease in HFD-fed mice in this study. This observation is in line with the others demonstrating that adiponectin levels are reduced in adults or rodents with obesity and type 2 diabetes [58]. High levels of adiponectin can predict enhanced insulin sensitivity of both glucose and lipid metabolism [59]. Following AnK administration, the mice display significantly increased blood levels of adiponectin, establishing that AnK could provide a unique therapeutic advantage associated with the regulation to improve insulin sensitivity. Moreover, studies have showed that there is an inverse relationship between plasma leptin or mRNA expression of leptin and insulin sensitivity [60]. In this study, leptin level is enhanced in HFD-fed mice, in accordance with a previous report [61]. Treatment with AnK markedly reduced the increase of leptin level. Thus, AnK prevented HFD-induced abnormalities in leptin levels and improved insulin resistance. Treatment with globular domain of adiponectin increased glucose uptake and AMPK activation [62]. Adiponectin is proposed to activate AMPK in the liver, enhance glucose utilization and fatty acid oxidation, and inhibit glucose production in the liver [63]. Administration of AnK significantly elevated phosphorylation of AMPK. On the basis of the previous reports [62, 64], the

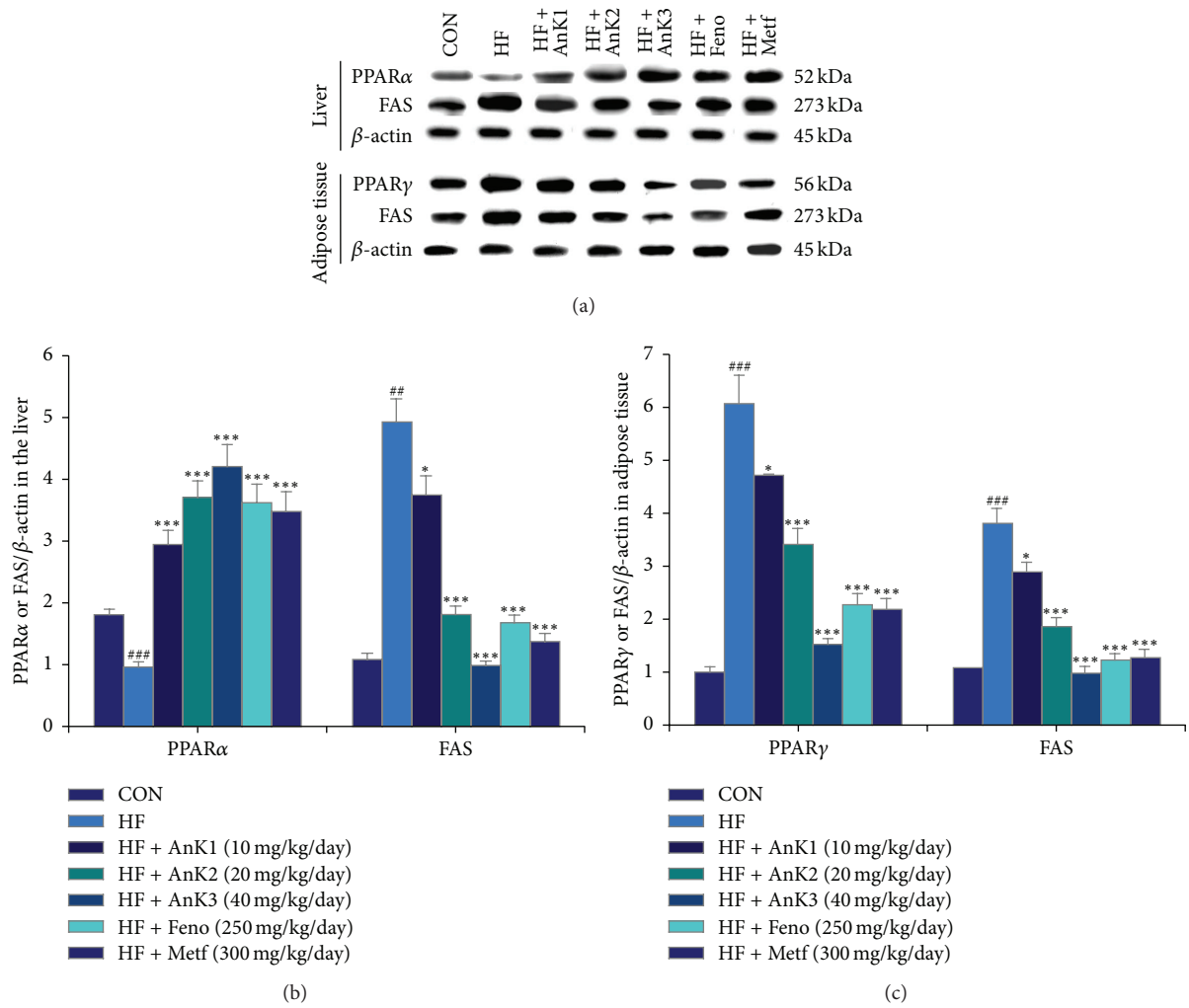


Figure 7: Expression levels of PPARα and FAS in the liver tissue and PPARγ and FAS in adipose tissue of mice by oral gavage antcin K: (a) representative image; (b, c) quantification of the expression levels of PPARα and FAS in the liver tissue and PPARγ and FAS in adipose tissue. Protein was separated by 12% SDS PAGE detected by Western blot. $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ compared with the control (CON) group; $^{*}P < 0.05$ and $^{***}P < 0.001$ compared with the high-fat-diet plus vehicle (distilled water) (HF) group. Antcin K (AnK1, AnK2, or AnK3, 10, 20, or 40 mg/kg body weight, resp.); Feno, fenofibrate (250 mg/kg body weight); Metf, metformin (300 mg/kg body weight).

AMPK phosphorylation by AnK may be associated with adiponectin and/or leptin secretion. Thus, there is possibility that AnK directly cause AMPK phosphorylation or act by adiponectin-mediated activation of AMPK and PPARα leads to a reduction in hepatic gluconeogenesis and increased muscle glucose uptake, resulting in reduced glucose levels *in vivo* and increased fatty acid oxidation in both tissues.

In conclusion, AnK-treated mice had not only lowered blood glucose and insulin, but also decreased triglyceride, total cholesterol levels, and finally ameliorated insulin resistance (Figure 8). Of interest, AnK at 40 mg/kg/day dosage displayed both antihyperglycemic effect comparable to Metf (300 mg/kg/day) and antihypertriglyceridemic effect comparable to Feno (250 mg/kg/day). The antidiabetic effect of AnK is due to significant increases in membrane GLUT4 expression levels in skeletal muscle to stimulate glucose uptake coincident with decreases in G6 Pase mRNA levels to inhibit hepatic glucose production, thus contributing to glucose-lowering efficacy. In both skeletal muscle and liver tissue, AnK-treated mice had increased AMPK activation. AnK treatment exhibited inhibition of hepatic lipogenic FAS expression but enhancement of fatty acid oxidation PPARα expression coincident with reduced SREBP1c mRNA levels in the liver, thus resulting in decreased plasma triglycerides and total cholesterol levels. AnK activates AMPK or Akt phosphorylation to increase GLUT4 translocation in muscles, which leads to a decrease in systemic insulin resistance and to fat accumulation in adipose tissue and liver. Additionally, the ameliorated insulin resistance also improved the liver insulin sensitivity (Akt activation). Our findings manifest that AnK has a favorable therapeutic potential for the management of type 2 diabetes associated with hyperlipidemia.

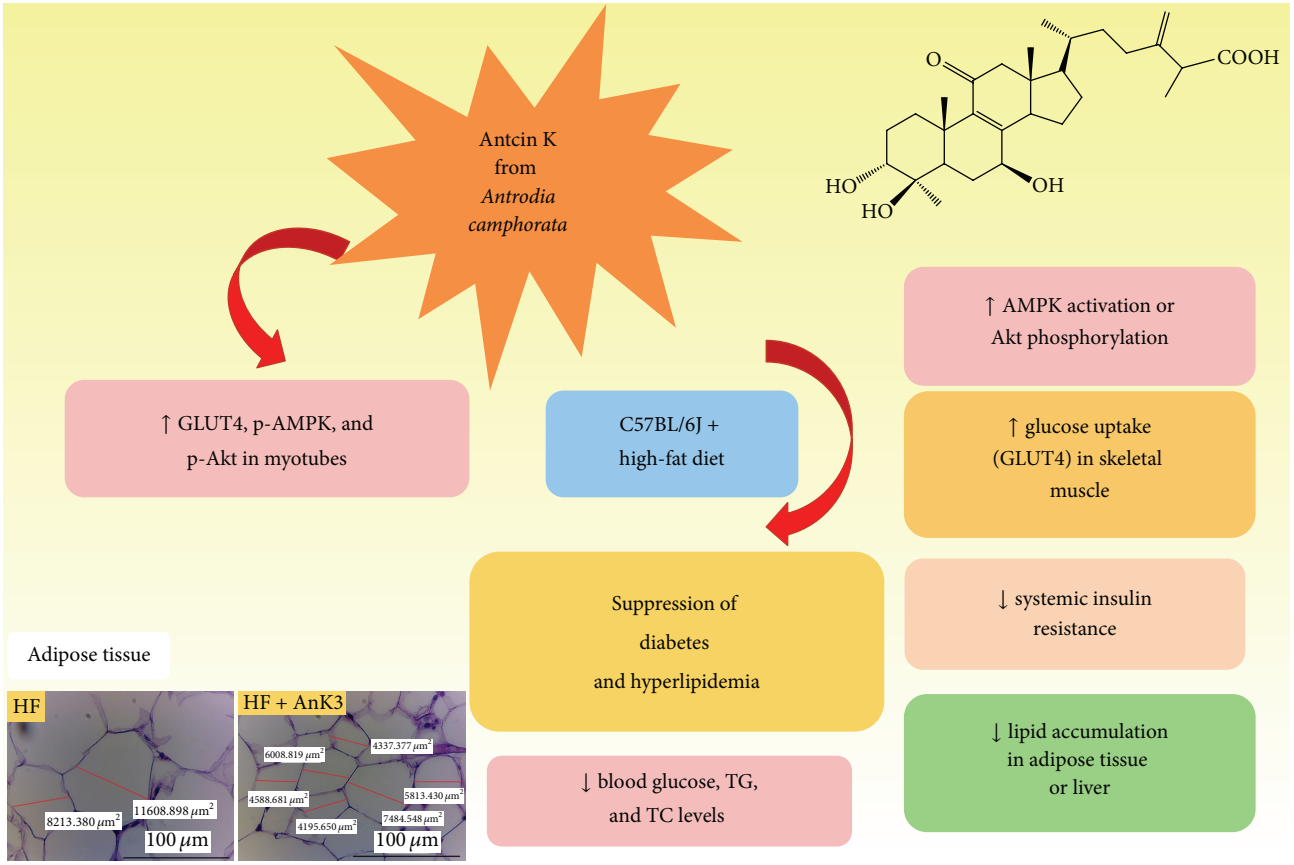


Figure 8: A proposed mechanism for AnK to improve diabetes and hyperlipidemia.

Abbreviations

AMPK: AMP-activated protein kinase
aP2: Adipocyte fatty acid binding protein 2
BAT: Brown adipose tissue
CON: Control
DGAT2: Acyl-coenzyme A: diacylglycerol acyltransferase 2
EWAT: Epididymal white adipose tissue
FAS: Fatty acid synthase
Feno: Fenofibrate
FFA: Free fatty acid
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
G6 Pase: Glucose-6-phosphatase
GLUT4: Glucose transporter 4
HF: High-fat control
HFD: High-fat diet
Metf: Metformin
MWAT: Mesenteric white adipose tissue
PPAR: Peroxisome proliferator-activated receptor
RT-PCR: Reverse transcription-polymerase chain reaction
RWAT: Retroperitoneal white adipose tissue
SREBP: Sterol regulatory element binding protein
TC: Total cholesterol
TG: Triglyceride
WAT: White adipose tissue.

Competing Interests

The authors wish to confirm that there are no known competing interests associated with this paper and there has been no significant financial support for this work that could have influenced its outcome.

Authors' Contributions

Yueh-Hsiung Kuo and Cheng-Hsiu Lin equally contributed to this paper.

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Research Article

A *Fomitopsis pinicola Jeseng* Formulation Has an Antiobesity Effect and Protects against Hepatic Steatosis in Mice with High-Fat Diet-Induced Obesity

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This study investigated the antiobesity effect of an extract of the Fomitopsis pinicola Jeseng-containing formulation (FAVA), which is a combination of four natural components: *Fomitopsis pinicola Jeseng*; *Acanthopanax senticosus*; *Viscum album coloratum*; and *Allium tuberosum*. High-fat diet- (HFD-) fed male C57BL/6J mice were treated with FAVA (200 mg/kg/day) for 12 weeks to monitor the antiobesity effect and amelioration of nonalcoholic fatty liver diseases (NAFLD). Body and white adipose tissue (WAT) weights were reduced in FAVA-treated mice, and a histological examination showed an amelioration of fatty liver in FAVA-treated mice without decreasing food consumption. Additionally, FAVA reduced serum lipid profiles, leptin, and insulin levels compared with the HFD control group. The FAVA extract suppressed lipogenic mRNA expression levels from WAT concomitantly with the cholesterol biosynthesis level in the liver. These results demonstrate the inhibitory effects of FAVA on obesity and NAFLD in the diet-induced obese (DIO) mouse model. Therefore, FAVA may be an effective therapeutic candidate for treating obesity and fatty liver caused by a high-fat diet.

1. Introduction

There is increasing consensus that obesity may be the main cause of various metabolic disorders. Obesity is caused by the combined effects of excess energy intake and reduced energy expenditure. It is one of the fastest growing disorders worldwide and is associated with various clinical symptoms in developed countries [1], such as hyperlipidemia, insulin resistance, and nonalcoholic fatty liver diseases (NAFLD) [2]. It is well known that excessive fat consumption is implicated in the development of obesity in mice [3], and long-term feeding with a high-fat diet (HFD) can induce obesity together with hyperlipidemia, insulin resistance, and NAFLD [4]. Hyperlipidemia is associated with high levels of lipids and lipoproteins in the blood and causes atherosclerosis and acute pancreatitis [5]. Although NAFLD is the second leading cause of death in the general population [6, 7], there is no pharmacological agent known to reverse NAFLD. Recently, effective medical interventions have been focused on the modification of risk factors, such as diet and weight reduction [8].

Adipose tissue, an important repository for energy storage, regulates energy homeostasis. Adipogenesis, a differentiation process of adipocytes, involves changes in gene expression and cellular morphology. Adipocyte hypertrophy results from an excessive accumulation of lipids from the intake of inordinate energy sources such as HFD. During adipogenesis, peroxisome proliferator-activated receptor-γ (PPAR-γ) and CCAAT/enhancer-binding protein-α (C/EBP-α) play key roles as major transcriptional factors [9]. Expression of PPAR-γ, a transcription factor of the nuclear-receptor superfamily, and C/EBP-α, a member of the C/EBP family of

basic leucine zipper class of transcription factors, increases during 3T3-L1 cell differentiation [10]. Lipin is also a central regulator of adipose tissue development. Mammalian lipin proteins have been shown to control gene expression and to enzymatically convert phosphatidate into diacylglycerol, an essential precursor in triacylglycerol and phospholipid synthesis [11]. Previous studies have established that lipin-1 is required at an early step in adipocyte differentiation for the induction of the adipogenic gene transcription program, including the key regulator PPAR-γ [12].

Acetoacetyl-CoA synthetase (AACS) regulation is related to cholesterol and lipid homeostasis [13]. Sterol response element binding protein-2 (SREBP-2) may play an essential role in the transcriptional regulation of AACS. SREBP-2 is a leucine zipper transcription factor that controls a rate-limiting enzyme in cholesterol synthesis, HMG-CoA reductase (HMGCR), when the factor binds sterol response element [14, 15].

The effects of *Acanthopanax senticosus*, *Allium tuberosum,* and *Viscum album coloratum* have been studied for inhibition of fatty acid synthase and prevention of obesity, as well as reducing hepatic steatosis [16–18]. *Fomitopsis pinicola Jeseng* has been reported for antihyperglycemic effect in diabetic rats [19]. In addition, it has been reported that β-glucan-rich extract, a major component of *Fomitopsis pinicola Jeseng*, effectively reduces adiposity [19, 20]. However, to our knowledge, no reports are available on the effect of *Fomitopsis pinicola Jeseng* on obesity.

In this study, we investigated the effect of FAVA in a high-fat diet-induced mouse model. The FAVA is a combination of herbal extracts (i.e., *Fomitopsis pinicola Jeseng*, *Acanthopanax senticosus*, *Allium tuberosum,* and *Viscum album coloratum*) at a ratio of 5 : 3 : 1 : 1. This study investigated the effect of a mixture containing dietary components on metabolic disorders including obesity, hyperlipidemia, and NAFLD using a high-fat diet-induced obesity mouse model and the molecular mechanism level of adipogenesis and cholesterol biosynthesis. Our results indicate the great potential of FAVA as a potential metabolic regulator of adipogenesis and cholesterol biosynthesis and as a potential therapeutic agent for preventing or treating obesity and NAFLD.

2. Materials and Methods

2.1. Preparation of FAVA. The oriental, medicinal, and herbal mixture used in this experiment (FAVA) was prepared as described previously [21–23]. Briefly, *Acanthopanax senticosus* and *Allium tuberosum* were extracted with 80% methanol while water extracted *Fomitopsis pinicola Jeseng* and *Viscum album coloratum* were purchased from commercial vendor (Mistle Biotech Co., Ltd., Korea). Each extraction of FAVA was resuspended in distilled water (DW) in a ratio of 50%, 30%, 10%, and 10%, respectively, and prepared in appropriate diluent for further *in vivo* study.

2.2. Animals and Diets. Lean, male C57BL/6J mice (7 weeks old) were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan). All animal experiments were approved by the Ethics Review Committee of the Pohang Center for the Evaluation of Biomaterials, Republic of Korea. All mice were housed for 1 week under a 12/12-h light/dark cycle in a temperature- (22 ± 1°C) and humidity- (55 ± 5%) controlled room and fed standard laboratory chow and water *ad libitum* while FAVA, orlistat, and saline supplementation were performed using oral gavage once a day. To induce obesity, the mice were fed a HFD (Rodent Diet D12492, Research Diet, New Brunswick, NJ, USA) consisting of 60% kcal fat. Control mice were fed a low-fat chow diet (Rodent Diet D12450B, Research Diet, New Brunswick, NJ, USA) consisting of 10% kcal fat. Experimental mice were given FAVA or orlistat as a positive control (Chongqing Zein Pharmaceutical Co., Ltd., Chongqing, China). The mice were randomly divided into four groups ($n = 8$ per group) that were fed a low-fat chow diet (CHOW), a high-fat diet (HFD), HFD plus FAVA (200 mg/kg/day), or HFD plus orlistat (60 mg/kg/day). Animals were fed via oral feeding needles for 12 weeks, and the CHOW and HFD group received an equivalent volume of saline. Body weight was measured once a week, and food intake was measured three times per week during the course of the study. At the conclusion of the *in vivo* experiment, the mice were sacrificed by cervical vertebral dislocation, and the epididymal, mesenteric, and subcutaneous fat pads and liver were collected and weighed. The epididymal fat pad samples were stored at −80°C until analysis.

2.3. Serum Analysis. Serum was collected by cardiac puncture, stored for 20 minutes at room temperature for coagulation, and then separated by centrifugation at 2,000 ×g for 20 minutes. The serum was stored at −70°C until analysis. The levels of triglycerides, total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, glucose, alanine transaminase (ALT), aspartate transaminase (AST), BUN, and creatinine in serum were measured by using an automated biochemical analyzer (BS-390, Mindray Bio-Medical Electronics Co., Ltd., China).

2.4. Measurement of Leptin and Insulin. The leptin and insulin concentrations in serum were determined by a mouse enzyme-linked immunosorbent assay (ELISA) kit (Morinaga Institute of Biological Science, Yokohama, Japan). The preparation of serum samples is described above.

2.5. Abdominal Computed Tomography Analysis. Experiments of micro-computed tomography (micro-CT) were performed with an animal positron emission tomography (PET)/CT/single photon emission computed tomography (SPECT) system (Inveon, Siemens, USA) prior to the sacrifice of animals under 1.5–2% isoflurane in O_2 anesthesia. Computed tomography pictures were further analyzed using Siemens Inveon software to calculate the three-dimensional volume of the fat mass between lumbar vertebrae one to five.

2.6. Liver Histology. Liver tissues were immediately isolated after sacrifice. For hematoxylin and eosin (H&E) staining, the tissues were fixed in 10% formalin, processed, and embedded in paraffin prior to sectioning (10 μm) and staining. The liver

Table 1: Primers used in the reverse transcriptase-polymerase chain reaction analysis.

