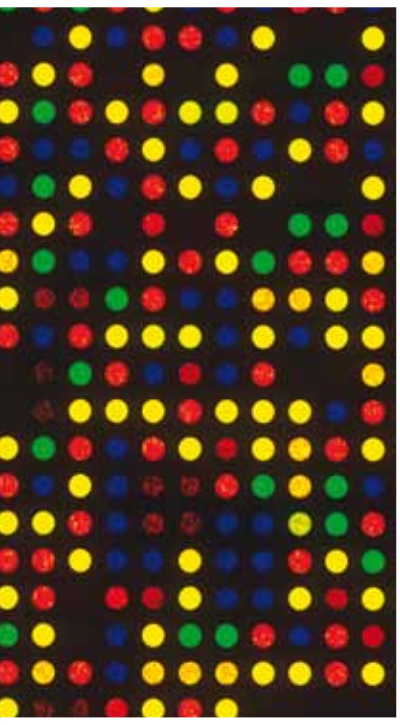
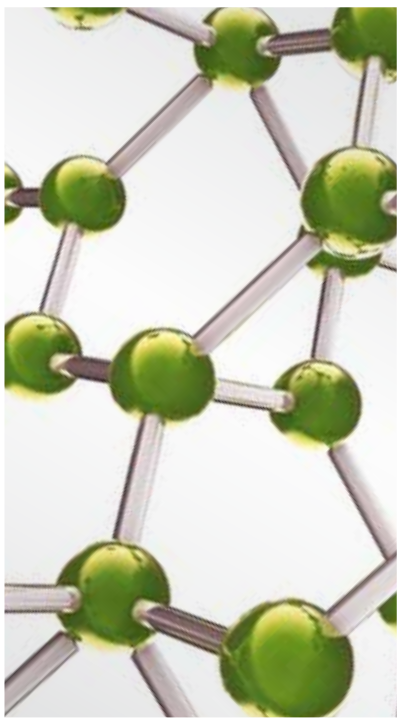
eCAM Evidence-Based Complementary and Alternative Medicine

Complementary and Alternative Medicine for Diabetes

Guest Editors: Wen-Chin Yang, Srinivas Nammi, Per Bendix Jeppesen, and William C. S. Cho



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Editorial Board

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Editorial
Complementary and Alternative Medicine for Diabetes

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Although diabetes was identified by a Greek physician, Aretaeus of Cappadocia, about 2,000 years ago, this old disease remains incurable. Diabetes is characterized by insulin deficiency, insulin resistance, and aberrant glucose, protein, and lipid metabolism. Genetic and environmental factors are the primary causes of diabetes. It is estimated that about 300 million people globally are afflicted with this disease. However, current oral antidiabetic agents using orthodox medicine have unmet efficacy and undesirable side effects in patients, leading to the development of microvascular and macrovascular complications. Research and development of new remedies for diabetes are, therefore, in great demand.

It is estimated that complementary and alternative medicine (CAM) is used by 80% of the world population for primary health care. Therefore CAM, including herbal medicines, acupuncture, moxibustion, and other therapies, represents an important area of exploration for diabetes therapy. In this special issue, we aimed to gather together updated information reflecting the considerable progress in basic and clinical research into CAM for diabetes and its complications.

The papers in this special issue cover a wide range of topics, including *in vitro* studies, preclinical studies, and clinical trials on CAM for diabetes and related diseases. One *in vitro* study by S.-C. Chang and W.-C. V. Yang titled "*Hyperglycemia induces altered expressions of angiogenesis associated molecules in the trophoblast*" describes the expression of perlecan and angiogenesis-related cytokines and growth factors in trophoblasts, one type of placenta cells. High glucose affected the expression level of cell-bound perlecan, angiogenesis-associated cytokines, and the matrix degradation on the cells, implying that hyperglycemia influences vessel formation during placentation. A. Nachar et al. ("*The action of antidiabetic plants of the Canadian James Bay Cree traditional pharmacopeia on key enzymes of hepatic glucose homeostasis*") report the antidiabetic action of seven Canadian plants as evidenced by glucose-6-phosphatase and glycogen synthase, two key enzymes, respectively, involved in gluconeogenesis and glycogenesis in hepatocytes. Among them, *Abies balsamifera* and *Picea glauca* decreased glucose-6-phosphatase activity. This decrease involved the Akt and AMPK pathways. In contrast, *Larix laricina* and *A. balsamifera* increased glycogen synthase activity. Preclinical studies were used to study the action and mechanism of CAM in rodents. Diabetes arises from a defect in β cell functions and insulin resistance. A small flavone-type molecule, swertisin, found in *Enicostemma littorale* was tested for its ability to promote the generation of pancreatic islets. N. Dadheech et al. ("*A small molecule swertisin from Enicostemma littorale differentiates NIH3T3 cells into islet-like clusters and restores normoglycemia upon transplantation in diabetic Balb/c mice*") showed that swertisin could promote the differentiation of NIH3T3 cells into pancreatic islet-like cell mass, which restored hypoglycemic status to normal. Another study by C. L.-T Chang et al. ("*Antidiabetic effect and mode of action of cytopiloyne*") reported that a small molecule, cytopiloyne, isolated from *Bidens pilosa*, improved type 2 diabetes (T2DM) in db/db mice via its modulation of

β-cell functions (insulin production and β-cell preservation) involving the calcium/DAG/PKCα cascade. J. Wang and colleagues ("*Improvement of liquid fructose-induced adipose tissue insulin resistance by ginger treatment in rats is associated with suppression of adipose macrophage-related proinflammatory cytokines*") show that treatment with ginger extract reduced fructose-induced insulin resistance in rats by suppression of adipose inflammatory cytokines (TNF-α, IL-6, MPC-1, CCR-2, etc.) and increased phosphorylation of IRS-2. K. K. Tan and K. H. Kim. ("*Alternanthera sessilis red ethyl acetate fraction exhibits antidiabetic potential on obese type 2 diabetic rats*") report that, despite their inability to identify active compounds, they observed that ethyl acetate fraction of *Alternanthera sessilis* ameliorated T2D via increased insulin content and decreased insulin resistance. Aside from blood glucose, this fraction reduced blood triglyceride and free fatty acids. S. H. Kim and coworkers ("*Citrus junos Tanaka peel extract exerts antidiabetic effects via AMPK and PPAR-γ both in vitro and in vivo in mice fed a high-fat diet*") indicate that the ethanol extract of the peel of *Citrus junos in vitro* stimulates glucose uptake in C2C12 myotube cells. This extract also augmented activity of PPAR-γ and AMPK in C2C12 cells. Consistent with the *in vitro* data, the extract diminished insulin resistance as well as body weight and adipokines and elevated AMPK phosphorylation in high-fat diet- (HFD-) induced mice. N. Hu et al. ("*Anti-diabetic activities of Jiaotaiwan in db/db mice by augmentation of AMPK protein activity and upregulation of GLUT4 expression*") demonstrate the antidiabetic action of a traditional Chinese medicine (TCM), Jiaotaiwan, as shown by reduction of blood glucose level and enhancement of islet protection, hepatic AMPK activity, and expression level of glucose transporter 4 in skeletal muscle and white fat. H.-Y. Huang and colleagues ("*Supplementation of Lactobacillus plantarum K68 and fruit-vegetable ferment along with high fat-fructose diet attenuates metabolic syndrome in rats with insulin resistance*") report that a mixture of fruit/vegetable ferment and one of its bacteria, *Lactobacillus plantarum* K68, reduced hyperglycemia, hyperinsulinemia, and hyperlipidemia as well as proinflammatory cytokines (TNF-α, IL-6, IL-1β, etc.) in HFD-induced rats. This reduction was associated with a decrease in insulin resistance. P. V. Rao and coworkers ("*Rhinacanthus nasutus ameliorates cytosolic and mitochondrial enzyme levels in streptozotocin-induced diabetic rats*") showed that glycolytic enzymes such as glucose-6-phosphate dehydrogenase, succinate dehydrogenase, glutamate dehydrogenase, and lactate dehydrogenase were upregulated in diabetic mice. In contrast, the methanol extract of *R. nasutus* reduced those enzymes, implying that this extract exerts antidiabetic action via reduction of metabolic enzymes. In addition, two articles delineate the impact of herbal medicine and compounds on diabetic complications in animals ("*Proanthocyanidin attenuation of oxidative stress and NF-κB protects apolipoprotein E-deficient mice against diabetic nephropathy*" and "*An aqueous extract of Radix Astragali, Angelica sinensis, and Panax notoginseng is effective in preventing diabetic retinopathy*"). One study by D. Gao and colleagues ("*An aqueous extract of Radix Astragali, Angelica sinensis, and Panax notoginseng is effective in preventing diabetic retinopathy*") reports that Dang Gui Bu Xue Tang, a TCM composed of *A. membranaceus*, *A. sinensis*, and *P. notoginseng*, reduced diabetic retinopathy in diabetic Goto-Kakizaki rats and/or streptozotocin- (STZ-) induced rats. This reduction was associated with retinal downregulation of proinflammatory cytokines and the reversal of glucose-induced inhibition of endothelial cell migration/proliferation *in vitro*. Another study shows that treatment with the polyphenolic compounds proanthocyanidins reduced nephropathy in STZ-treated apolipoprotein E-deficient mice ("*Proanthocyanidin attenuation of oxidative stress and NF-κB protects apolipoprotein E-deficient mice against diabetic nephropathy*"). This reduction is relevant to its attenuation of oxidative stress and NF-κB activation.