Gene name	Accession number		Sequence
Lipin-1	NM_172950	Forward	5′-TCA GAC ACT TTC AGT AAC TTC AC-3′
		Reverse	5′-TAT CAG CCT TCC CAG CAG-3′
C/EBP-α	NM_007678	Forward	5′-CGT CTA AGA TGA GGG AGT C-3′
		Reverse	5′-GGC ACA AGG TTA CTT CCT-3′
PPAR-γ	NM_001127330	Forward	5′-GAA AGA CAA CGG ACA AAT CAC-3′
		Reverse	5′-GAA ACT GGC ACC CTT GAA-3′
AACS	NM_030210	Forward	5′-AAG CCC AGA GTT ACG AGT AT-3′
		Reverse	5′-ACA CAG GAA TAG AGG AGT TCT-3′
HMGCR	NM_008255	Forward	5′-AGA ATA ATG TGC TAA GTA GTG CTA A-3′
		Reverse	5′-GCC TCT CTG AAC AAA GAC TC-3′
SREBP-2	NM_033218	Forward	5′-GCG ACC AGG AAG AAG AGA-3′
		Reverse	5′-ACA AAT CCC ACA GAG TCC A-3′
β-actin	NM_007393	Forward	5′-GGG AAG GTG ACA GCA TTG-3′
		Reverse	5′-ATG AAG TAT TAA GGC GGA AGA TT-3′

C/EBP-α: CCAAT/enhancer-binding protein-α; PPAR-γ: peroxisome proliferator-activated receptor-γ; AACS: acetoacetyl-CoA synthetase; HMGCR: HMG-CoA reductase; and SREBP-2: sterol regulatory element binding protein-2.

samples of 3 mice from each group (CHOW, HFD, HFD + FAVA, and HFD + ORLISTAT) were measured. Briefly, the following criteria were used for scoring hepatic steatosis: grade 0 (no fatty liver) and grade 1 (mild fatty liver), if hepatocytes occupied <33% of the hepatic parenchyma [24].

2.7. RNA Preparation and Real-Time PCR. Total RNA was extracted by ReliaPrep RNA Tissue Miniprep System (Promega) according to the manufacturer's instructions. RNA integrity was assessed by an automated microfluidics-based system (Bioanalyzer 2100, Agilent, Palo Alto, CA, USA). First-strand cDNA was synthesized with the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA), and real-time PCR was performed using an iCycler iQ Real-Time Detection System (Bio-Rad). PCR reactions were conducted with iQ SYBR Green Supermix (Bio-Rad). Real-time PCR analysis was performed using an iCycler iQ Real-Time Detection System (Bio-Rad). Amplification of real-time PCR was performed according to the protocols of Jung et al. [25]. The reaction was performed at 95°C for 3 min, followed by 39 cycles of amplification (95°C for 10 s, 58°C for 10 s, and 72°C for 30 s). A melting curve was produced to confirm a single gene-specific peak and detect primer/dimer formation by heating the samples from 65 to 95°C in 0.5°C increments with a dwell time at each temperature of 10 s, while continuously monitoring fluorescence. The mRNA levels of specific genes were normalized to those of β-actin. The primers used are listed in Table 1.

2.8. Statistical Analyses. The data (mean ± SE) were analyzed using GraphPad Prism (version 5.04, GraphPad Software, USA). Unpaired two-tailed Student's t-tests were used to evaluate differences between means as indicated and p values < 0.05 were considered significant.

3. Results

3.1. Effects of FAVA on Body Weight, Dietary Intake, and Fat Mass in White Adipose Tissue in HFD-Fed Mice. The effects of FAVA on body weights are shown in Figure 1(a). During the 12-week experiment, body weight was measured weekly, and food intake was measured every other day. After 9 weeks, the body weight of the mice in the HFD group was significantly higher than that of the mice in the CHOW group ($p < 0.0005$). The FAVA-treated group showed a significant decrease in body weight compared with the HFD group. At the end of the experiment, the body weight of the mice fed FAVA was 9.7 ± 2.0% lower ($p < 0.05$) than that of the mice in the HFD group, whereas HF diet plus orlistat-fed mice weighed almost the same as the mice that were fed FAVA (Figure 1(a)). These effects of FAVA on body weight were not due to decreased food intake, because the amount of kcal consumed per mouse over a 24-h period remained unchanged (Figure 1(b)). These data indicate that FAVA might have antiobesity effects *in vivo*, without affecting food intake. To investigate whether body weight loss was caused by decreased adiposity, the animals were sacrificed, and the epididymal fat pad, the mesenteric fat pad, and the subcutaneous fat pad were dissected and weighed. FAVA supplementation significantly suppressed the increase of fat mass in all white adipose tissues, including mesenteric, subcutaneous, and epididymal adipose tissue (Figures 1(c)–1(e)).

3.2. Effect of FAVA on Adiposity in HFD-Fed Mice. We performed micro-CT imaging to assess the effect of FAVA on adiposity. CT imaging showed a significant reduction in body fat profiles with FAVA treatment (Figure 2(a)). There was a significant reduction in fat volume (Figure 2(b)) and total body fat percentage in FAVA-fed groups compared with the HFD group (Figures 2(b) and 2(c)).

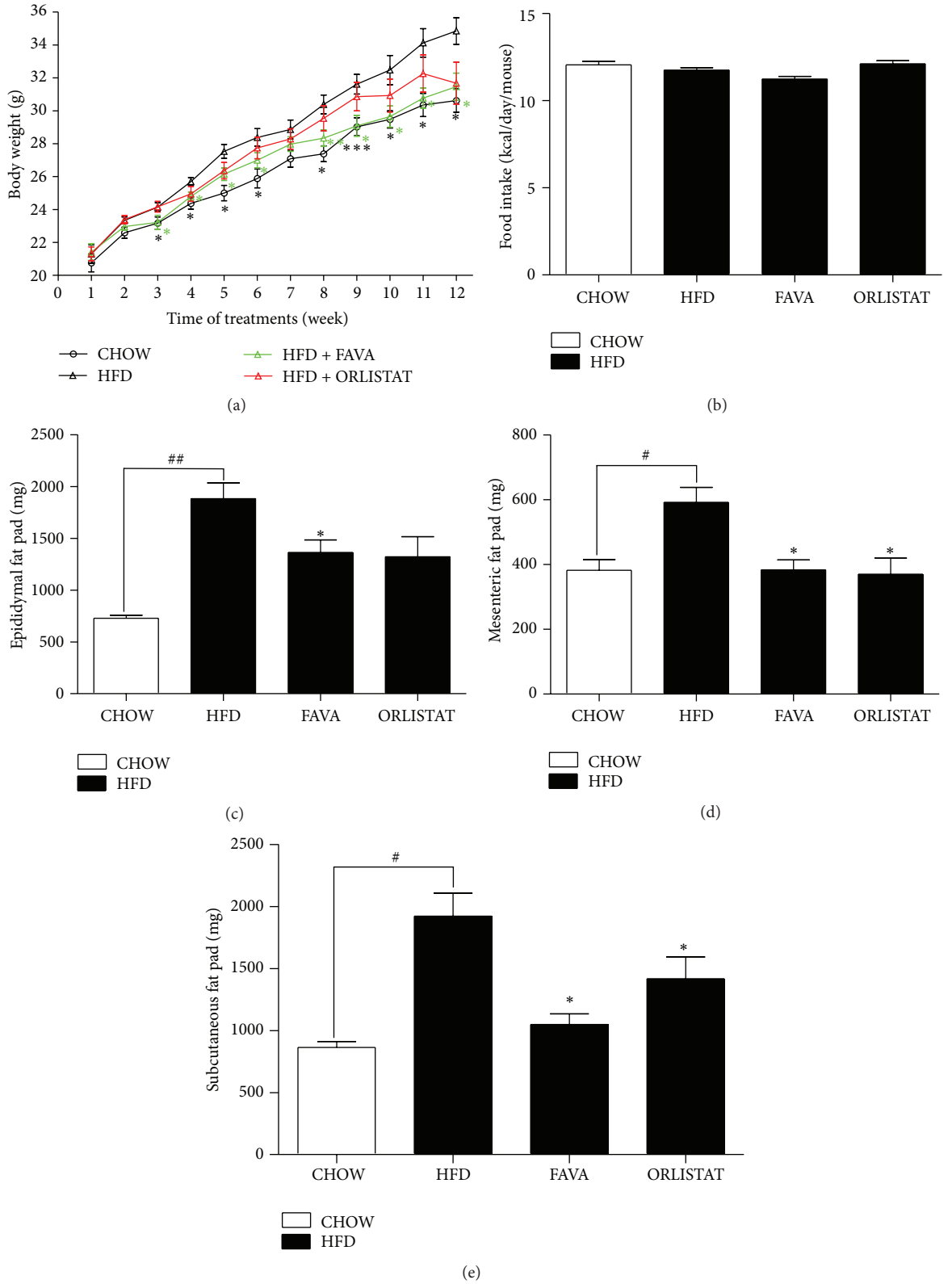

Figure 1: Effect of FAVA on body weight, food intake, and white fat pad in mice fed a high-fat diet for 12 weeks. (a) Changes in body weight gain at each treatment period are shown: (circle) CHOW: chow diet; (black triangle) HFD: high-fat diet; (red triangle) HFD + ORLISTAT: high-fat diet plus orlistat 60 mg/kg; and (green triangle) HFD + FAVA: high-fat diet plus FAVA 200 mg/kg. (b) Average food intake expressed as kcal/mouse/day. ((c)–(e)) Epididymal fat pad (c), mesenteric fat pad (d), and subcutaneous fat pad (e) weights expressed. The values represent the mean ± standard error of mean (SEM) ($^{\#}p < 0.05$ and $^{\#\#}p < 0.005$ versus the CHOW group; $^{*}p < 0.05$, $^{**}p < 0.005$, and $^{***}p < 0.0005$ versus the HFD group, $n = 8$ per group).

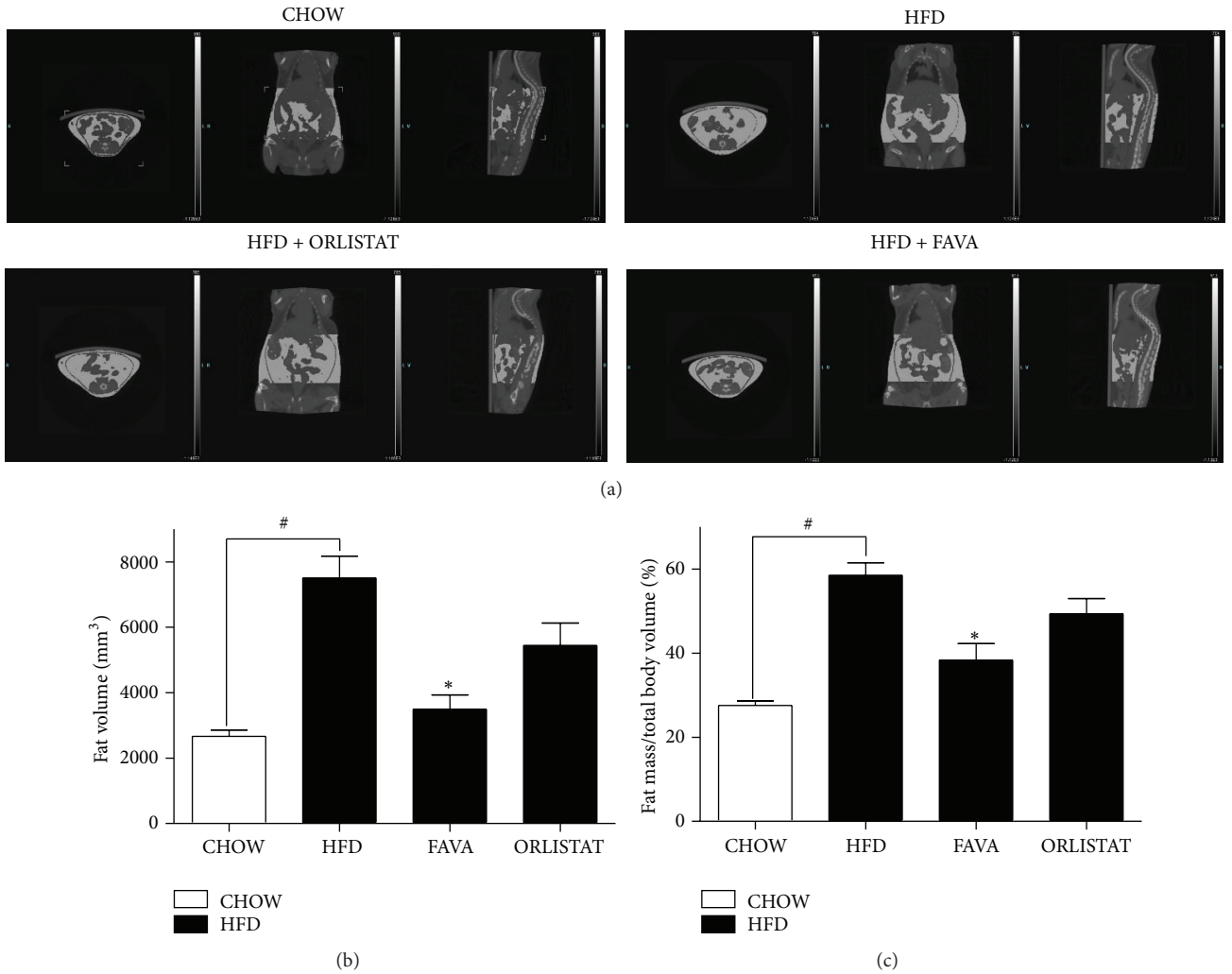

Figure 2: Effect of FAVA on high-fat diet-induced adiposity. (a) Micro-computed tomography (micro-CT) pictures were analyzed using Siemens Inveon software to calculate the three-dimensional volume of the fat mass between vertebrae number one to five of mice fed a chow diet (CHOW), high-fat diet (HFD), HFD with 60 mg/kg/day orlistat (ORLISTAT), or HFD with 200 mg/kg/day FAVA (FAVA). (b) Fat volumes (mm^3) in mice are shown. (c) Fat pad mass expressed as percentage of total body weight. The values represent the mean ± SEM ($^{\#}p < 0.05$ versus the CHOW group; $^{*}p < 0.05$ versus the HFD group, $n = 3$).

3.3. Effects of FAVA on Serum Insulin, Leptin, and Lipid Profiles in the Serum of HFD-Fed Mice. The changes in the blood plasma parameters are shown in Figures 3(a)–3(e). As shown in Figures 3(a) and 3(b), HFD-induced obese mice showed significantly higher levels of serum insulin and leptin, whereas the FAVA group showed significantly decreased levels of serum insulin and leptin by 60.9 ± 8.1% and 40.4 ± 3.0%, respectively. Concomitant reductions of serum insulin and leptin levels were monitored in the orlistat group in a similar manner. Additionally, the FAVA group showed lower levels of serum total cholesterol and the ratio of LDL cholesterol/total cholesterol than those of the HFD group.

3.4. Effects of FAVA on mRNA Levels of Transcriptional Factors in Epididymal Fat Pad. Because FAVA extract reduced fat mass in all white adipose tissues and serum insulin levels (Figures 1 and 3), we evaluated the effect of FAVA on the expression of various adipogenic and lipogenic genes [26]. PPARγ and C/EBPα are known to have roles in insulin sensitivity, lipogenesis, and lipolysis [27]. Lipin-1 is also thought to regulate the transcription of genes involved in adipocyte differentiation and fat synthesis and storage [28]. To investigate the antiadipogenic mechanism, the effects of FAVA on mRNA expression levels of PPARγ, C/EBPα, and lipin-1 were determined in the epididymal fat pad. The expression of both adipogenic genes, PPARγ and C/EBPα, was significantly decreased by FAVA (Figures 4(b) and 4(c)). Additionally, FAVA significantly suppressed lipin-1 expression by 95.6 ± 0.5% compared with that of the HFD group and was greater than that of the orlistat group as a positive control.

3.5. Effects of FAVA on Hepatic Histology of HFD-Fed C57BL/6 Mice and mRNA Levels of Cholesterol Biosynthesis in Liver. A common characteristic among people with obesity is

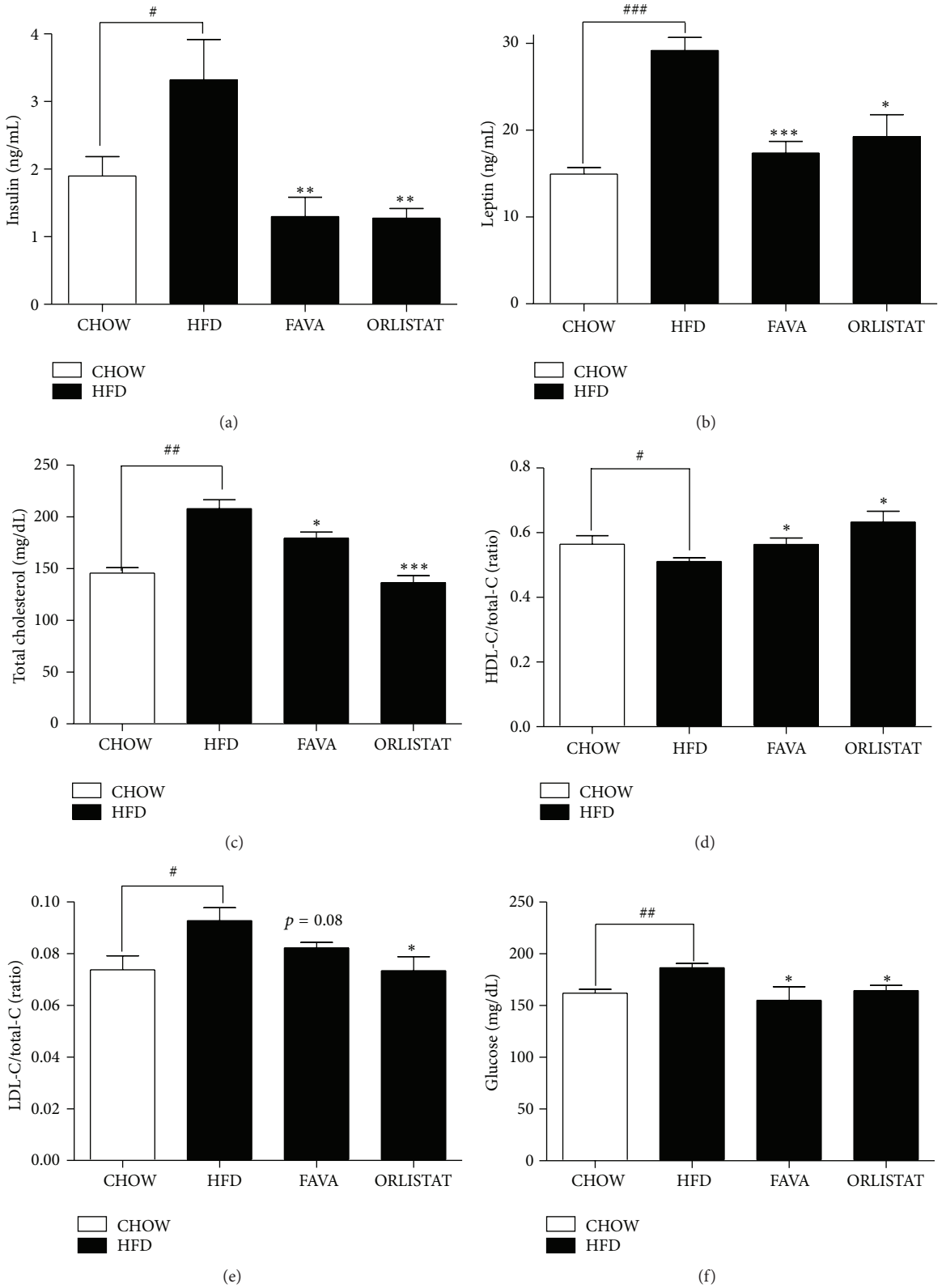

Figure 3: Effect of FAVA on serum insulin, leptin, and lipid profiles in mice fed a high-fat diet. Changes in insulin (a), leptin (b), total cholesterol (c), the ratio of HDL cholesterol/total cholesterol (d), and the ratio of LDL cholesterol/total cholesterol (e) of the mice were measured. The values represent the mean ± SEM ($^{\#}p < 0.05$, $^{\#\#}p < 0.005$, and $^{\#\#\#}p < 0.0005$ versus the CHOW group; $^{*}p < 0.05$, $^{**}p < 0.005$, and $^{***}p < 0.0005$ versus the HFD group, $n = 5$~7 per group).

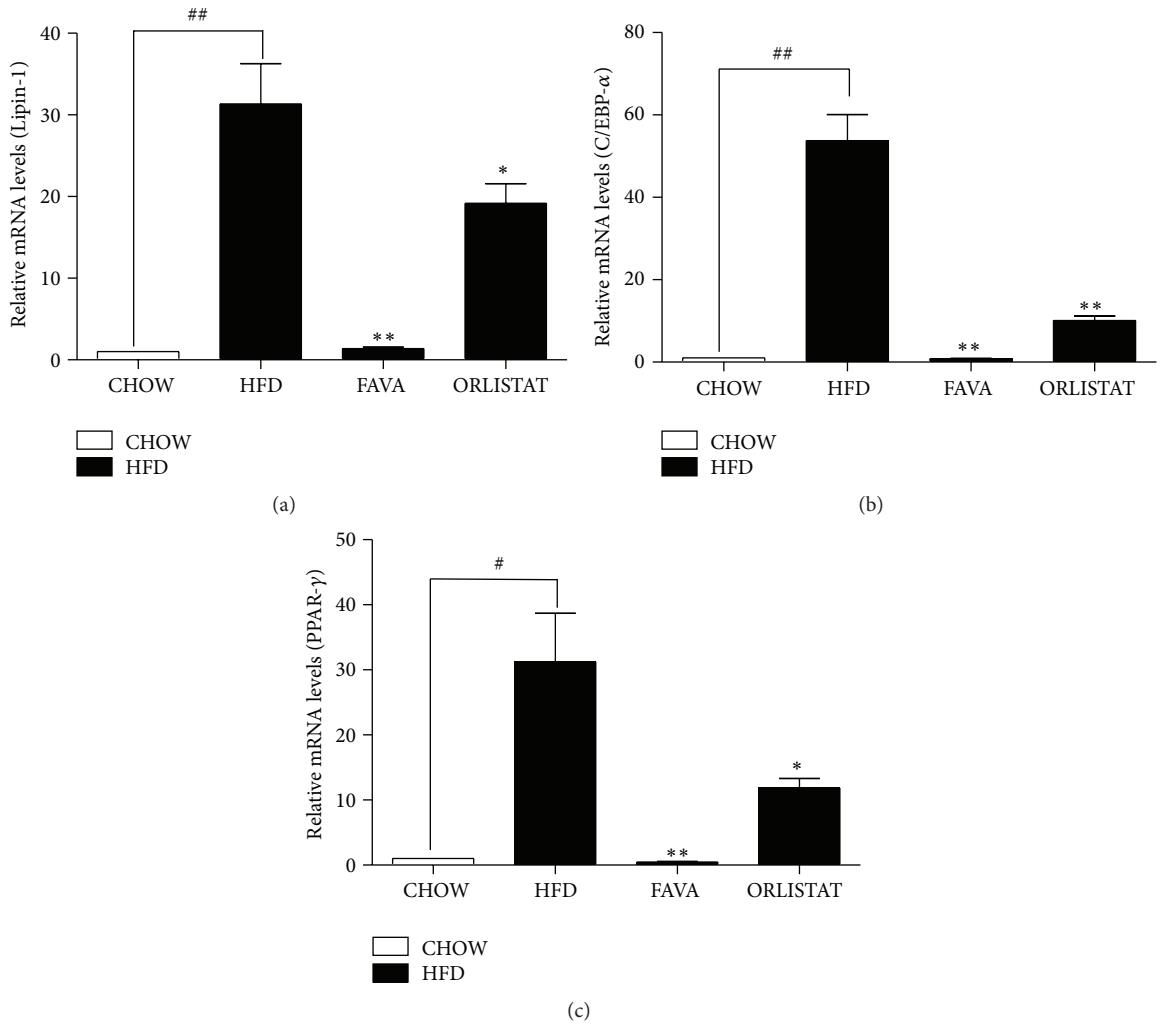

Figure 4: mRNA expressions of transcription factors in the epididymal fat pad of animals treated with HFD or HFD + ORLISTAT (high-fat diet plus orlistat 60 mg/kg) or FAVA (high-fat diet plus FAVA 200 mg/kg) or chow as quantified by real-time PCR. The graphs represent mRNA expression of transcription factors Lipin-1 (a), ACC and C/EBP-α (b), and PPAR-γ. The data represent the mean ± SEM ($^{\#}p < 0.05$ and $^{\#\#}p < 0.005$ versus the CHOW group; $^{*}p < 0.05$ and $^{**}p < 0.005$ versus the HFD group, $n = 5$).

the development of fatty liver [29, 30]. Therefore, we also analyzed the effect of FAVA on fatty liver development. Histological evaluation is regarded as the "gold standard" for assessing the presence and severity of NAFLD [31]. We histologically evaluated liver sections to determine the extent to which FAVA attenuated hepatic steatosis development. As shown in Figure 5(a), mild fatty liver was observed in mice that were fed a high-fat diet without FAVA. However, a marked reduction in the degree of steatosis was shown in livers from high-fat diet mice treated with FAVA. Moreover, FAVA treatment also decreased total serum cholesterol in mice to 13.7 ± 3.4% (Figure 3(c)). Therefore, we investigated whether SREBP-2, AACS, and HMGCR RNA in the mouse liver were induced by FAVA. Total RNA was prepared from mouse livers, and SREBP-2, AACS, and HMGCR mRNA levels were quantified using real-time PCR. SREBP-2, AACS, and HMGCR mRNA levels were dramatically suppressed in the mice that were fed FAVA (Figures 5(b) and 5(c)).

4. Discussion

Our study is the first to demonstrate that FAVA prevents weight gain in HFD-induced obesity in C57Bl/6 mice. Our results showed that body weight gain in groups fed a diet supplemented with FAVA was reduced compared with control HFD mice (Figure 1(a)). Epididymal, mesenteric, and subcutaneous fat pads in C57BL/6 mice were significantly reduced by FAVA supplementation (Figures 1(c)–1(e)). There was a significant reduction in subcutaneous and abdominal fat mass in FAVA-fed groups compared with the HFD group (Figures 2(a)–2(c)). Subcutaneous fat and abdominal fat are the major types of white adipose tissue. Abdominal obesity is associated with an increased risk of cardiovascular diseases and insulin resistance [32]. This study also provides evidence that dietary supplementation of FAVA protects against hepatic steatosis development (Figure 5(a)). We have considered the possibility that the effect of FAVA

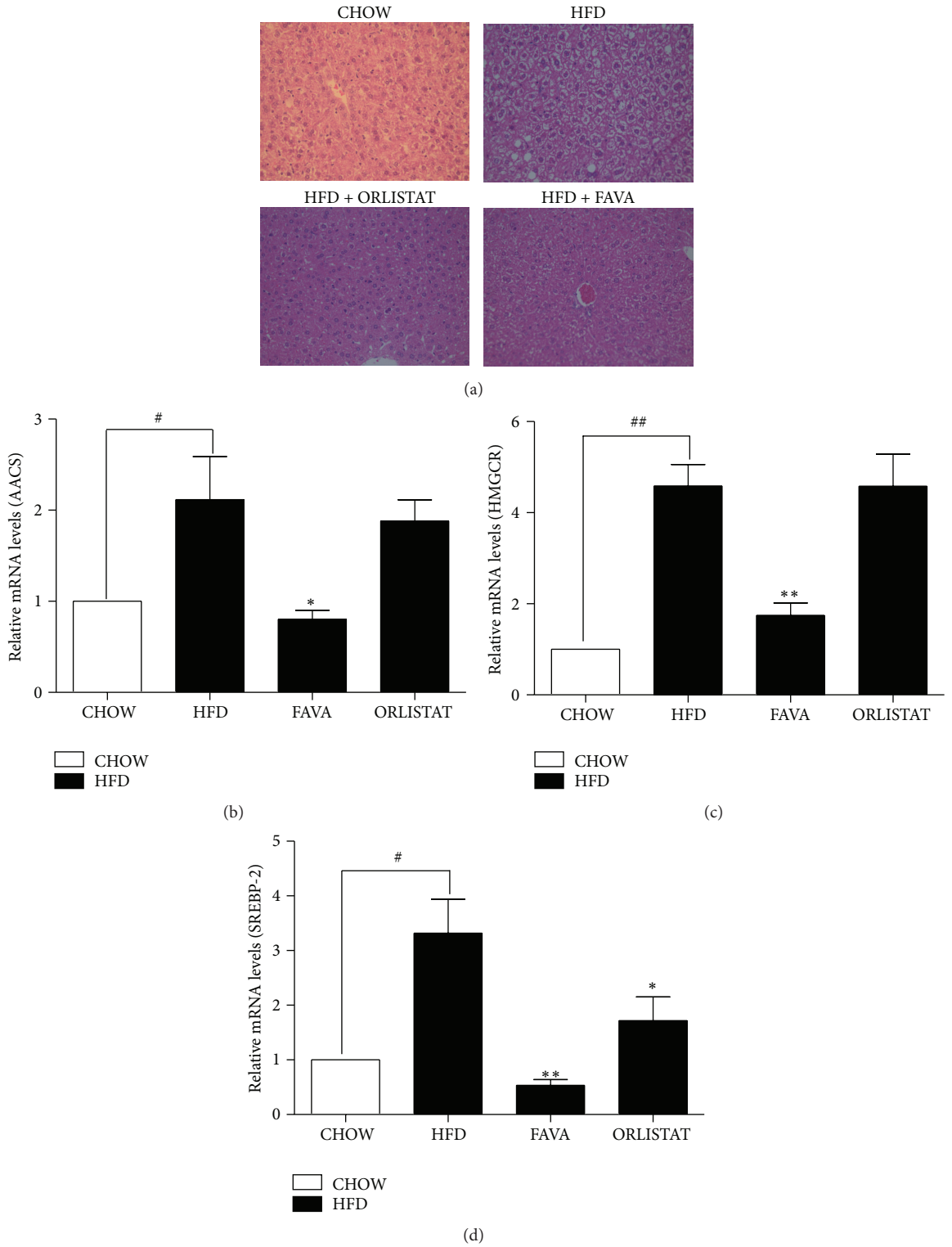

Figure 5: Effect of FAVA on hepatic steatosis and mRNA expressions of cholesterol biosynthesis in the liver of mice. (a) Hematoxylin and eosin staining of liver from mice fed chow diet (CHOW), high-fat diet (HFD), or high-fat diet supplemented with orlistat at 60 mg/kg/day (HFD + ORLISTAT) or high-fat diet supplemented with FAVA at 200 mg/kg/day (HFD + FAVA) (40x magnification). ((b)–(d)) The graphs represent mRNA expression of cholesterol synthesis factors AACS (b), HMGCR (c), and SREBP2 (d), which was analyzed by real-time PCR. The data represent the mean ± SEM ($^{\#}p < 0.05$ and $^{\#\#}p < 0.005$ versus the CHOW group; $^{*}p < 0.05$ and $^{**}p < 0.005$ versus the HFD group, $n = 5$).

may be mediated through food intake because decreased food intake would be expected to significantly affect body weight, which influences hepatic steatosis. In this study, however, there was no difference in food intake-induced increase of body weight between the FAVA-fed and non-FAVA-fed groups (Figure 1(b)). This result suggests that FAVA directly protected against obesity and hepatic steatosis independent of food intake.

Obesity is most likely to cause hyperlipidemia, which is considered the leading cardiovascular risk. The hallmark of dyslipidemia in obesity is hypertriglyceridemia in combination with the preponderance of high LDL and low HDL cholesterol [33]. This study shows that, in high-fat diet-fed mice, FAVA supplementation significantly reduced serum levels of cholesterol and the ratio of LDL cholesterol/total cholesterol (Figures 3(c) and 3(d)). Furthermore, insulin levels were increased in the HFD group and were decreased significantly by FAVA supplementation (Figure 3(a)). In the case of prediabetes, increases of blood glucose stimulate the secretion of insulin and subsequently induce hyperinsulinemia to a normal blood glucose range. Hyperinsulinemia, which is a biomarker of insulin resistance, is frequently accompanied by obesity [34]. Leptin is a fat-derived hormone that plays an important role in appetite control and energy expenditure [35]. It has been reported that the concentration of serum leptin is associated with general adiposity and reflects the body fat content [36]. In this report, it was demonstrated that FAVA treatment suppressed the plasma leptin level in mice fed with HFD (Figure 3(b)). Moreover, the weight of adipose tissues strongly correlated with the plasma leptin level. These results confirm that FAVA treatment exerted an antiobesity effect in the diet-induced obesity C57BL/6 mouse model.

PPAR-γ, a transcription factor predominantly expressed in adipose tissue, plays an essential role in adipocyte differentiation, lipid storage, and glucose homeostasis [37]. Additionally, adipogenesis is highly regulated by two primary adipogenic transcription factors, PPAR-γ and C/EBPs [38]. Among those factors, PPAR-γ is well known as the key regulator of adipogenic transcription [10]. PPAR-γ is also known to bind to the C/EBP-α promoter region that induces the expression of C/EBP-α [39]. C/EBP-α is a promising candidate transcription factor for directly controlling adipocyte differentiation [40]. We found that FAVA significantly downregulated PPAR-γ and C/EBP-α mRNA levels in the epididymal fat pad. This effect might be explained in two ways: FAVA either inhibited PPAR-γ and C/EBP-α or suppressed the upstream molecules. Lipin-1 is also required in adipocyte differentiation for the induction of the adipogenic gene transcription [12]. We found that FAVA could inhibit adipocyte differentiation through the suppression of lipin-1.

Acetoacetyl-CoA synthetase (AACS) can facilitate the incorporation of ketones into lipogenesis [13]. Hasegawa et al. [13] demonstrated that the AACS gene, which encodes the ketone body-utilizing enzyme, is transcriptionally regulated by SREBP-2 and the knockdown of SREBP-2 induced downregulation of AACS and HMGCR gene expression. Additionally, ketone body metabolism via AACS plays an essential role in cholesterol homeostasis. In this study, we showed that the treatment of mice with FAVA resulted in a decrease of SREBP2, AACS, and HMGCR mRNA levels. Therefore, our results suggest that FAVA improves obesity, hyperlipidemia, and NAFLD and that FAVA treatment might be a promising adjuvant therapy in the management of these metabolic disorders.

5. Conclusions

FAVA had a marked inhibitory effect on the development of obesity and NAFLD in a high-fat diet-induced obesity mouse model. Inhibiting transcription factors and adipocyte-specific lipogenic genes and decreasing cholesterol synthesis are two possible mechanisms for the antiobesity effect of FAVA. This study suggests that FAVA might be a potential dietary supplement for preventing obesity and NAFLD.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

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Research Article

Metabolomic Analysis of Biochemical Changes in the Plasma of High-Fat Diet and Streptozotocin-Induced Diabetic Rats after Treatment with Isoflavones Extract of Radix Puerariae

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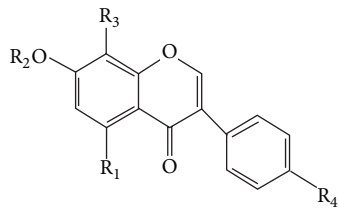
The main purpose of this study was to investigate the protective effects of total isoflavones from Radix Puerariae (PTIF) in diabetic rats. Diabetes was induced by a high-fat diet and intraperitoneal injection of low-dose streptozotocin (STZ; 40 mg/kg). At 26 weeks onwards, PTIF 421 mg/kg was administrated to the rats once daily consecutively for 10 weeks. Metabolic profiling changes were analyzed by Ultraperformance Liquid Chromatography-Quadrupole-Exactive Orbitrap-Mass Spectrometry (UPLC-Q-Exactive Orbitrap-MS). The principal component discriminant analysis (PCA-DA), partial least-squares discriminant analysis (PLS-DA), and orthogonal partial least-squares discriminant analysis (OPLS-DA) were used for multivariate analysis. Moreover, free amino acids in serum were determined by high-performance liquid chromatography with fluorescence detector (HPLC-FLD). Additionally, oxidative stress and inflammatory cytokines were evaluated. Eleven potential metabolite biomarkers, which are mainly related to the coagulation, lipid metabolism, and amino acid metabolism, have been identified. PCA-DA scores plots indicated that biochemical changes in diabetic rats were gradually restored to normal after administration of PTIF. Furthermore, the levels of BCAAs, glutamate, arginine, and tyrosine were significantly increased in diabetic rats. Treatment with PTIF could regulate the disturbed amino acid metabolism. Consequently, PTIF has great therapeutic potential in the treatment of DM by improving metabolism disorders and inhibiting oxidative damage.

1. Introduction

Diabetes mellitus (DM), a typical complex metabolic disease characterized by insulin deficiency or insulin resistance, can induce a series of complications such as nephropathy, cardiovascular diseases, and diabetic retinopathy. DM was known as "Xiaoke" disease in traditional Chinese medicine. Herbal medications play a particularly important role in the treatment of diabetes for thousands of years and their curative mechanism has been gradually recognized nowadays [1]. For example, isoflavones-containing herbs, also commercially available as dietary supplements, have many reported benefits to cardiovascular and diabetes treatments [2, 3]. Meanwhile, accumulated clinical evidence shows that the conjunction usage of traditional Chinese medicine with western medicine could largely minimize the occurrence of diabetic complications [4].

Puerariae Radix, the dried root of *Pueraria lobata* (Willd.) Ohwi, is also known as Gegen and Kudzu. It is an important source of isoflavones, including puerarin, daidzin, genistin, daidzein, and genistein (Figure 1), which are considered to be the principal bioactive ingredients [5]. Gegen has been used widely in traditional Chinese classical prescriptions for diabetes treatment, such as Gegen Qinlian decoction, Huangqi Gengen decoction, and Yuquan Wan. Emerging evidence also indicates that the extract of Puerariae Radix can improve antioxidant defense system by reducing the plasma level of coenzyme Q9 and MDA [6]. Puerarin has shown great potential benefits in diabetes treatment, not only protecting islets cells from oxidative stress by activating antioxidant enzymes [7], but also greatly improving insulin responsiveness in diabetic mice when used as an adjuvant for metabolic disease [8]. However, the exact therapeutic



	R_1	R_2	R_3	R_4
Puerarin	H	H	Glu	OH
Daidzin	H	Glu	H	OH
Genistin	OH	Glu	H	OH
Daidzein	H	H	H	OH
Genistein	OH	H	H	OH

Figure 1: Chemical structure of isoflavonoids from Puerariae Radix.

mechanism of isoflavones extract of Radix Puerariae on diabetes has not yet been well explained.

Metabolomics can provide a systematic overview on the impact of the disease processes and drug interventions, which is well coincident with the holistic theory of Chinese medicine [9]. Recently, metabonomics has been increasingly applied in the diagnosis and evaluation of the therapeutic effects of DM and its complications [10, 11]. For instance, 12 biomarkers were identified using metabolomics in KKay mice, and a water-soluble extract from *Ophiopogon japonicus* has potential activity against diabetes [12]. A recent metabolomic study suggested that 20(S)-ginsenoside Rg3, an active ingredient of *Panax ginseng*, may be involved with the regulation of nucleic acid metabolism, energy metabolism, and gut flora metabolism in type 2 diabetes [13]. Another famous natural product, berberine, might play a pivotal role through downregulating the high level of free fatty acids as the metabolomics study showed for type 2 diabetes treatment [14]. Taken together, these studies unveiled that diabetic diseases have a direct relationship with a disorder in the lipids, fatty acids, and energy metabolism. Additionally, as one kind of metabolites, amino acids especially the branched-chain amino acids (BCAAs) are strongly associated with diabetes and its complications [15] and can presage the development of diabetes [16].

In the development of diabetes and its complications, oxidative stress or imbalance between prooxidants and antioxidants is often thought to be involved [17]. Oxidative stress reflects an imbalance between reactive oxygen species (ROS) and the antioxidants, and high level of this can cause severe cellular dysfunction including membrane lipid peroxidation, DNA fragmentation, and protein damage [18]. On the mechanism of oxidative damage, many studies have identified that the overproduction of superoxide causes the high gene expression of inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and vascular endothelial growth factor (VEGF) [19]. In addition, inflammation may cause pancreatic β-cell dysfunction, the development of insulin resistance, and so on [20, 21]. In fact, as some experimental and clinical studies have strongly suggested, the treatment based on antioxidant and anti-inflammatory is effective in DM and its complications therapeutic [22, 23].

Considering the DM is associated with metabolic disorders, advanced oxidative stress, and chronic inflammation, we evaluated the therapeutic effects of Puerariae Radix on diabetic rats model induced by a high-fat diet and low-dose streptozotocin and explored the metabolomics profiles using Ultraperformance Liquid Chromatography-Quadrupole-Exactive Orbitrap-Mass Spectrometry and liquid chromatography with fluorescence detector.

2. Materials and Methods

2.1. Chemicals and Reagents. Puerarin with the batch number of 110752-200912 was bought from NICPBP (China). Daidzin (MUST-15031507), daidzein (MUST-1505-1511), genistin (MUST-14110908), and genistein (MUST-15021802) were bought from the Chengdu Must Bio-Technology Co., Ltd. (Chengdu, China). STZ with the batch number of SLBB7526V were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Water used in this study was prepared by ULUP Ultrapure Water System (Ulupure, Chengdu, China). Methanol and acetonitrile with HPLC-grade were purchased from Thermo Fisher Scientific Inc. (Iowa, USA).

2.2. Animals and Experimental Design. Healthy adult male Sprague-Dawley rats weighing (250 ± 20) g were purchased from the Animal Breeding Center of DaShuo Biotechnology Co., Ltd. (Chengdu, China). All rats were maintained on an alternating 12 h light/dark cycle, at a temperature of 22–25°C and humidity of 55%–60%. This study was carried out in China State Administration of TCM, Chengdu University of TCM (TCM Pharmacology P3 laboratory, number TCM2032043). Experimental procedures were strictly in accordance with the Guide for the Care and Use of Laboratory Animals. The animal protocol was approved by the Animal Ethical Committee of Chengdu University of Traditional Chinese Medicine. Rats were divided into normal group and high-fat diet group. Normal group was fed with standard diet. High-fat diet group was fed with high-fat diet for six weeks and then intraperitoneally injected with a single dose of a freshly prepared solution of streptozotocin (40 mg/kg in citric acid-sodium citrate buffer, pH 4.2) [24, 25]. Normal rats received citric acid-sodium citrate buffer only. After STZ injection, all rats were fed with standard diet. Animals with blood glucose levels ≥16.7 mmol/L 72 h after administration of STZ were considered as diabetic rats. Diabetic rats were randomly divided into two groups: the diabetic group and diabetic + PTIF group. At 26 weeks onwards, PTIF was administrated to the treatment rats by intragastric gavage (i.g.) at a dosage of 421 mg/kg. The normal rats and untreated diabetic rats received distilled water the same way. PTIF treatment was performed once daily consecutively for 10 weeks. All animals were allowed to eat and drink freely. At the end of the experiment, plasma/serum samples from each one were isolated by centrifugation at 3500 rpm for 10 min at 4°C. The samples were immediately stored at −20°C until the measurement of oxidative stress and inflammatory cytokines and at −80°C until the metabolomics and amino acid analysis were performed.

2.3. Preparation of PTIF. PTIF was provided by Taiji Group, Chongqing Fuling Pharmaceutical Factory Co., Ltd. (China). Isoflavones extract of Radix Puerariae: *Pueraria* powder was extracted with 10 times or 8 times volume of 60% ethanol for 1 h, respectively. The solvent was recovered under reduced pressure. Macroporous resin was used for the purification of the concentrated extraction solution. Isoflavones extract of Radix Puerariae was obtained with vacuum concentration, decompression drying and finally through a 100-mesh sieve.

2.4. Standardization of PTIF. A high-performance liquid chromatography method was established for the identification of the major compounds in PTIF (Agilent 1260 HPLC system, Agilent, CA, USA). A Capcell PAK C-18 analytical column (100 mm × 2.0 mm, 3 μm, Shisedo, Japan) was used with the column temperature maintained at 35°C. 0.1% formic acid in water and 0.1% formic acid in acetonitrile were treated as mobile phases A and B, respectively. The mobile phase gradient elution was programmed as follows: 90% A (0.01–12 min), 90%–80% A (12.1–20 min), and 80%–75% A (20.1–30 min). The flow rate was set at 0.5 mL/min, and the sample injection volume was set at 50 μL.

2.5. Measurement of Oxidative Stress and Inflammatory Cytokines. Superoxide dismutase (SOD_1) levels and hypoxia inducible factor 1 alpha (HIF-1α) were measured by a sandwich enzyme immunoassay method using a kit from Uscn Life Science Inc. (Wuhan, China). Plasma Malondialdehyde (MDA) was measured by competitive inhibition enzyme immunoassay technique using a kit from Uscn Life Science Inc. (Wuhan, China). Serum vascular endothelial growth factor (VEGF) and intercellular adhesion molecule -1 (ICAM-1) were measured by a sandwich enzyme immunoassay technique using a kit from R&D Systems, Inc. (USA). The concentration of nitric oxide (NO) in serum was quantified based on the enzymatic conversion of nitrate to nitrite by nitrate reductase using a Nitrotyrosine-EIA kit from R&D Systems, Inc. (USA). All data were carried out by Varioskan Multifunctional full wavelength microplate reader (Thermo Fisher Scientific, USA) according to the manufacturers.

2.6. UPLC-Q-Exactive Orbitrap-MS Analysis. The plasm samples were reconstituted to room temperature before analysis. Then, 400 μL acetonitrile was added to 100 μL of sample for protein precipitation. The mixture was vortexed for 3 min and then centrifuged at 12,000 rpm for 10 min at 4°C. Finally, the supernatant was transferred to an autosampler vial for further determination.

All the LC/MS data were acquired using Dionex™ Chromeleon™ 6.8 and Thermo Xcalibur software. The plasma samples were performed with a C18 column (Acquity UPLC BEH C18, 2.1 mm × 100 mm, 1.7 μm, Waters, United States). 0.1% formic acid in water and 0.1% formic acid in acetonitrile were considered as mobile phases A and B, respectively. The flow rate of 0.4 mL/min at 35°C was used in linear gradients as follows: 95–90% A (0–3 min), 90–85% A (3–6 min), 85–60% A (6–20 min), 60–35% A (20–25 min), 35–5% A (25–30 min), and 5% A (30–35 min). The standard positive ion mode was selected under the following conditions: full scan range, 80 to 1200m/z; scan resolution, 7,000m/z/s; sheath gas flow rate, 30 arbitrary units; aux gas flow rate, 10 arbitrary units; spray voltage, 3.5 KV; capillary temperature, 350°C; aux gas temperature, 200°C. The background ion 445.12503(+) was used as lock mass to ensure the mass calibration accuracy. A sample volume of 5 μL was used for injection.

2.7. Identification of Potential Biomarkers. The raw MS data obtained by X calibur were exported to MZ mine 2.14.2 using Thermo MS File Reader program. The main MZ mine parameters were set as follows: mass detector was exact mass at noise level 6.0$E4$ intensity; min time span was 0.1 min with m/z tolerance 5.0 ppm; chromatogram deconvolution used baseline cut-off algorithm and min peak height with 1.0$E5$; isotopic peaks grouper used lowest m/z representative isotope and setting maximum charge 1. Each sample data was normalized to total area to correct for the MS response shift prior to multivariate analysis. The information of potential biomarkers was obtained by searching databases of KEGG, Lipidmaps, and Metlin based on the accurate molecular weights and isotope shapes.

2.8. Amino Acid Analysis. Free amino acids in serum samples were measured by a HPLC-FLD method using an AccQ·Tag precolumn derivatization technology with waters AccQ·Tag chemistry package (Waters Chemical Co., USA). 70 μL borate extraction agent was added to 10 μL serum, and then 20 μL AccQ-Flour derived reagent was added to the mixture. After incubating at 55°C for 10 min, supernatants were collected for the analysis. The amino acid concentration was measured with high-performance liquid chromatography using an amino acid analysis column (3.9 mm × 150 mm, 4 μm, Waters, USA) and a mobile phase consisting of (A) water containing 10% AccQ·Tag Eluent A and (B) water containing 40% acetonitrile at a flow rate of 1 mL/min at 37°C with an excitation wavelength at 250 nm and an emission wavelength at 395 nm. The mobile phase gradient elution was programmed as follows: 5–98% A (0–0.5 min), 98–93% A (0.5–15 min), 93–90% A (15–19 min), 90–67% A (19–32 min), 67% A (32-33 min), 67–0% A (33-34 min), 0% A (34–37 min), 0–100% A (37-38 min), and 100% A (38–64 min). The sample injection volume was set at 10 μL.

2.9. Data Analysis. Data was analyzed using R version 3.1.2 (R Core Team; 2014 R: A Language and Environment for Statistical Computing; R Foundation for Statistical Computing, Vienna, Austria; URL: https://www.r-project.org/.), PCA-DA, PLS-DA, and OPLS-DA in package MUMA (Metabolomics Univariate and Multivariate Analysis, Ver 1.4; Edoardo G, Francesca C, Dimitrios S, Silvia M, Andrea S, and Michela G, 2012). R package version 1.4. https://cran.r-project.org/web/packages/muma/index.html) of R was used for multivariate analysis. All results were presented as the mean ± SD. One-way analysis of variance (ANOVA) was used for significance analysis. Values of $P < 0.05$ were considered statistically significant.

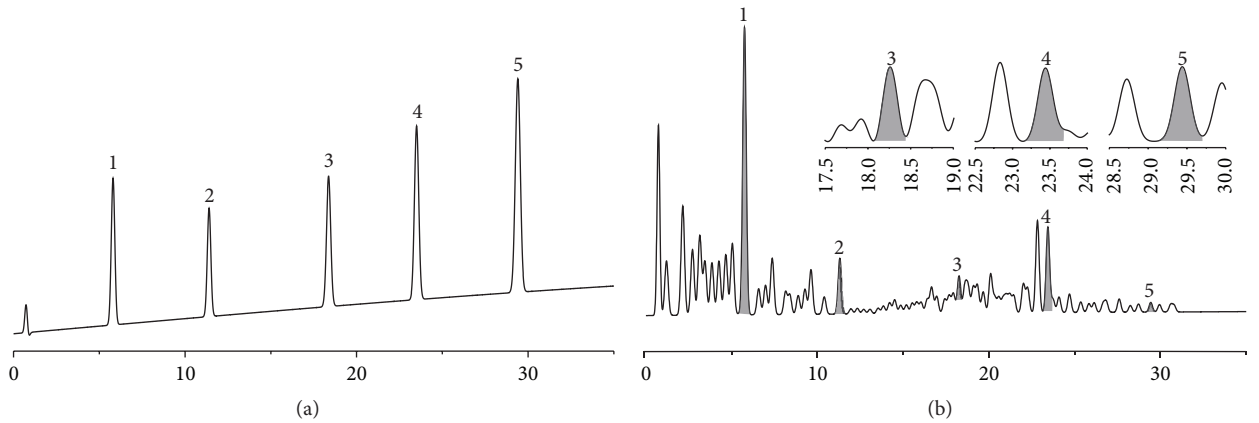


Figure 2: Typical high-performance liquid chromatography profile of five standard mixtures of isoflavonoids (a) and root extract of Puerariae Radix (b) at an absorbance of 254 nm. 1, puerarin (5.8 min); 2, daidzin (11.7 min); 3, genistin (18.2 min); 4, daidzein (23.7 min); 5, genistein (29.3 min).

Table 1: Effects of PTIF (421 mg/kg) on body weight in high-fat diet and STZ-induced diabetic rats (g).

Group	N	0 weeks	26 weeks	30 weeks	34 weeks	36 weeks
Normal	10	283.4 ± 7.8	522.2 ± 22.0	553.6 ± 24.4	586.2 ± 30.4	601.8 ± 30.5
Diabetic	10	280.1 ± 5.2	404.1 ± 49.6*	375.3 ± 50.3**	378.0 ± 54.8**	386.4 ± 57.5**
Diabetic + PTIF	9	285.3 ± 7.3	426.8 ± 39.5	406.8 ± 48.1	431.7 ± 48.0$^{\#}$	438.9 ± 49.4$^{\#}$

All data were expressed as mean ± SD. One-way analysis of variance (ANOVA) was used for significance analysis. $^{*}P < 0.05$ and $^{**}P < 0.01$ indicate a significant difference as compared with normal rats; $^{\#}P < 0.05$ indicate a significant difference as compared with diabetic rats.

Table 2: Effects of PTIF (421 mg/kg) on blood glucose in high-fat diet and STZ-induced diabetic rats (mmol/L).

Group	N	0 weeks	26 weeks	30 weeks	34 weeks	36 weeks
Normal	10	6.4 ± 0.5	6.8 ± 0.7	6.2 ± 0.5	6.4 ± 0.4	6.6 ± 0.6
Diabetic	10	6.5 ± 0.7	25.1 ± 2.9**	25.0 ± 3.5**	23.9 ± 4.0**	25.1 ± 3.4**
Diabetic + PTIF	9	6.2 ± 0.6	25.0 ± 2.1	23.6 ± 1.9	23.5 ± 3.0	22.5 ± 3.3

All data were expressed as mean ± SD. One-way analysis of variance (ANOVA) was used for significance analysis. $^{**}P < 0.01$ indicate a significant difference as compared with normal rats.

3. Results

3.1. Standardization of PTIF. A typical HPLC fingerprint of PTIF was shown in Figure 2. Five major components were determined by a HPLC method. They were well separated and the retention times are 5.8, 11.7, 18.2, 23.7, and 29.3 min for puerarin, daidzin, genistin, daidzein, and genistein, respectively. As a result, the total isoflavonoids content was 48.00%. As the main constituent, the concentration of puerarin was 19.8%. Daidzin was the next highest at 2.7%, and genistin and daidzein were present at 1.53% and 0.33%, respectively, while genistein has the lowest level at 0.12%.

3.2. Effect of PTIF on Body Weight and Blood Glucose Level. As shown in Table 1, compared with the normal group, STZ treatment resulted in a significant decrease in body weight during the 36 weeks of observation. STZ treatment also induced a sustained high blood glucose level as shown in Table 2. While, compared with diabetic group, rats in diabetic + PTIF group showed a significant increase in body weight at weeks 34 and 36 ($P < 0.05$), there was no significant difference in blood glucose with the treatment of PTIP.

3.3. Effect of PTIF on Oxidative Stress and Inflammatory Cytokines in STZ-Induced Diabetic Rats. To investigate the effect of PTIF on antioxidant and anti-inflammatory, we examined the concentration of oxidative stress and inflammatory cytokines. As illustrated in Figure 3, compared with the normal group, the activation of SOD_1 in diabetic rats was obviously decreased ($P < 0.01$) and significantly increased by PTIF treatment ($P < 0.05$). The concentrations of HIF-α and MDA were both remarkably increased and were restored to normal values in PTIF-treated rats. In diabetic rats, we found increased levels of VEGF and ICAM-1 ($P < 0.05$) (Figure 3). Moreover, there were no visible differences in NO levels in the three groups ($P > 0.05$). Treatment with PTIF has no significant influences on inflammatory cytokines, such as VEGF, ICAM-1, and NO in this study ($P > 0.05$).

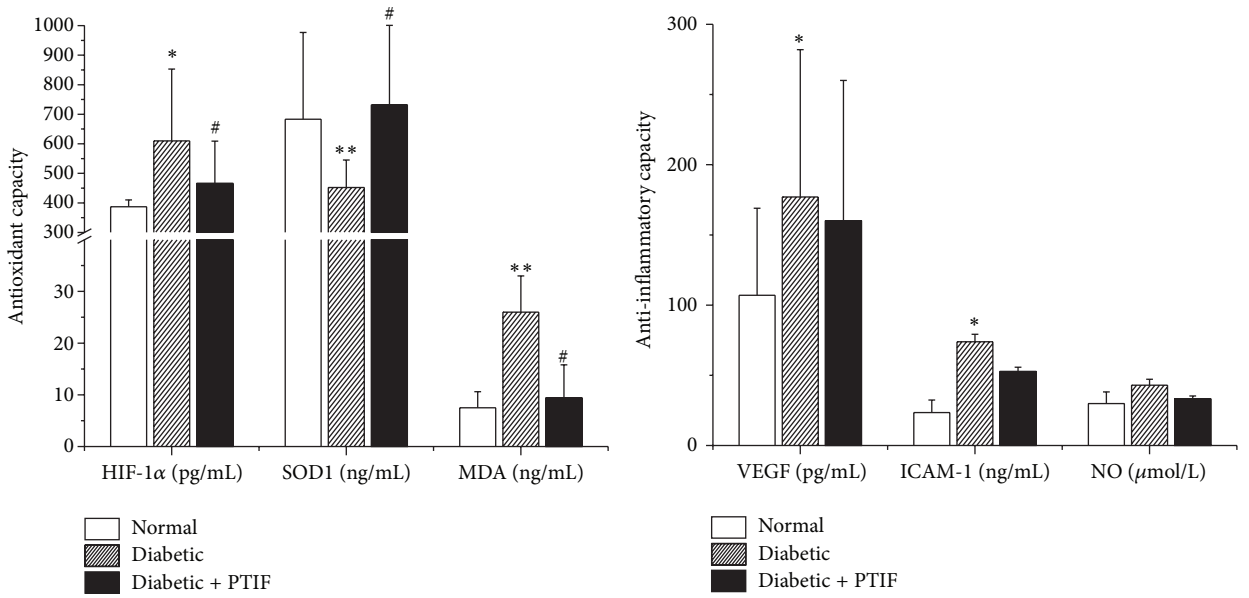


FIGURE 3: The level of HIF-1α, SOD_1, MDA, VEGF, ICAM-1, and NO in the sample from normal rats, diabetic rats treated with distilled water, and diabetic rats treated with PTIF (421 mg/kg). All data were expressed as mean ± SD. One-way analysis of variance (ANOVA) was used for significance analysis. $^{*}P < 0.05$ and $^{**}P < 0.01$ indicate a significant difference as compared with normal rats; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ indicate a significant difference as compared with diabetic rats.

TABLE 3: Identification results of main potential biomarkers changes.

Number	RT (min)	Formula	Mass (m/z)	Potential biomarkers	Diabetic versus Normal	Treatment versus Diabetic
1	8.83	$C_{15}H_{31}N_5O_5$	362.24	Lys-Lys-Ser	$\uparrow^{**}$	$\downarrow^{\#\#}$
2	9.27	$C_{21}H_{42}N_6O_6$	475.33	Leu-Ser-Lys-Lys	$\uparrow^{**}$	$\downarrow^{\#\#}$
3	9.65	$C_{29}H_{60}NO_7P$	566.43	PC(21:0/0:0)[U]	$\downarrow^{**}$	$\uparrow^{\#\#}$
4	15.79	$C_9H_{20}O_3$	177.15	2-Hexylglycerol	$\downarrow^{*}$	$\uparrow^{\#}$
5	16.65	$C_8H_{14}O_3$	159.1	7-Keto-n-caprylic acid	$\downarrow^{**}$	$\uparrow^{\#}$
6	20.65	$C_{11}H_{15}N_5O_3S$	298.1	5′-Methylthioadenosine	$\uparrow^{**}$	$\downarrow^{\#\#}$
7	21.18	$C_7H_{12}O_3$	145.08	7-Oxoheptanoic acid	$\downarrow^{*}$	$\uparrow^{\#\#}$
8	21.59	$C_{31}H_{46}O_2$	451.36	Phylloquinone	$\uparrow^{**}$	$\downarrow^{\#\#}$
9	28.38	$C_{42}H_{84}NO_8P$	784.58	PC(22:0/12:0)	$\uparrow^{*}$	$\downarrow^{\#}$
10	28.79	$C_{16}H_{27}N_9O_5$	425.21	Gly-His-Arg-Gly	$\uparrow^{*}$	$\downarrow^{\#\#}$
11	29.45	$C_{46}H_{82}NO_8P$	808.58	PC(20:4(5Z,8Z,11Z,14Z)/18:1(11Z))	$\uparrow^{*}$	$\downarrow^{\#\#}$

"↑" and "↓" represent upregulation and downregulation. $^{*}P < 0.05$ and $^{**}P < 0.01$ indicate a significant difference as compared with normal rats; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ indicate a significant difference as compared with diabetic rats.

3.4. Validation of UPLC-Q-Exactive Orbitrap-MS Conditions. The plasma samples were analyzed by UPLC-Q-Exactive Orbitrap-MS according to the method below. Representative UPLC-Exactive Plus Orbitrap-Mass TIC chromatograms of the plasma samples from the three groups are presented in Figure 4. The precision, repeatability of sample preparation, and system stability were validated before the experimental sample analysis. The results indicated that this method could be used in subsequent metabolomics analysis of plasma samples.

3.5. Pattern Recognition and Identification of Potential Biomarkers. The principal component discriminant analysis (PCA-DA), partial least-squares discriminant analysis (PLS-DA), and orthogonal partial least-squares discriminant analysis (OPLS-DA) are frequently adopted for multivariate analysis. The discovery of potential biomarkers in the plasma enhances our understanding of significant metabolic variations associated with DM rats. As shown in Figure 5, PLS-DA scores plots indicated that there is a satisfactory classification between the normal and diabetic rats, which indicated that the plasma metabolic fingerprint was significantly disturbed by the treatment of streptozotocin. Moreover, biochemical changes of model rats were gradually restored to normal after administration of PTIF, which means that PTIF have a significant efficacy in the improvement of metabolism disorders in STZ-induced diabetic rats.

Furthermore, eleven potential metabolite biomarkers were identified and their functional pathways have been analyzed (shown in Table 3). Boxplots of identical

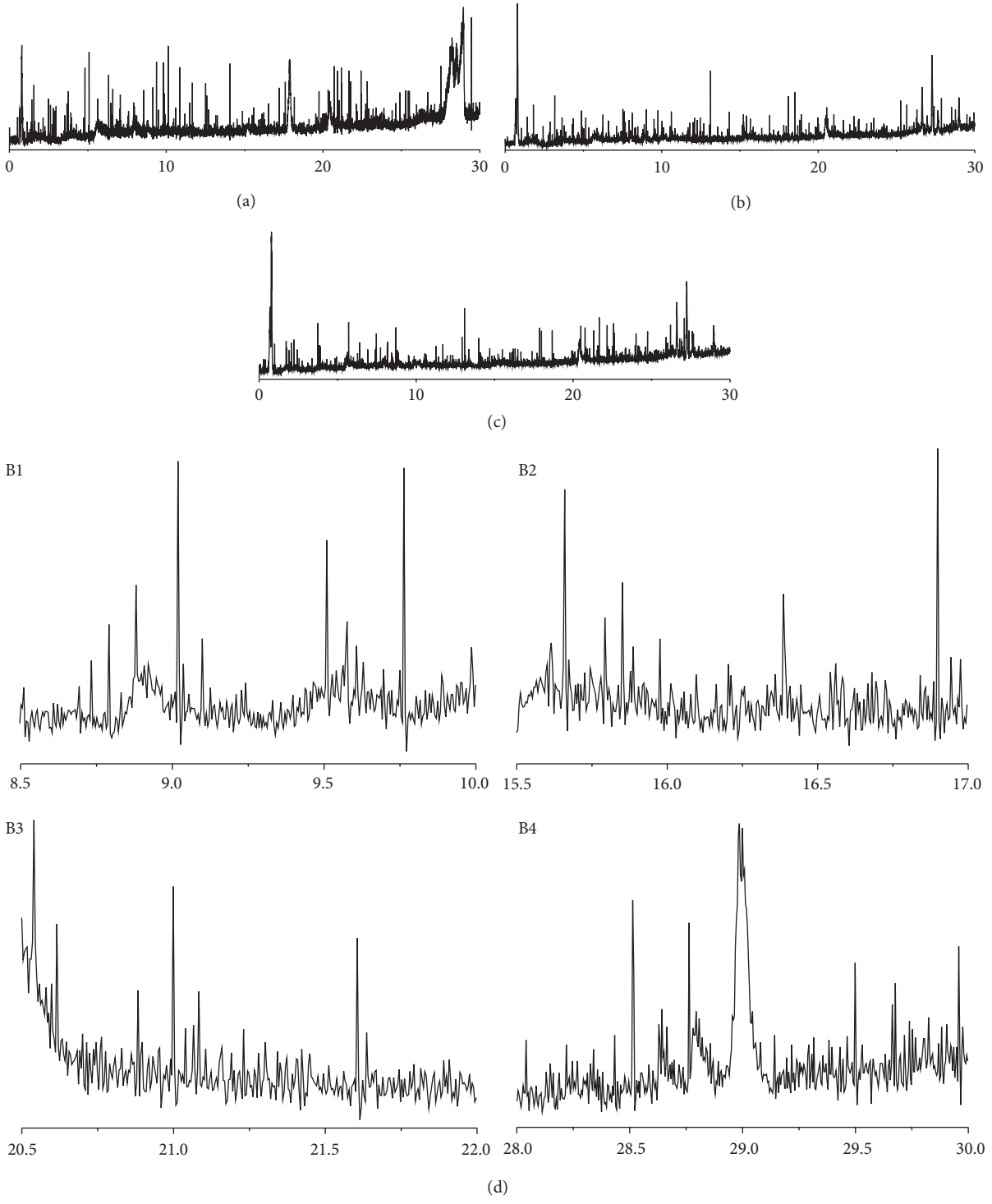


Figure 4: Representative UPLC-Exactive Plus Orbitrap-Mass TIC chromatograms of the plasma samples from the three groups. (a) Normal group, (b) diabetic group, and (c) diabetic + PTIP (421 mg/kg/day) group. (d) B1, B2, B3, and B4 detailed chromatogram B in corresponding time intervals.

biomarkers intensity of rat plasma were shown in Figure 6. In particular, the lipid metabolism-related metabolites, including PC(21:0/0:0)[U], 2-hexylglycerol, 7-keto-n-caprylic acid, 7-oxoheptanoic acid, PC(22:0/12:0), and PC(20:4(5Z, 8Z,11Z,14Z)/18:1(11Z)), have been identified in diabetic rats, indicating a dysregulation of lipid metabolites in diabetic rats. Compared with normal control group, the phylloquinone level was significantly increased in diabetic model ($P < 0.01$). Moreover, the metabolomic results also illustrated that PTIF possess a therapeutic influence on

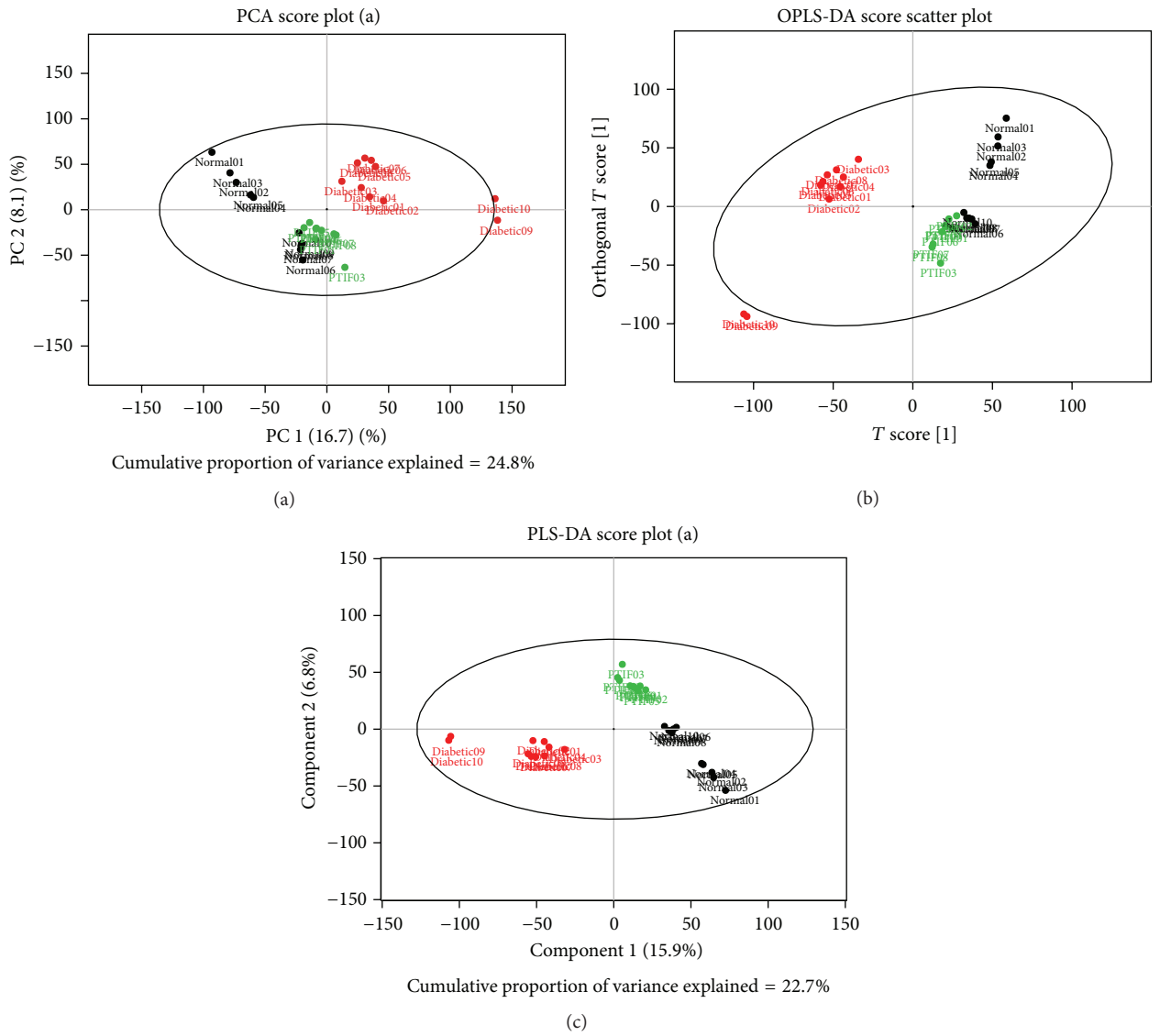


FIGURE 5: Scores plots from PCA_DA (a), OPLS_DA (b), and PLS_DA (c) classifying normal control group (black dot, $n = 10$), diabetic group (red dot, $n = 10$), and diabetic rats treated with PTIF group (green dot, $n = 9$).

DM by partially ameliorating the coagulopathy. In addition, 5′-methylthioadenosine, Lys-Lys-Ser, Leu-Ser-Lys-Lys, and Gly-His-Arg-Gly were found to be significantly increased in model rats ($P < 0.01$). As shown in Figure 6, the marker metabolites with dark gray background possibly relate with amino acids metabolism. Treatment with PTIF exhibited a benefit effect on regulating the amino acid metabolism disorders to normal state.

3.6. Amino Acid Analysis Conditions. Free amino acids in serum samples were measured by a HPLC-FLD using an AccQ·Tag precolumn derivatization method. A typical high-performance liquid-fluorescent detection chromatography profile of standard mixtures of amino acids and extract of rat plasma was shown in Figure 7. In the chromatographic conditions established, the calibration curves showed good linearity over the studied concentration range. The within- and between-day variation coefficients were lower than 9.28% and 8.72%, respectively. The spiked recoveries were greater than 75.02%. The results showed that this method was satisfactory for the analysis of amino acids.

3.7. Plasma Amino Acid Concentration. Aspartate and cysteine were not detectable in some samples, so the statistical analyses of aspartate and cysteine were not involved. As shown in Table 4, compared with normal control group, the concentrations of the BCAAs (leucine, valine, and isoleucine) were significantly increased in diabetic rats ($P < 0.01$). Furthermore, the levels of glutamate, arginine, and tyrosine in serum were also significantly increased ($P < 0.01$). PTIF supplementation in diabetic rats can lower the level of glutamate and leucine ($P < 0.05$).

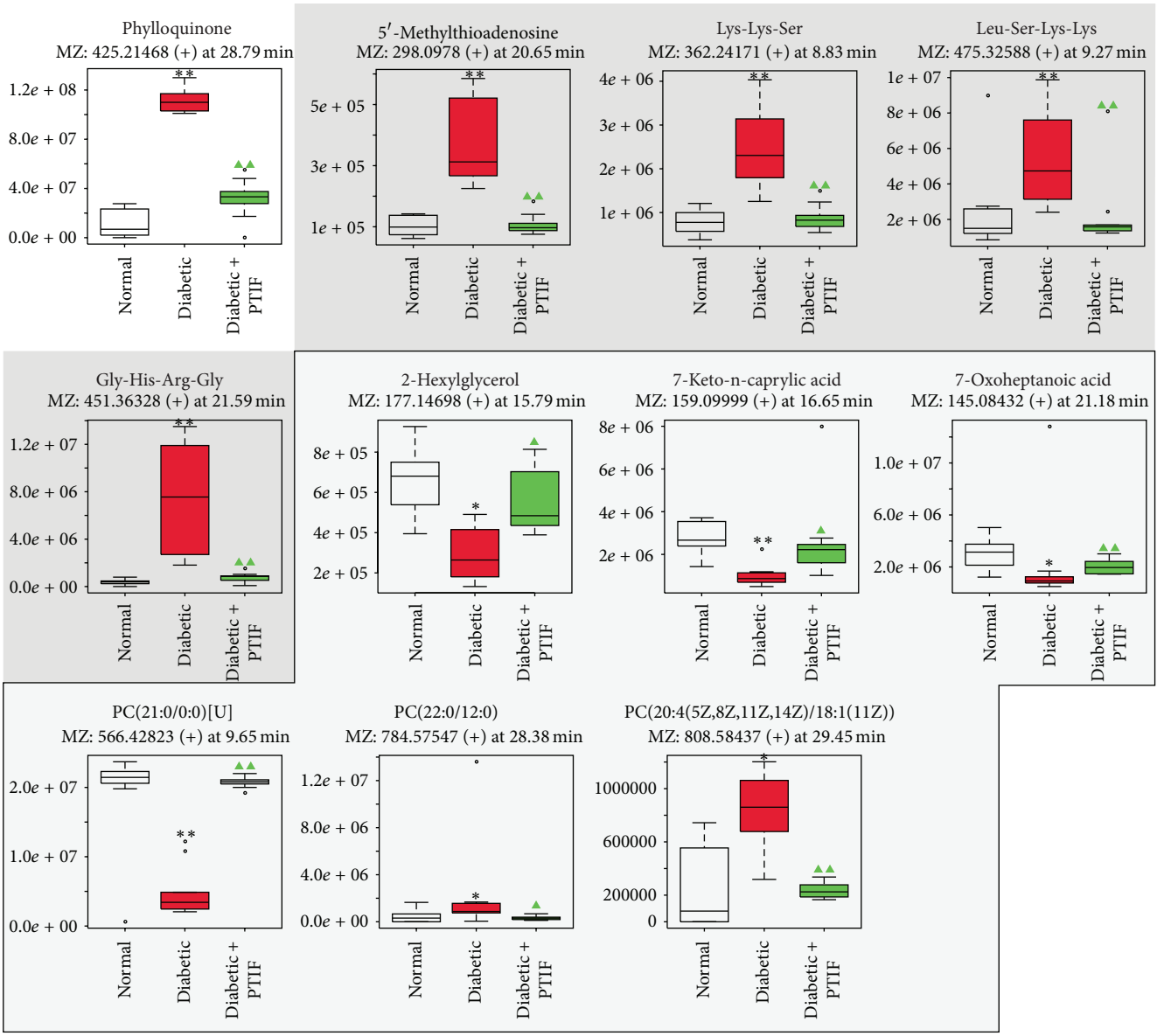


Figure 6: Boxplots of identical biomarkers intensity of rat plasma. For all figures, normal = white bars; diabetic = red bars; diabetic treated with PTIF = green bars; the vertical axis represents the chromatography peak intensity. The biomarkers are possibly related with amino acids, fatty acids, and PCs metabolism. $^{*}P < 0.05$ and $^{**}P < 0.01$ indicate a significant difference as compared with normal rats; ▲ $P < 0.05$, ▲▲ $P < 0.01$ indicate a significant difference as compared with diabetic rats.

4. Discussion

It is known that the traditional Chinese medicine therapy was focused on the system function of the body. Therefore, there are many advantages in the treatment of metabolic diseases like diabetes. For the concern about the hypoglycemic effect, the majority of diabetic patients commonly choose a combination therapy of certain antidiabetic drugs and adjuvants such as traditional Chinese medicine, instead of taking antidiabetic drugs alone. The present study shows that PTIF have no significant effect in restoring the disordered blood glucose in DM rats. Thus, it suggests that the therapeutic effect of PTIF might be achieved without improvement of the hyperglycemia of high-fat diet and STZ-induced diabetic rats.

Studies have pointed out that the abnormal metabolic and hyperglycemia-induced oxidative stress leads to a substantial increase in superoxide production, which can activate the protein kinase C, polyol, and hexosamine pathway, thus increasing the conformation of the advanced glycation end products (AGEs) and the expression of the AGEs receptor [19]. Studies have shown that, among the oxidative stress markers, SOD values and lipid peroxidation products such as MDA were highly correlative in diabetic patients, possibly causing further damage to metabolic systems [26]. Some

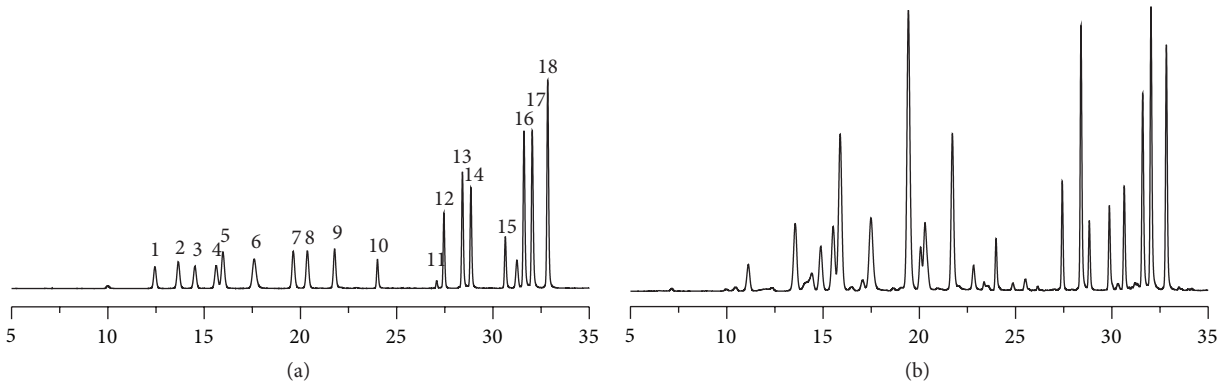


Figure 7: High-performance liquid-fluorescent detection chromatography profile of standard mixtures of amino acids (a) and extract of rat serum (b) by excitation wavelength at 250 nm and the emission wavelength at 395 nm. 1: Asp; 2: Ser; 3: Glu; 4: Gly; 5: His; 6: NH_3; 7: Arg; 8: Thr; 9: Ala; 10: Pro; 11: Cys; 12: Tyr; 13: Val; 14: Met; 15: Lys; 16: Ile; 17: Leu; 18: Phe.

Table 4: Effects of PTIF (421 mg/kg) on amino acids in high-fat diet and STZ-induced diabetic rats (μmol/L).

Amino acid	Normal	Diabetic	Diabetic + PTIF
Serine	103.1 ± 30.4	117.7 ± 35.8	115.3 ± 18.2
Glutamate	29.3 ± 7.3	61.0 ± 22.3**	42.9 ± 8.1#
Glycine	111.4 ± 28.3	111.4 ± 20.4	104.6 ± 17.1
Histidine	158.7 ± 27.0	173.1 ± 24.9	208.6 ± 42.8
Arginine	226.0 ± 56.0	347.9 ± 99.8**	349.4 ± 59.1
Threonine	89.7 ± 24.1	81.0 ± 18.4	96.3 ± 16.7
Alanine	185.4 ± 37.0	213.2 ± 42.7	171.4 ± 35.6
Proline	84.7 ± 28.0	99.0 ± 34.7	69.1 ± 9.4
Tyrosine	38.0 ± 10.6	59.4 ± 18.9**	45.9 ± 8.1
Valine	50.6 ± 12.7	107.2 ± 32.2**	123.0 ± 22.6
Methionine	29.4 ± 9.3	33.5 ± 12.8	28.9 ± 5.2
Lysine	86.0 ± 14.5	70.9 ± 11.1	78.4 ± 17.1
Isoleucine	25.7 ± 6.5	68.5 ± 11.3**	56.9 ± 14.1
Leucine	42.1 ± 10.7	104.8 ± 17.8**	84.0 ± 21.9#
Phenylalanine	40.5 ± 9.8	52.9 ± 13.6	56.6 ± 10.1

All data were expressed as mean ± SD. One-way analysis of variance (ANOVA) was used for significance analysis. $^{**}P < 0.01$ indicate a significant difference as compared with normal rats; $^{\#}P < 0.05$ indicate a significant difference as compared with diabetic rats.

previous research suggests that the beneficial effect of PTIF might result from its intervention in superoxide production. In our research, an obvious decrease in the level of SOD_1 and a concomitant increase in the concentration of MDA and HIF-1α were observed in diabetic rats. This is in agreement with those reported earlier suggesting that the oxidative stress is one of the important events in diabetes. Further, after the administration of PTIF, the plasma levels of MDA, HIF-1α, and SOD_1 were restored, indicating the possible mechanism of PTIF on DM may be correlated with the inhibition of chronic oxidative damage.

Inflammation is a multicomponent response to tissue stress, injury, and infection, which is considered to be the foreground of the acute and chronic pathological conditions [27]. It is now increasingly appreciated that diabetes is a typical disease associated with such chronic inflammatory changes [28]. It is recognized that the low-term chronic inflammation increases insulin resistance leading to hyperglycemia [29]. Various studies have demonstrated that the levels of inflammatory cytokines, including ICAM-1, TNF-α, and IL-6, are increased in patients and in diabetic rats [30, 31]. In the present study, a significantly increased concentration of ICAM-1 and VEGF in the diabetic rats probably suggests that the inflammatory processes may contribute to the development of diabetes mellitus. However, we did not see obvious effect of PTIF on VEGF, ICAM-1, and NO, implying that the antidiabetic effect of PTIF may be unrelated to lowering inflammatory cytokines.

In this study, some lipid metabolism-related metabolites, including PC(21:0/0:0)[U], 2-hexylglycerol, 7-keto-n-caprylic acid, 7-oxoheptanoic acid, PC(22:0/12:0), PC(20:4(5Z,8Z,11Z,14Z)/18:1(11Z)), have been identified in diabetic rats, which indicate a dysregulation of lipid metabolites in diabetic rats. Metabolites of the phospholipids and fatty acid pathway are associated with diabetes. Phosphatidylcholines (PCs) products or metabolites are the important components of

lipid bilayer of cells, as well as being involved in metabolism and signaling. Previous studies have shown that Radix Puerariae flavones can improve lipid metabolism in adipose tissue and liver in ovariectomized rats due to its estrogen-like effect [32]. Similar to previous studies, this study also shows that the administration of PTIF may contribute to improving the lipid metabolism in diabetic organism. An earlier study suggested that the peroxisome-proliferator activated receptors (PPAR), involved in lipid homeostasis and metabolism, were activated by isoflavones (genistein or daidzein) [33]. Thus, we can conclude that the improvement of PTIF on lipid metabolism may be due to these active ingredients.

As a purified form of vitamin K, phylloquinone was found to play an essential role in the coagulation process. It was widely used for the treatment of disease characterized by reduced levels of prothrombin. It has been shown that endothelial dysfunction, coagulative activation, and platelet hyperreactivity contribute to the hypercoagulable and prothrombotic state in diabetes mellitus, resulting from the interaction among hyperglycemia, insulin resistance, inflammation, and oxidative stress [34, 35]. Moreover, the high blood viscosity, tortuous microvessels, and large platelets induced by diabetes are an important factor in thrombosis in microvessels [36]. Compared with normal control group, the phylloquinone level was significantly increased in diabetic model. Moreover, the metabolomic results also illustrated that PTIF possess a therapeutic effect on DM through partially regulating the coagulopathy. Previous studies revealed that puerarin, as one of the major isoflavonoids compounds of Gegen, could efficiently lower the erythrocyte aggregation index, RBC aggregation, plasma viscosity, and blood yield stress in the acute blood-stasis model rats [37, 38].

Additionally, some potential biomarkers related to amino acid metabolism, including 5′-methylthioadenosine, Lys-Lys-Ser, Leu-Ser-Lys-Lys, and Gly-His-Arg-Gly, have been identified, which suggest an occurrence of amino acid metabolism disorder in diabetic rats. In addition to being an important energy source, amino acids (AAs), especially branched-chain amino acids, (BCAAs) are important intracellular signal molecules that regulate gene transcription and translation [39]. Adenine and yield 5-methylthioribose-1-phosphate are two main metabolites of 5′-methylthioadenosine (MTA). The metabolism of MTA plays a key role in the purine salvage and methionine pathways [40]. Moreover, it is also reported that increased human urinary MTA is associated with severe combined immunodeficiency syndrome [41]. In our research, compared with normal control group, the concentrations of these biomarkers were found to be significantly increased. However, the increase was significantly reversed by PTIF, which indicated PTIF could distinctly regulate the metabolism of amino acid in DM.

Furthermore, 17 kinds of free amino acid content in serum were measured in order to investigate the effect of PTIF on amino acid metabolism in diabetic rats. Recent studies suggest that higher level of branched-chain amino acid directly contributes to insulin resistance by decreasing the activity of AMP-activated protein kinase and eventually leads to type 2 diabetes [42, 43]. However, on the other hand, the activity of branched-chain alpha-keto acid dehydrogenase complex (BCKDC) is reduced markedly in diabetes rats [44], finally leading to the catabolism barriers and an increasing plasma level of BCAAs. Our results showed that the concentrations of the BCAAs (valine, leucine, and isoleucine), glutamate, arginine, and tyrosine were significantly increased, consistent with the previous reports [45]. The beneficial effects of PTIF on improved amino acid metabolism might be explained by its benefit effect on circulating insulin concentrations and insulin responsiveness by activating the signaling pathway of cAMP/PKA-dependent ERK1/2 [46, 47].

Glutamate was a central junction in both amino acid synthesis and degradation [48]. Studies have shown that in pancreatic β-cells glutamic acid oxidation and the glutamine-to-glutamate ratio are closely related to the insulin resistance [49]. Additionally, the glutamate level was found to be significantly increased in obese syndrome animals [50], which is similar to the results of our study. Our data also indicate that concentration of glutamate was significantly decreased with the oral administration of PTIF. Arginine is the precursor of nitric oxide (NO) and insulin can stimulate NO(x) synthesis from arginine [51]. Therefore, we can conclude that the increased level of arginine in this study may cause further damage on blood vessels and cell function.

5. Conclusion

In summary, a metabolomics method based on UPLC-Q-Exactive Orbitrap-MS has been established to investigate the metabonomic profiles of high-fat diet and STZ-induced diabetic rat. Eleven potential metabolite biomarkers in plasma were identified. The protective effect of PTIF has been reliably confirmed by intervening in lipid metabolism, amino acid metabolism, and coagulopathy. Moreover, we considered that oxidative stress and inflammatory reactions play a key role in diabetic rats. PTIF has great therapeutic potential in the treatment of diabetes mellitus by inhibition of oxidative damage.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

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Research Article

A 70% Ethanol Extract of Mistletoe Rich in Betulin, Betulinic Acid, and Oleanolic Acid Potentiated β-Cell Function and Mass and Enhanced Hepatic Insulin Sensitivity

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We investigated that the long-term consumption of the water (KME-W) and 70% ethanol (KME-E) mistletoe extracts had antidiabetic activities in partial pancreatectomized (Px) rats. Px rats were provided with a high-fat diet containing 0.6% KME-E, 0.6% KME-W, and 0.6% dextrin (control) for 8 weeks. As normal-control, Sham-operated rats were provided with 0.6% dextrin. In cell-based studies, the effects of its main terpenoids (betulin, betulinic acid, and oleanolic acid) on glucose metabolism were measured. Both KME-W and KME-E decreased epididymal fat mass by increasing fat oxidation in diabetic rats. KME-E but not KME-W exhibited greater potentiation of first-phase insulin secretion than the Px-control in a hyperglycemic clamp. KME-E also made β-cell mass greater than the control by increasing β-cell proliferation and decreasing its apoptosis. In a euglycemic-hyperinsulinemic clamp, whole-body glucose infusion rate and hepatic glucose output increased with potentiating hepatic insulin signaling in the following order: Px-control, KME-W, KME-E, and normal-control. Betulin potentiated insulin-stimulated glucose uptake via increased PPAR-γ activity and insulin signaling in 3T3-L1 adipocytes, whereas oleanolic acid enhanced glucose-stimulated insulin secretion and cell proliferation in insulinoma cells. In conclusion, KME-E prevented the deterioration of glucose metabolism in diabetic rats more effectively than KME-W and KME-E can be a better therapeutic agent for type 2 diabetes than KME-W.

1. Introduction

There has been a marked increase in the prevalence of type 2 diabetes among individuals older than 40 years of age, and approximately 20% of people in Korea older than 65 years of age have type 2 diabetes, which is over two-fold times greater than the average rate for Organization for Economic Cooperation and Development countries [1]. Type 2 diabetes has been associated with incremental increases in insulin resistance due to Westernized lifestyles and diets [1–3]. Among Caucasians in Western countries, a high-fat diet leads to hyperinsulinemia, which is intended to compensate for increased insulin resistance [3]; however, it has been reported that Asians do not have a sufficient capacity for insulin secretion to compensate for this type of increase in insulin resistance [4, 5]. Asians also exhibit higher levels of endogenous basal glucose production, which suggests that this population has higher levels of hepatic insulin resistance [6]. Moreover, because Asians are less likely to develop hyperinsulinemia, they are more susceptible to the development of type 2 diabetes. These differences may be related to β-cell mass because Asian patients with type 2 diabetes, especially Koreans and Chinese, have a lower β-cell mass [5]. Aging is also an important contributor to this process. Thus, the identification and development of herbs that can attenuate insulin resistance as well as potentiate β-cell function and mass are becoming increasingly important, because these herbs can be ingested as food and/or drinks that may prevent the development of type 2 diabetes.

Mistletoe (*Viscum album coloratum*) is a general name for the woody parasites of several plant families, and most genera of Korean mistletoe belong to the family Santalaceae.

Korean mistletoe tends to grow on oak trees and has traditionally been used as a herbal medicine in European, African, and Asian countries, including Korea. Korean mistletoe extract in water (KME-W) and Korean mistletoe extract in ethanol (KME-E) are not associated with any toxicity [7], and it has been confirmed that mistletoe is characterized by immunomodulatory, antidiabetic, antimicrobial, anticarcinogenic, antioxidant, and hypolipidemic activities [8–10]. This plant also contains various terpenoids, alkaloids, lectins, viscotoxins, phenylpropanoids, tannins, lignans, and polyphenols [11, 12]; however, its content varies somewhat based on the host trees from which they are collected and the area in which they grow. Similarly, the components and bioactivities of various mistletoe extracts differ according to the extraction solvents used to produce the solution. For example, triterpene acids have been quantified in aqueous mistletoe extracts (pH: 7.3), and the analyses revealed that oleanolic acid (1.1 μg/mL) and betulinic acid (0.9 μg/mL) are extracted with yields of less than 5% [12]. Furthermore, a high-performance liquid chromatography (HPLC) analysis conducted by our research group [10] demonstrated that KME-E contains betulin, betulinic acid, and oleanolic acid but that KME-W does not. On the other hand, KMW-W contains lectins and viscothionins [10]. These findings indicate that two different extracts of mistletoe possess different bioactive components. This result suggests that these compounds should be assessed to identify differences in their antidiabetic activities.

Thus, the present study aimed to determine whether the long-term consumption of KME-W or KME-E would have different antidiabetic activities in an animal model of nonobese type 2 diabetic rats and whether any differences would be associated with the primary terpenoids (betulin, betulinic acid, and oleanolic acid) found in the mistletoe extracts. These hypotheses were tested using partially pancreatectomized (Px) rats that received a high-fat diet. Additionally, the mechanisms underlying the antidiabetic activities of the extracts were assessed via the investigation of betulin, betulinic acid, and oleanolic acid in adipocytes and insulinoma cells. The primary aim of the present study was to determine which elements among the major terpenoids (betulin, betulinic acid, and oleanolic acid) in the extracts were most prominent and aided in the attenuation of insulin resistance and the potentiation of insulin secretion and cell proliferation.

2. Materials and Methods

2.1. Water Extracts of Mistletoe and Terpenoid Contents. Fresh mistletoe grown on oak trees was collected in January 2011 from Taebaek mountain in Gangwon-Do, Republic of Korea, and stored at −70°C until used. To prepare the mistletoe extract, mistletoe (100 g) was washed, dried at room temperature, freeze-dried, and powdered. The powder was extracted in distilled water at 100°C for 12 h or in 70% ethanol at 70°C for 12 h and each of them was centrifuged at 10,000 g at 4°C for 20 min. The supernatants were lyophilized in freeze-dryer.

The contents of total phenolic compounds in water or 70% ethanol extracts were measured using Folin-Ciocalteu reagent and expressed as mg gallic acid equivalents $\cdot$ g^{-1}. The contents of total flavonoids were measured by the modified methods reported by modified Davis method and rutin was used as the standard. Each of free-dried extracts was dissolved in 70% ethanol and it had syringe filter to remove the undissolved contents. Terpenoids in each extract were analyzed by HPLC using Luna C18 column (4.6 mm × 250 mm ID 5 μm). The mobile phase solvents consisted of acetonitrile and 0.2% acetic acid in water (8 : 2, v : v) with isocratic elution with a flow rate of 0.5 mL/min and 40°C in column temperature and UV detection was at 210 nm. The terpenoid contents were calculated from each of the standards such as betulinic acid, oleanolic acid, and betulin.

2.2. Animals and Ethics. Eight-week-old male Sprague-Dawley rats (weighing 218 ± 23 g) were housed individually in stainless steel cages in a controlled environment (23°C and with a 12 h light/dark cycle). All surgical and experimental procedures were performed according to the guidelines of the Animal Care and Use Review Committee of Hoseo University, Korea (2013-01). The rats had a 90% pancreatectomy using the Hosokawa technique [13] or received a sham pancreatectomy (Sham) under anesthesia induced by intramuscular injection of a mixture of ketamine and xylazine (100 and 10 mg/kg body weight, resp.). Px rats exhibited characteristics of type 2 diabetes (random glucose levels over 180 mg/dL), whereas the Sham rats did not [13, 14].

2.3. Experimental Design. The dosage of KME in the present study was based on a previous study [10] that provided diets containing 0.2% or 0.6% of 70% KME-E or KME-W to Px rats. Our preliminary study has demonstrated that the low dosage (0.5–2 μg/mL) treatment with Korean mistletoe water extracts lower tumor necrosis factor-α expression in a dose-dependent manner in RAW 264.7 cells activated with lipopolysaccharides but higher dosage up to 10 μg/mL does not change the efficacy [10]. In the present study, 30 Px rats were randomly assigned to the following three groups that differed according to diet: (1) 0.6% of KMW-W, (2) 0.6% of 70% KME-E, and (3) 0.6% of dextrin (Px-control). Additionally, 10 sham-operated rats (normal-control) received a high-fat diet containing 0.6% dextrose. All experimental animals were given free access to water and a high-fat diet containing either the assigned extracts or dextrose over the 8-week experimental period. The high-fat diet was a modified semipurified AIN-93 formulation for experimental animals [15] that consisted of 40% energy from carbohydrates, 20% energy from protein, and 45% energy from fats. The major carbohydrate, protein, and fat sources were starch and sugar, casein (milk protein), and lard (CJ Co., Seoul, Korea), respectively.

Overnight fasted serum glucose levels, food and water intakes, and body weight were measured every Tuesday at 10 AM. An oral glucose tolerance test (OGTT) was performed every three weeks in overnight fasted animals by orally administering 2 g glucose/kg body weight. Serum glucose

and insulin were measured by tail bleeding at 0, 10, 20, 30, 45, 60, 90, and 120 min after glucose loading. Serum glucose levels were analyzed with a Glucose Analyzer II (Beckman, Palo Alto, CA), and serum insulin and leptin levels were measured by radioimmunoassay kit (Linco Research, Billerica, MA). Serum alanine aminotransferase (ALT) and aspartate aminotransferase activity (AST) were measured by standard colorimetric methods using commercial kits (Asan Pharmaceutical, Seoul, Republic of Korea).

2.4. Hyperglycemic Clamp. After seven weeks of the treatment, catheters were surgically implanted into the right carotid artery and left jugular vein of conscious and overnight fasted ten rats from each group after the anesthetization with ketamine and xylazine. After 5-6 days of implantation, a hyperglycemic clamp was performed in free-moving and overnight fasted rats to determine insulin secretion capacity as described in previous studies [13, 16, 17]. During the clamp, glucose was infused to maintain serum glucose levels of 5.5 mM above baseline and serum insulin levels were measured at designated time. After the clamp, rats were freely provided with foods and water for 2 days and next day they were deprived of food for 16 hours. The rats were anesthetized with the mixture of ketamine and xylazine and human regular insulin (5 U/kg body weight) was injected through the inferior vena cava of the rats. Ten min later, they were killed by decapitation and tissues were rapidly collected, frozen in liquid nitrogen, and stored at −70°C for further experiments. In order to determine the glycogen content in the liver, its lysates were centrifuged at 3000 rpm for 10 minutes and the supernatants deproteinized with 1.5 N perchloric acid. The glycogen content was calculated from glucose concentrations derived from glycogen hydrolyzed by α-amyloglucosidase in an acid buffer [18]. Triglyceride was extracted with a chloroform-methanol (2 : 1, vol/vol) from the liver and resuspended in pure chloroform [19]. Triacylglycerol concentration was determined using a Trinder kit (Young Dong Pharm., Seoul, Korea).

2.5. Euglycemic Hyperinsulinemic Clamp. After the catheterization of the right carotid artery and left jugular vein at the seventh week of the experimental periods, a euglycemic hyperinsulinemic clamp was performed on fasted conscious rats to determine insulin resistance as previously described [19, 20]. [3-^{3}H] glucose (NEN Life Science, Boston, MA) was continuously infused during a 4-hour period at the rate of 0.05 μCi/min. Basal hepatic glucose output was measured in blood collected at 100 and 120 minutes after initiation of the [3-^{3}H] glucose infusion. Then a primed continuous infusion of human regular insulin (Humulin, Eli Lilly, Indianapolis, IN) was initiated at a rate of 20 pmol × kg^{-1} × min^{-1} to raise plasma insulin concentration to approximately 1100 pM at 210–240 min. Blood samples from arteries were collected at 10-minute intervals for glucose estimation, and 25% glucose was infused at variable rates as needed to clamp glucose levels at approximately 6 mM. For the determination of plasma [3-^{3}H] glucose concentrations, plasma was deproteinized with $ZnSO_4$ and $Ba(OH)_2$, dried to remove ^3H_2O, and resuspended in water, and disintegrations per min (dpm) of ^{3}H were recorded. The plasma concentration of ^3H_2O was determined by the difference between ^{3}H counts without and with drying. Rates of whole body glucose uptake and basal glucose turnover were determined as the ratio of the [^{3}H] glucose infusion rate to the specific activity of plasma glucose (dpm/μmol) during the final 30 minutes of the respective experiments. Hepatic glucose production at hyperinsulinemic clamped state was determined by subtracting the glucose infusion rate from the whole body glucose uptake.

2.6. Immunoblot Analysis. The liver collected from rats stimulated with insulin for 10 min was lysed with lysis buffer containing a 20 mM Tris buffer (pH 7.4) containing 2 mM EGTA, 137 mM NaCl, 1% NP40, 10% glycerol, and 12 mM α-glycerol phosphate and protease inhibitors. After 30 min on ice, the lysates were centrifuged for 10 min at 12,000 rpm at 4°C. After measuring protein contents in lysate by Bio Rad protein assay kit (Hercules, CA), lysates with equivalent amounts of protein (30–50 μg) were resolved with SDS-PAGE and immunoblotted with antibodies of phospho-Akt^{ser478}, Akt, phospho-glycogen synthase kinase- (GSK-) 1β, GSK-1β (Cell Signaling Technology, Beverly, MA), and phosphoenolpyruvate carboxykinase (PEPCK), generously provided by Dr. Garner of Vanderbilt University [16, 19]. The intensity of protein expression was determined using ImageQuant TL (Amersham Biosciences, Piscataway, NJ). These experiments were repeated three times for each group.

2.7. In Vitro Insulin-Stimulated Glucose Uptake and Insulin Signaling. Insulin-stimulated glucose uptake was analyzed by measuring the uptake of 2-deoxy-D-[^{3}H] glucose in 3T3-L1 adipocytes, as previously described [21]. Briefly, the adipocytes were seeded in 24-well plates (4×10^4 cells per well) in high glucose Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Carlsbad, CA) containing fetal bovine serum (FBS) for 6 hours. The media were switched to low glucose DMEM (Invitrogen) containing 0.3% bovine serum albumin (BSA) and two concentrations (5 and 50 μM) of betulin, betulinic acid, or oleanolic acid; they were then incubated for 16 hours at 37°C. The antidiabetic drug rosiglitazone (0.5 or 2 μM) was used as a positive control. The media were then switched to a Krebs-Ringer-Hepes buffer (KRH) containing the respective compounds and either 0.2 or 10 nM insulin and incubated for 30 minutes at 37°C. Following the incubation period, glucose uptake was measured for 10 minutes using 0.1 μCi 2-deoxy-D-[^{3}H] glucose and 1 mM glucose as the final concentrations. Nonspecific glucose uptake was measured following treatment with the extracts in the absence of insulin, and the radioactivity retained by the cell lysates was determined with a Wallac Liquid Scintillation Counter (Perkin Elmer, Waltham, MA).

After treatment with 50 μM of betulin, betulinic acid, or oleanolic acid for 16 hour, the cells were lysated with the lysis buffer and then immunoblotted with antibodies for phospho-Akt^{ser478}, Akt, phospho-GSK-1β, GSK-1β, phospho-AMPK, and AMPK (Cell Signaling Technology).

2.8. Peroxisome Proliferator-Activated Receptor- (PPAR-) γ Agonist Activity. Human embryonic kidney 293 cells were transiently transfected with a peroxisome proliferator responsive element- (PPRE-) luciferase construct (firefly pGL3-DR-1-luciferase; 0.12 μg DNA·well^{-1}), pSV-SPORT-PPAR-γ expression vector (0.12 μg DNA·well^{-1}), pSV-SPORT-retinoid X receptor-α vector (0.08 μg DNA·well^{-1}), and renilla phRL-TK vector (10 ng DNA·well^{-1}) with a Lipofectamine PLUS reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. PPAR-γ activity was measured as described in previous studies [21, 22]. After 2 h of transfection, vehicles (DMSO) or 5 or 50 μM betulinic acid, oleanolic acid, or betulin was added to media for 40 h and the media were changed to serum-free DMEM containing 0.1% BSA, which also contained the respective extracts, for 12 h [22]. Both firefly (PPRE-luciferase) and renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega) in an Aureon PhL luminometer (Aureon Biosystems, Vienna, Austria). Ratios of firefly luciferase activity and renilla luciferase activity were calculated.

2.9. Glucose-Stimulated Insulin Secretion and Cell Viability. Mouse insulinoma cells (Min6 cells) were grown as previously described by Park et al. [22] in a 24-well plate at 6×10^4 cells per well with high glucose DMEM containing 0.3% BSA and either vehicle or 5 or 50 μm of betulinic acid, oleanolic acid, or betulin for 16 h. Exendin-4 (2.5 nM, Sigma Co., St. Louis, MO) treated cells were used as a positive control. After washing the cells with PBS, the Min6 cells were treated with vehicle or respective terpenoids in low (2 mM) or high glucose (20 mM) KRH buffer containing 20 mM Hepes pH 7.4 for 30 min. Insulin concentrations in supernatants from all treatments were measured using a radioimmunoassay kit (Linco Research, St. Charles, MO) and a Packard Cobra gamma-counter (Packard Instrument Co. Inc., Meriden, CT).

Cell viability was measured in Min6 cells treated with vehicle (DMSO) or 5 or 50 μm of betulinic acid, oleanolic acid, and betulin and 2.5 nM of exendin-4 with a Cell Proliferation WST-1 reagent assay kit from Roche Diagnostic Co. (Indianapolis, IN) in an Aureon plate reader (Aureon Biosystems, Vienna, Austria). The kit is a modified tetrazolium salt that can be cleaved by metabolically active cells to a water soluble formazan, which was quantitated at 450 nm with an ELISA plate reader [23].

2.10. Statistical Analyses. All data are expressed as means ± standard deviations (SDs), and all statistical analyses were performed using SAS version 9.1 (SAS Institute, Cary, NC). In the animal studies, significant differences among the Px-control, KME-W, KME-E, and normal-control groups were identified with a one-way analysis of variance (ANOVA). In the cell-based studies, significant differences among the control, single compound, and positive control conditions were also identified with a one-way ANOVA. In both sets of studies, significant differences in the main effects among the groups were identified using post hoc Tukey's tests. A P value < 0.05 was considered to indicate statistical significance.

Table 1: The contents of phenolic compounds and flavonoids (unit: mg/g dry weight).

	Water extract of Korean mistletoe	70% methanol extract of Korean mistletoe
Polyphenols	3.53 ± 0.54	5.86 ± 0.63
Flavonoids	1.32 ± 0.22	2.38 ± 0.31
Betulin	ND	0.11 ± 0.01
Betulinic acid	ND	0.51 ± 0.00
Oleanolic acid	ND	0.89 ± 0.01

Values are means ± SD. ND, nondetectable.

3. Results

3.1. Total Phenolic Compounds and Flavonoids. The 70% KME-E contained 1.7- and 2.7-fold higher levels of total polyphenols and flavonoids, respectively, than the KME-W. Additionally, the KME-E contained betulin, betulinic acid, and oleanolic acid, whereas KME-W did not (Table 1).

3.2. Energy Metabolism. During the 8-week experimental period, the Px-control group gained less body weight and had a lower epididymal fat mass than the normal-control group (Table 2). However, the caloric intake was higher in the Px-control than in the normal-control group even though their daily energy expenditures did not differ. This suggests that the lesser amount of weight gained by the Px-control rats was related to urinary glucose loss. The KME-W and KME-E groups had a tendency to gain more weight than the Px-control group, but these differences were not significant (Table 2). The daily energy intakes and energy expenditures of the KME-W and KME-E groups did not significantly differ from each other, but the cumulative energy intake of the KME-W group was lower than that of the KME-E group. Thus, treatment with KME-W and KME-E may reduce the urinary loss of glucose.

Interestingly, the epididymal fat masses of the KME-W and KME-E groups were lower than that of the Px-control group (Table 2); in fact, the fat mass serum leptin levels were highest in the normal-control group and descended in the order of the Px-control, KME-W, and KME-E groups. These differences were likely related to the energy sources used by the body. Rats in the Px-control and normal-control groups typically used fat and carbohydrates as their primary energy sources, whereas rats in the KME-W and KME-E groups used fat as their main energy source (Table 2). Thus, diabetic Px rats exhibited a somewhat deteriorated energy metabolism compared with the nondiabetic normal-control (sham) rats. Both KME-W and KME-E prevented this impairment in diabetic rats, albeit in an incomplete manner.

Since KME may have potential adverse effects on liver, serum ALT and AST activities which are known indicators of liver damage were measured in the present study. The levels were in a normal range and they rather decreased in KME-W and KME-E treatment. They were not significantly different in diabetic and nondiabetic controls.

Table 2: Metabolic changes at the end of 8-week treatment.

	Control ($n = 16$)	KME-W ($n = 16$)	KME-E ($n = 16$)	Normal-control ($n = 16$)
Body weight (g)	337 ± 31^{b}	342 ± 32^{b}	358 ± 34^{b}	407 ± 37^{a}
Epididymal fat pads (g)	4.2 ± 0.6^{b}	3.3 ± 0.6^{c}	3.6 ± 0.5^{c}	5.9 ± 0.8^{a}
Relative epididymal fat pad (g/kg bw)	12.4 ± 1.7^{b}	9.6 ± 1.4^{c}	10.1 ± 1.6^{c}	14.5 ± 1.9^{a}
Caloric intakes (kcal/day)	128 ± 16^{a}	118 ± 14^{a}	117 ± 15^{a}	101 ± 14^{b}
Energy expenditure (kcal/kg$^{0.75}$/day)	92.1 ± 12.1	97.9 ± 12.1	92.7 ± 11.6	94.3 ± 11.6
Carbohydrate oxidation (mg/kg$^{0.75}$/min)	3.9 ± 0.5^{a}	3.7 ± 0.5^{ab}	3.4 ± 0.4^{b}	4.2 ± 0.5^{a}
Fat oxidation (mg/kg$^{0.75}$/min)	5.9 ± 0.8^{b}	6.8 ± 0.8^{a}	6.5 ± 0.7^{ab}	5.8 ± 0.7^{b}
Overnight fasted serum leptin levels (ng/mL)	5.5 ± 0.8^{b}	4.4 ± 0.7^{c}	4.2 ± 0.8^{c}	6.5 ± 0.9^{a}
Overnight fasted serum glucose (mmol/L)	8.0 ± 1.0^{a}	7.5 ± 0.9^{a}	6.6 ± 0.8^{b}	5.4 ± 0.6^{c}
Overnight fasted serum insulin (ng/mL)	0.82 ± 0.14^{b}	0.88 ± 0.15^{b}	1.07 ± 0.19^{a}	1.14 ± 0.20^{a}
Serum AST (IU/L)	130 ± 13.0^{a}	112 ± 12^{b}	118 ± 13^{b}	125 ± 13^{a}
Serum ALT (IU/L)	45 ± 4.8^{a}	36.4 ± 4.2^{b}	38.4 ± 4.4^{b}	44.2 ± 4.6^{a}

Values are mean ± SD. Diabetic Px rats were fed with high-fat diets supplementing (1) 0.6% water extract of Korean mistletoe (KMW-W), (2) 0.6% of 70% ethanol extract (KME-E), or (3) 0.6% dextrose (Control). Sham-operated rats were provided with a high-fat diet containing 0.6% dextrose as a normal-control.
[a,b,c]Values in the same row with different superscripts were significantly different in Tukey test at $P < 0.05$.

3.3. Glucose Tolerance. Following an overnight fast, the serum glucose level of the Px-control group was significantly higher than that of the nondiabetic normal-control group, whereas the normal-control group had a significantly higher serum insulin level than the Px-control group. These results were related to the partial removal of the pancreas. After glucose loading, the serum glucose levels of the diabetic groups increased for 50 minutes, but they peaked at 40 minutes in the nondiabetic groups (Figure 1); these levels were lowest in the Px-control group and decreased in the order of the KME-W, KME-E, and normal-control groups. However, at the peak, the serum glucose level of the KME-E group was not as low as that of the normal-control group (Figure 1). The results of oral glucose tolerance test (OGTT) suggest that glucose-stimulated insulin secretion and insulin sensitivity may have been modified by the ingestion of KME-W and KME-E.

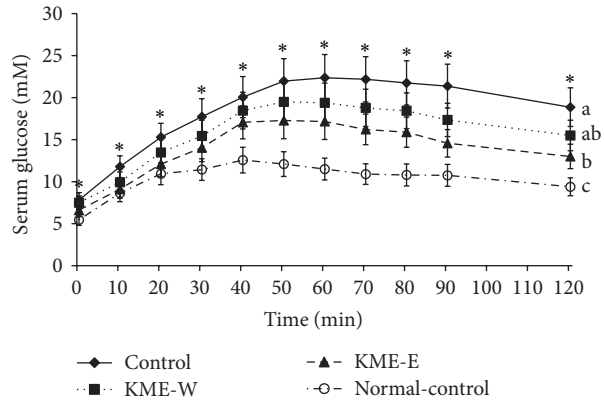

Figure 1: Changes in serum glucose concentrations during the oral glucose tolerance test. Oral glucose tolerance tests were performed on Px rats fed high-fat diets supplemented with either (1) 0.6% water extract of Korean mistletoe (KMW-W), (2) 0.6% of 70% ethanol extract of Korean mistletoe (KME-E), or (3) 0.6% dextrose (Px-control). As a normal-control group, sham-operated rats were provided with a high-fat diet containing 0.6% dextrose. After 8 weeks of treatment, glucose (2 g/kg body weight) was administered orally, blood samples were taken at the indicated time points, and serum glucose levels were measured. The sample size of each group was the same as in Table 2. The dots and error bars represent mean ± SD. [a,b,c]Values on the bars with different superscripts were significantly different in a Tukey post hoc test at a significance of $P < 0.05$.

3.4. Glucose-Stimulated Insulin Secretion by Hyperglycemic Clamp. In the present study, the glucose-stimulated insulin secretion capacity indicated the presence of β-cell function. During the hyperglycemic clamp procedure, serum insulin levels peaked between 2 and 5 minutes after the infusion of glucose into the jugular vein and then declined to a nadir at 10 minutes; this is referred to as first-phase insulin secretion. The serum glucose levels were sustained at more than 100 mg/dL above the baseline serum glucose levels for 60–90 minutes, and the serum insulin levels also increased from their nadir at 10 minutes and were then maintained at certain levels, known as second-phase insulin secretion (Figure 2). The diabetic Px-control group exhibited insulin levels that were approximately 59% and 81% of the first- and second-phase insulin secretion levels, respectively, of the nondiabetic normal-control group (Table 2). These findings indicate that the reduction of β-cell mass following the removal of the pancreas primarily resulted in a reduction in first-phase insulin secretion. KME-E, but not KME-W, prevented these reductions during the first- and second-phase insulin secretion in the Px-control group, but it did not do so to the same degree as it did in the normal-control group (Table 2).

The glucose infusion rates that were necessary to maintain serum glucose levels at 5.5 mM above baseline levels during the hyperglycemic clamp were lower in the Px-control group than in the normal-control group, whereas insulin sensitivity during the hyperglycemic state decreased in the Px-control

Table 3: Insulin secretion capacity during hyperglycemic clamp.

	Control ($n = 8$)	KME-W ($n = 8$)	KME-E ($n = 8$)	Normal-control ($n = 8$)
Serum insulin at basal state (ng/mL)	0.84 ± 0.11^{c}	0.90 ± 0.12^{c}	1.09 ± 0.16^{b}	1.46 ± 0.21^{a}
Serum insulin at first phase (ng/mL)	2.74 ± 0.30	3.11 ± 0.35^{c}	3.87 ± 0.43^{b}	4.75 ± 0.51^{a}
Serum insulin at second phase (ng/mL)	3.02 ± 0.31^{b}	3.05 ± 0.32^{b}	3.43 ± 0.36^{a}	3.78 ± 0.40^{a}
AUC of insulin at first phase (ng/mL∗min)	19.3 ± 2.1^{c}	20.5 ± 2.3^{c}	26.2 ± 3.1^{b}	32.8 ± 3.6^{a}
AUC of insulin at second phase (ng/mL∗min)	186 ± 20^{bc}	176 ± 20^{c}	202 ± 22^{b}	230 ± 24^{a}
Glucose infusion rate (mg/kg bw/min)	12.2 ± 1.5^{c}	14.0 ± 1.7^{bc}	16.2 ± 2.1^{b}	26.5 ± 3.2^{a}
Insulin sensitivity (μmol glucose· min^{-1}·100 g^{-1} per μmol insulin/L)	10.9 ± 1.4^{c}	13.3 ± 1.7^{b}	13.4 ± 1.7^{b}	19.2 ± 2.3^{a}

Values are mean ± SD. Diabetic Px rats were fed with high-fat diets supplementing (1) 0.6% water extract of Korean mistletoe (KMW-W), (2) 0.6% of 70% ethanol extract (KME-E), or (3) 0.6% dextrose (Control). Sham-operated rats were provided with a high-fat diet containing 0.6% dextrose as a normal-control.
[a,b,c]Values in the same row with different superscripts were significantly different in Tukey test at $P < 0.05$.

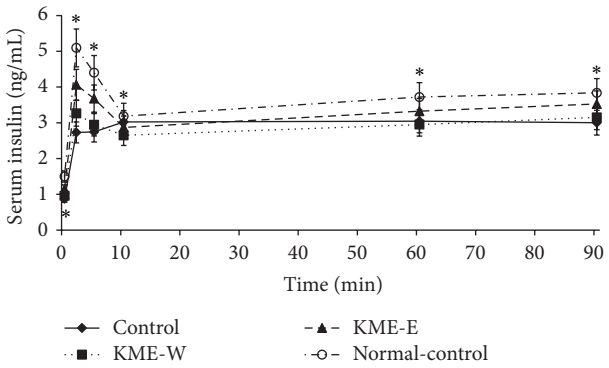

Figure 2: Insulin secretion capacity during hyperglycemic clamp. At the end of the experimental periods, hyperglycemic clamp was performed on Px rats fed high-fat diets supplemented with either (1) 0.6% water extract of Korean mistletoe (KMW-W), (2) 0.6% of 70% ethanol extract of Korean mistletoe (KME-E), or (3) 0.6% dextrose (Px-control). As a normal-control group, sham-operated rats were provided with a high-fat diet containing 0.6% dextrose. During hyperglycemic clamp, serum insulin levels were measured in free-moving and overnight-fasted diabetic rats as serum glucose levels at 5.5 mM above fasting levels were maintained. The sample size in each group was the same as in Table 3. The dots and error bars represent mean ± SD. *Significantly different among the groups in one-way ANOVA at a significance of $P < 0.05$.

group (Table 3). During the hyperglycemic state, KME-E increased the glucose infusion rates and insulin sensitivity to levels higher than those found in the Px-control group but lower than those observed in the normal-control group, whereas KME-W enhanced insulin sensitivity as much as KME-E (Table 3). These results suggest that KME-E promotes glucose-stimulated insulin secretion capacity and insulin sensitivity under hyperglycemic conditions in diabetic rats and that KME-W enhanced insulin sensitivity but does not influence insulin secretion capacity.

3.5. β-Cell Mass. Insulin secretion capacity is associated with pancreatic β-cell mass [24, 25]. In the present study, pancreatic β-cell mass was calculated by multiplying the weight of the pancreas by the β-cell area. We found that the Px-control group had a greater β-cell area than the normal-control group, and this increase was associated with a remarkable expansion of β-cell mass due to the role of insulin as the pancreas was regenerated. Due to removal of the pancreas, the pancreatic β-cell mass of the Px-control group was 56.4% of that of the normal-control group. The KME-E group, but not the KME-W group, exhibited an increase in β-cell area and β-cell mass compared with the Px-control group, but this increase was not as substantial as it was in the normal-control group (Table 4). This increase in β-cell area was associated with the number and size of individual β-cells. Indeed, individual β-cells were larger in size in the Px-control group than in the normal-control group, which indicates that there were fewer cells in the Px-control than in the normal-control group in the same-sized β-cell area (Table 4). This was confirmed by assessing β-cell proliferation and apoptosis. β-cell proliferation was greater in the normal-control group than in the Px-control group, but apoptosis was much higher in the Px-control group, which means that the Px-control group had fewer β-cells.

Both the KME-W and KME-E groups showed smaller-sized individual β-cells relative to those in the Px-control group. On the other hand, the KME-E, but not the KME-W, group showed increased proliferation of β-cells compared with the Px-control group, whereas both the KME-W and KME-E groups exhibited reduced β-cell apoptosis (Table 4). These results suggest that KME-E increased β-cell mass by hyperplasia and could maintain insulin secretion and sufficiently compensate for insulin resistance by sustaining β-cell function.

3.6. Insulin Sensitivity during the Euglycemic Hyperinsulinemic Clamp. During the euglycemic hyperinsulinemic clamp, measures of whole-body glucose utilization indicated the presence of whole-body insulin resistance under euglycemia. During the clamp, insulin was infused into the jugular vein to achieve serum insulin levels of approximately 1100 pM, and the infused glucose resulted in insulin levels of 100 mg/dL

Table 4: The modulation of islet morphometry at the end of experiment.

	Control ($n = 6$)	KME-W ($n = 6$)	KME-E ($n = 6$)	Normal-control ($n = 6$)
β-cell area (%)	7.5 ± 0.9^{b}	7.7 ± 0.8^{b}	8.7 ± 0.9^{a}	6.3 ± 0.7^{c}
Individual β-cell size (μm^2)	242 ± 30^{a}	193 ± 25^{b}	201 ± 24^{b}	189 ± 23^{b}
Absolute β-cell mass (mg)	32.3 ± 3.4^{c}	33.5 ± 3.7^{c}	39.8 ± 4.0^{b}	57.3 ± 6.2^{a}
$BrdU^+$ cells (% $BrdU^+$ cells of islets)	0.97 ± 0.13^{a}	1.01 ± 0.13^{b}	1.14 ± 0.15^{a}	0.75 ± 0.11^{c}
Apoptosis (% apoptotic bodies of islets)	1.08 ± 0.12^{a}	0.92 ± 0.09^{b}	0.88 ± 0.12^{b}	0.73 ± 0.08^{b}

Values are mean ± SD. Diabetic Px rats were provided with high-fat diets supplementing (1) 0.6% water extract of Korean mistletoe (KMW-W), (2) 0.6% of 70% ethanol extract (KME-E), or (3) 0.6% dextrose (Control). Sham-operated rats were provided with a high-fat diet containing 0.6% dextrose as a normal-control.
[a,b,c]Values in the same row with different superscripts were significantly different in Tukey test at $P < 0.05$.

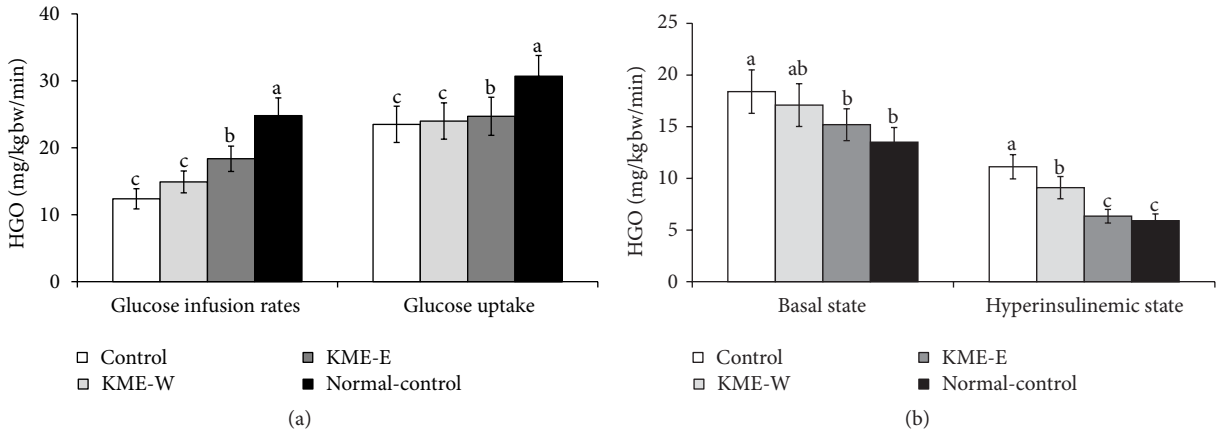

Figure 3: Serum glucose and insulin levels and glucose infusion rates during a euglycemic hyperinsulinemic clamp. Px rats fed high-fat diets supplemented with either (1) 0.6% water extract of Korean mistletoe (KMW-W), (2) 0.6% of 70% ethanol extract of Korean mistletoe (KME-E), or (3) 0.6% dextrose (Px-control). As a normal-control group, sham-operated rats were provided with a high-fat diet containing 0.6% dextrose. After an 8-week treatment period, the animals had a euglycemic hyperinsulinemic clamp assay in conscious, free-moving, and overnight fasted rats to determine whole body and hepatic insulin resistance. Glucose infusion rates and glucose uptake at a clamped steady-state (a) and hepatic glucose output at baseline and hyperinsulinemic state about 1100 pM of serum insulin (b) were measured. The sample size in each group was the same as in Table 3. The bars and error bars represent mean ± SD. [a,b,c]Values on the bars with different superscripts were significantly different in a Tukey post hoc test at a significance of $P < 0.05$.

or a euglycemic state. At approximately 1100 pM, the glucose infusion rate and whole-body glucose uptake were lower in the diabetic Px-control group than in the nondiabetic normal-control group (Figure 3(a)). Glucose uptake was altered by both KME-E and KME-W, but glucose infusion rates under the hyperinsulinemic state increased to the greatest degree in the control group; this was followed by the KME-W, KME-E, and normal-control groups, in ascending order (Figure 3(a)). Hepatic glucose output levels under the basal and hyperinsulinemic states were not suppressed in the diabetic Px-control group relative to the nondiabetic normal-control group (Figure 3(b)). KME-E, but not KME-W, reduced hepatic glucose output under the basal state by approximately 27.4% relative to the comparable figure in the Px-control group. In a hyperinsulinemic state, the hepatic glucose output of the Px-control group showed a 1.9-fold increase compared with that of the normal-control group, whereas KME-E and KME-W suppressed this increase by 18.2% and 43.0%, respectively. The KME-E-induced suppression resulted in values similar to those of the normal-control group (Figure 3(b)).

3.7. Hepatic Insulin Signaling. The diabetic Px-control group had less glycogen storage but increased triglyceride content in the liver compared with the nondiabetic normal-control group. KME-E prevented the deterioration of hepatic glycogen and triglyceride storage (Figure 4(a)), and these changes were related to hepatic insulin signaling, which was determined by assessing the phosphorylation of Akt and GSK-1β and the expression of PEPCK in the liver. The serine phosphorylation rates of Akt and GSK-1β were attenuated in the Px-control group compared with the normal-control group (Figure 4(b)). This phosphorylation was potentiated in the ascending order of KME-W and KME-E and, moreover, the phosphorylation was increased in the KME-E group as much as in the normal-control group. In contrast to Akt phosphorylation, PEPCK expression was higher in the Px-control group than in the normal-control group, and it was reduced in the KME-E group (Figure 4(b)).

3.8. Insulin-Stimulated Glucose Uptake in 3T3-L1 Adipocytes and PPAR-γ Activity. The most likely effective components in

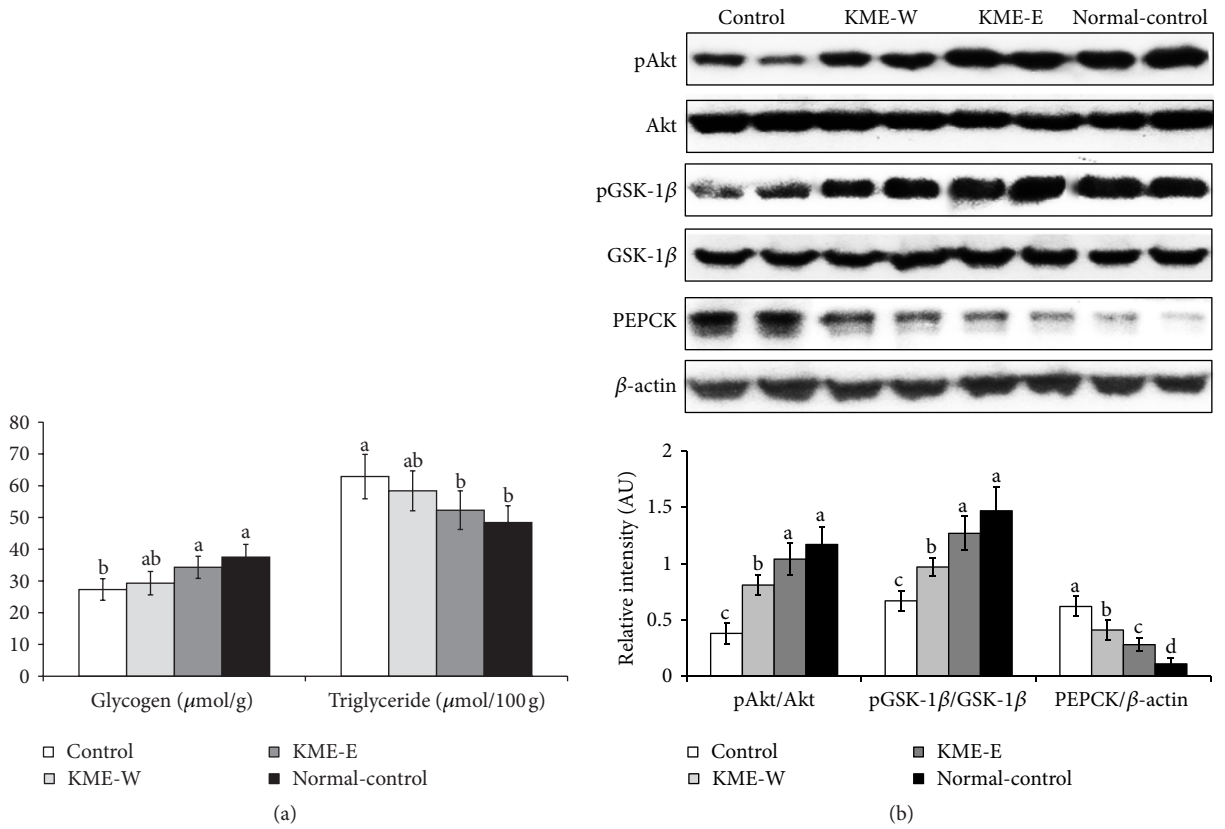

Figure 4: The modulation of insulin signaling in the liver at the end of experimental periods. After 10 min of insulin (5 U/kg body weight) stimulation through the inferior vena cava at the end of each experimental period, the liver was collected from the Px rats fed high-fat diets supplemented with either (1) 0.6% water extract of Korean mistletoe (KMW-W), (2) 0.6% of 70% ethanol extract of Korean mistletoe (KME-E), or (3) 0.6% dextrose (Px-control) and sham-operated rats fed a high-fat diet containing 0.6% dextrose (a normal-control). The liver was immediately lysed with a lysis buffer. Hepatic glycogen and triglyceride contents were also determined (a). The phosphorylation and expression levels of the Akt, GSK-1β, and PEPCK, involved in insulin sensitivity, were determined by immunoblotting with specific antibodies. The intensity of protein expression was determined using ImageQuant TL (b). These experiments were repeated four times for the liver, and the bars and error bars represent mean ± SD ($n = 4$). ^{a,b,c,d}Values on the bars with different superscripts were significantly different in a Tukey post hoc test at a significance of $P < 0.05$.

the KME solutions were betulin, betulinic acid, and oleanolic acid, and the insulin-sensitizing effects of these compounds were tested via treatment with a low dose of insulin (0.2 nM). Following administration of 0.2 nM of insulin, glucose uptake was 10.1 ± 1.4 disintegrations per minute (dpm)/μg protein. Furthermore, betulin concentrations of 5 and 50 mM increased insulin-stimulated glucose uptake by 2.1- and 3.2-fold, respectively, but these increases were less than those induced by rosiglitazone treatment. Treatment with betulinic acid also elevated insulin-stimulated glucose uptake in a dose-dependent manner but to a lesser degree than betulin treatment. In contrast, oleanolic acid only minimally enhanced insulin-stimulated glucose uptake. Thus, betulin and betulinic acid exhibited moderate insulin-sensitizing activity (Figure 5(a)). All of betulin, betulinic acid, and oleanolic acid improved insulin-stimulated insulin signaling (the phosphorylation of Akt and GSK-1β) in 3T3-L1 adipocytes and betulinic acid potentiated it the most (Figure 5(b)). In addition, betulinic acid enhanced the phosphorylation of AMPK (Figure 5(b)).

Betulin and betulinic acid increased PPAR-γ activity, but betulin was a more effective agonist of PPAR-γ activity than betulinic acid. However, the PPAR-γ-related agonistic activity of betulin was not as high as that of rosiglitazone (2 μM), which is a known commercial PPAR-γ agonist (Figure 6). Thus, betulinic acid and betulin resulted in mild and moderate PPAR-γ agonistic activity, respectively.

3.9. Glucose-Stimulated Insulin Secretion and Cell Viability in Min6 Cells. In insulinoma Min6 cells, insulin secretion was increased by 5.1 ± 0.7-fold in the high-glucose (20 mM) DMEM media compared with the low-glucose (2 mM) media. Betulin, betulinic acid, and oleanolic acid did not stimulate insulin secretion in the low-glucose media (data not shown), but oleanolic acid potentiated insulin secretion in dose-dependent manner in the high-glucose media. However, this increase was not to the same degree as that initiated by exendin-4 (2.5 nM), which is a known insulinotropic agent (Figure 7(a)).

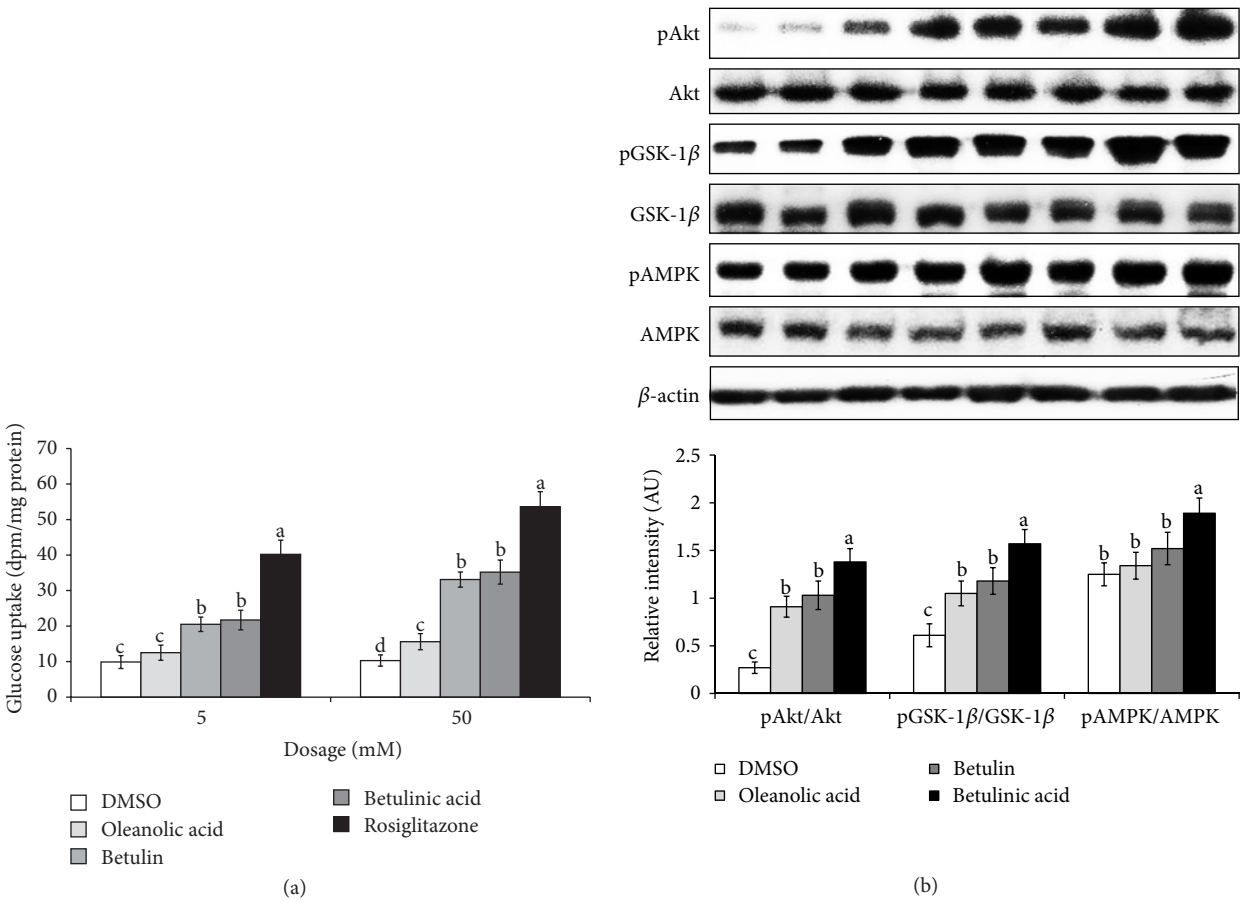

Figure 5: Insulin-stimulated glucose uptake in 3T3-L1 adipocytes and PPAR-γ activity by betulin, betulinic acid, and oleanolic acid. 3T3-L1 adipocytes were treated with low (0.5 μg/mL) or high (5 μg/mL) doses of vehicle (DMSO) or low (5 μM) or high (50 μM) doses of betulin, betulinic acid, and oleanolic acid for 16 hours. At the end of the incubation, insulin (0.2 nM) was administered for 30 minutes to determine insulin-stimulated glucose uptake, which would represent insulin-sensitizing activities. Vehicle and 10 nM insulin treatments were used as negative and positive controls, respectively. The data are presented as ^{3}H-deoxyglucose content per mg of protein in the cells, which indicates the degree of glucose uptake ($n = 7$). After 16 hours of treatment with betulin, betulinic acid, or oleanolic acid, the cells were immediately lysed with a lysis buffer, and the phosphorylation and expression levels of Akt, GSK-1β, and AMPK were determined by immunoblotting with specific antibodies (b). ^{a,b,c,d}Values on the bars with different superscripts were significantly different in a Tukey post hoc test at a significance of $P < 0.05$.

Relative to the control group, oleanolic acid increased cell proliferation in a dose-dependent manner, and betulinic acid also enhanced cell proliferation, but it was less than that induced by 50 μM of oleanolic acid (Figure 7(b)). Thus, oleanolic acid exerted insulinotropic action.

4. Discussion

KME-W has higher concentrations of lectins and viscothionins, which are associated with immune modulation, whereas KME-E has a concentration of triterpenoids, which are related to metabolic diseases. In the present study, triterpenoids were detected in KME-E but not KME-W, and this difference resulted in different antidiabetic activities in diabetic rats. Type 2 diabetes is induced when enhanced insulin secretion cannot compensate for increasing levels of insulin resistance. Thus, the tight regulation of insulin resistance and β-cell function play important roles in the prevention of type 2 diabetes as well as in delaying its progression.

The present findings demonstrated that, in a hyperglycemic clamp, glucose-stimulated insulin secretion, particularly first-phase insulin, was potentiated in the KME-E, but not in the KME-W, group compared with the Px-control group. KME-E also enhanced β-cell mass to a greater degree than observed in the Px-control group. Additionally, KME-E reduced whole-body insulin resistance relative to that observed in the Px-control group, whereas hepatic insulin resistance was attenuated in the Px-control, KME-W, and KME-E groups, in descending order. Therefore, KME-E prevented the deterioration of glucose metabolism in diabetic Px rats via the actions of betulin, betulinic acid, and oleanolic acid. Betulin potentiated insulin-stimulated glucose uptake by increasing PPAR-γ activities in 3T3-L1 adipocytes, whereas oleanolic acid enhanced glucose-stimulated insulin

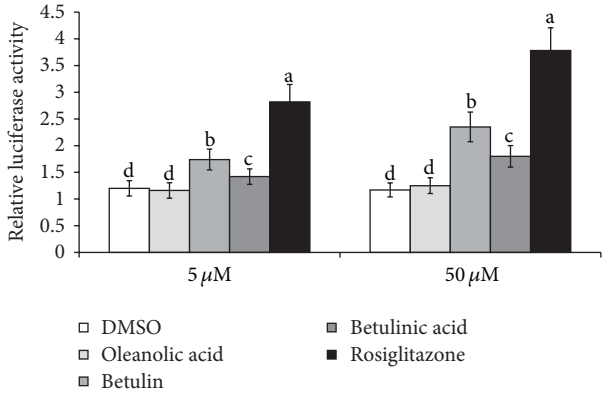

Figure 6: PPAR-γ activity of betulin, betulinic acid, and oleanolic acid as determined by luciferase ligands. The cells were transiently transfected with four vectors such as a PPRE-luciferase construct, pSV-SPORT-PPAR-γ expression vector, pSV-SPORT-retinoid X receptor- (RXR-) α vector, and renilla phRL-TK vector with a Lipofectamine PLUS reagent. After 2 h of transfection, the cells were treated with serum-free DMEM containing 0.1% BSA for 40 h and were exposed to betulin, betulinic acid, and oleanolic acid (5 and 50 μM) for 12 hours. At the end of the incubation, the cells were solubilized in 1x passive lysis buffer and assayed for both firefly (PPRE-luciferase) and renilla luciferase activities using the Dual-Luciferase Reporter Assay System. Ratios of firefly luciferase activity and renilla luciferase activity were used for the results. The bars and error bars represent mean ± SD ($n = 7$). ^{a,b}Values on the bars with different superscripts were significantly different in a Tukey post hoc test at a significance of $P < 0.05$.

secretion and cell proliferation in Min6 insulinoma cells. Therefore, KME-E may be a potential therapeutic agent for patients with type 2 diabetes. To the best of our knowledge, no studies have evaluated the modulation of insulin secretion and insulin resistance in terms of antidiabetic activity.

KME has traditionally been used as a herbal medicine in European, African, and Asian countries, including Korea. Analyses of various crude alcoholic extracts and purified fractions of KME have revealed that these products possess hypotensive, hypoglycemic, antilipidemic, antioxidative, anti-inflammatory, and antimicrobial activities and tend to ameliorate health problems such as diabetes mellitus, hypertension, arthritis, pain, and cancer [26]. Adaramoye et al. [8] reported that 3 weeks of treatment with African mistletoe methanolic extract reduced fasting blood glucose concentrations and glycated hemoglobin (HbA1c) levels to the same degree as glibenclamide treatment in streptozotocin-induced diabetic rats. However, these findings did not reveal any changes in insulin secretion and insulin resistance. Orhan et al. [27] found that the antidiabetic activities of mistletoe extracts were highly dependent on the host plant species and the extraction solvents that are used. For example, these authors demonstrated that 7 days of treatment with an ethanolic extract of European mistletoe grown on pine trees produced the greatest reduction in serum glucose levels during an OGTT in normal and streptozotocin-induced diabetic rats. Furthermore, the maximum effects of a methanolic extract (400 mg/kg) of African mistletoe from *Persea americana*, the avocado tree, reduced blood glucose levels in alloxan-induced diabetic rats as much as glibenclamide treatment 24 hours after administration [28]. This study also found no toxicity among the mistletoe extracts obtained from five different host trees. The present study is the first to use KME grown on oak trees to investigate the antidiabetic activities of the extract. The majority of previous studies have used streptozotocin or alloxan to induce diabetes in experimental animals, and these compounds are known to destroy β-cells via the generation of free radicals [8, 27, 28]. Thus, mistletoe likely reduces blood glucose levels by reducing the level of free radicals and partly preventing β-cell damage.

In the present study, KME-E exerted superior antidiabetic activities relative to KME-W by potentiating β-cell function and mass and reducing insulin resistance in type 2 diabetic Px rats. These effects were most likely related to the actions of triterpenoids, such as betulin, betulinic acid, and oleanolic acid, which were present in KME-E. In the cell culture analyses of the present study, betulin and betulinic acid enhanced insulin-stimulated glucose uptake via PPAR-γ activity in adipocytes, whereas oleanolic acid potentiated glucose-stimulated insulin secretion in insulinoma cells. Consistent with these results, several studies have shown that betulin and betulinic acid improve glucose metabolism. Using a diet-induced obesity animal model, Tang et al. [29] demonstrated that betulin reduces the biosynthesis of cholesterol and fatty acids by inhibiting sterol regulatory element-binding protein (SREBP) pathways. It has also been shown that betulin dramatically enhances the expressions of adiponectin, lipoprotein lipase, and PPAR-γ in white adipose tissues, which may improve insulin sensitivity and glucose metabolism [29]. Additionally, Wan et al. [30] reported that betulin and betulinic acid suppress the expression of SREBP-1 and its target genes related to fatty acid synthesis in hepatocytes, which results in protection against acute ethanol-induced fatty liver. Similarly, betulinic acid alleviates nonalcoholic fatty liver disease by potentiating the AMPK/mammalian target of the rapamycin/SREBP signaling pathway in mice with diet-induced obesity [31]. In the present study, betulinic acid activated AMPK via phosphorylation in 3T3-L1 adipocytes. Thus, the betulin and betulinic acid components of KME-E may play important roles in the improvement of glucose metabolism by enhancing insulin sensitivity in the adipose tissues and liver of diabetic Px rats.

Type 2 diabetes is strongly related to pancreatic β-cell dysfunction, which is influenced by β-cell mass. In the present study, KME-E, but not KME-W, potentiated glucose-stimulated insulin secretion and β-cell proliferation in diabetic Px rats, and it was shown that this resulted from the oleanolic acid component of KME-E. Oleanolic acid exerts antidiabetic activities in diabetic animals; these activities are primarily explained by enhanced pancreatic β-cell function and reduced β-cell apoptosis [32, 33]. Additionally, oleanolic acid attenuates hepatic insulin resistance via antioxidant, hypolipidemic, and anti-inflammatory activities [34, 35]. As a result, oleanolic acid can ameliorate diabetic symptoms by potentiating β-cell function, resulting in improved insulin resistance, especially in the liver.

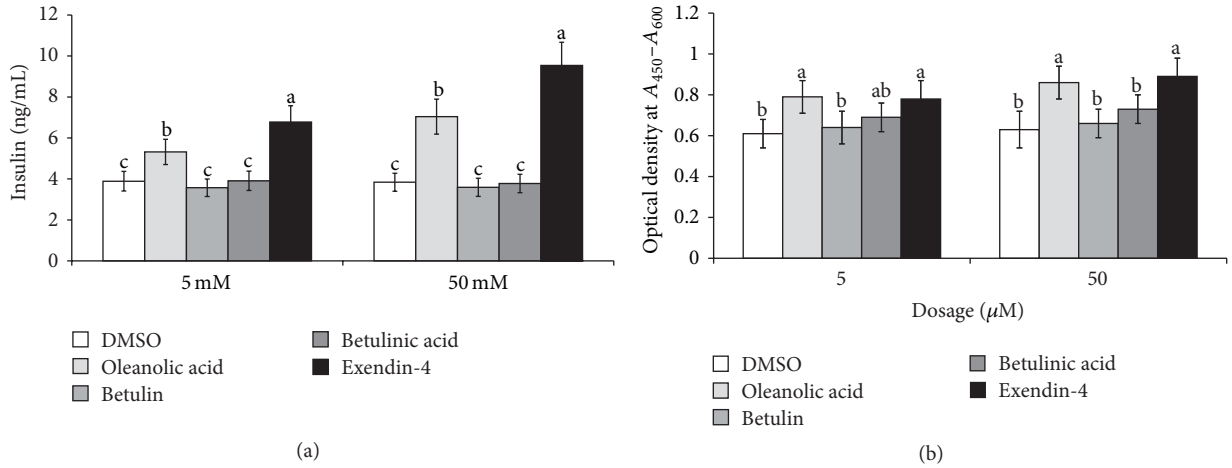

Figure 7: Glucose-stimulated insulin secretion and cell viability in insulinoma Min6 cells. Min6 insulinoma cells were treated with low (0.5 μg/mL) or high (5 μg/mL) doses of vehicle (DMSO) or low (5 μM) or high (50 μM) doses of betulin, betulinic acid, and oleanolic acid for 16 hours. At the end of the incubation, media were changed into high glucose (20 mM) Krebs-Ringer-Hepes buffer containing assigned compound for 30 min and insulin concentrations in the buffer were measured by RIA insulin kit (a). After treatment with vehicle (DMSO) or low (5 μM) or high (50 μM) doses of betulin, betulinic acid, and oleanolic acid for 48 h and glucose-stimulated insulin secretion measured for 30 min, cell viability was measured with a cell proliferation WST-1 reagent assay kit (b). The bars and error bars represent mean ± SD ($n = 7$). [a,b,c]Values on the bars with different superscripts were significantly different in a Tukey post hoc test at a significance of $P < 0.05$.

It has been suggested that KME is toxic to the liver due to several of its components, including lectins [7]. Preliminary findings from our research group have shown that KME is generally nontoxic when it is boiled for more than 10 hours because the toxic compounds may be denatured; we also found that KME-W contains lectins but that KME-E does not [10]. Therefore, KME-E has a much lower chance of inducing liver toxicity. Kim et al. [36] have also shown that extracts of mistletoe grown on oak tree have LD50 of above 5,000 mg/kg in rats and no extract-related adverse effects are revealed with up to 1,000 mg/kg body weight/day in rats of chronic consumption study. Korean mistletoe is accepted to be safe to consume. The present study also demonstrated that both KME-W and KME-E did not lead to an increase in aspartate aminotransferase and alanine aminotransferase activities in the blood, which indicates that the liver was not damaged by either of the extracts.

5. Conclusion

KME-E improved glucose tolerance via improvements in hepatic insulin resistance and alterations of β-cell function and mass in diabetic Px rats. The superior antidiabetic effects of KME-E compared with KME-W were likely due to the presence of betulin, betulinic acid, and oleanolic acid in the ethanolic extract. It was also shown that betulin and betulinic acid acted as PPAR-γ agonists and increased insulin-stimulated glucose uptake and potentiated the phosphorylation of AMPK in 3T3-L1 adipocytes. Furthermore, oleanolic acid enhanced β-cell function and mass. Therefore, KME-E can be a potential therapeutic agent for the treatment of patients with type 2 diabetes.

Conflict of Interests

The authors declare that there is no conflict of interests.

Authors' Contribution

Byoung-Seob Ko and Sunmin Park designed the study protocol, analyzed statistical analysis, and prepared the final paper. Suna Kang and Bo Reum Moon performed all experiments and analyze the data. Jin Ah Ryuk participated in discussion and revised the paper. All authors approved the final paper.

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