In human clinical studies, X. Tu et al. ("*Fructus mume formula in the treatment of type 2 diabetes mellitus: a randomized controlled pilot trial*") demonstrate that 12-week treatment with a monofactorial formula, *F. mume*, reduced blood glucose in 41 patients of T2DM. X. Li and colleagues ("*The rs1142345 in TPMT affects the therapeutic effect of traditional hypoglycemic herbs in prediabetes*") concluded that a single nucleotide polymorphism (rs1142345) in thiopurine *S*-methyltransferase could affect the therapeutic outcome of the patients with T2DM receiving the TCM, Tianqi Jiang Tang. C. X. Huang et al. ("*Prescription pattern of Chinese herbal products for diabetes mellitus in Taiwan: a population-based study*") present the use and likely mechanisms of action of multifactorial formulae such as Liu-Wei-Di-Huang-Wan and its derivatives. Their antidiabetic mechanisms are multifaceted and include increase in insulin secretion, insulin sensitivity, peripheral glucose uptake, diminution of intestinal glucose absorption, hepatic glucose, production and insulin resistance. Despite some evidence in favor of their use, the data could be confounded by the placebo effect, suggesting that well-conducted, double-blind, randomized, placebo-controlled studies are required for further investigations. Further, C. I. Tsai et al. ("*Chinese medicinal formula (MHGWT) for relieving diabetic neuropathic pain: a randomized, double-blind, placebo-controlled trial*") investigated the therapeutic effect of a modified TCM formula, Hungqi Guizhi Wuwu Tang, on neuropathic pain in 112 diabetic patients, as assessed by 15-item short-form brief pain inventory and the 17-item short-form McGill pain questionnaire. This TCM significantly improved neuropathic pain in the patients.

In addition to original research articles, this special issue also features review articles. One review entitled "*Herbal therapies for type 2 diabetes mellitus: chemistry, biology, and potential application of selected plants and compounds*" describes a variety of antidiabetic herbal products from chemical, biological, pharmacological, and clinical aspects. And another review entitled "*Adjunct methods of the standard diabetic foot ulceration therapy*" summarizes three adjunct therapies, hyperbaric oxygen therapy, maggot therapy, and platelet-rich plasma therapy in one of the most serious diabetic complications, foot ulcers. The authors discuss preclinical and clinical studies of those therapies together with their effects and modes of action in animals and humans. Both review articles shed light on further directions for CAM in diabetes and diabetic complications.

We envision that this special issue will attract broad interest in the field of diabetes and encourage the perusal of more in-depth investigations into the use of CAM-based therapies for diabetes and the related complications.

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Research Article

Chinese Medicinal Formula (MHGWT) for Relieving Diabetic Neuropathic Pain: A Randomized, Double-Blind, Placebo-Controlled Trial

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Objective. To investigate the effects of modified *Hungqi Guizhi Wuwu Tang* (MHGWT), a formula that comprises Chinese medicinal herbs, in relieving neuropathic pain in diabetics. *Method*. Between March 2008 and April 2009, 112 participants were randomly assigned to either the MHGWT group, whose members received MHGWT ($n = 56$), or the control group, whose members received a placebo ($n = 56$). Diabetic neuropathic pain (DNP) was rated using the 15-item Short-Form Brief Pain Inventory (SF-BPI), the 17-item Short-Form McGill Pain Questionnaire (SF-MPQ), the 13-item Modified Michigan Neuropathy Screening Instrument (MMNSI), and the 36-item "SF-36." Nerve conduction studies (NCSs) were performed before and after treatment. *Results*. After 12 weeks of treatment, the SF-MPQ and SF-BPI scores of the MHGWT group were significantly ($P < 0.05$) reduced and a significant difference between the groups was observed ($P < 0.05$). The levels of NCS in the MHGWT group were nonsignificantly ($P > 0.05$) reduced, and no significant difference in NCS level was observed between the groups ($P > 0.05$). *Conclusions*. MHGWT shows promise in relieving DNP and deserves further investigation.

1. Introduction

Neuropathy is the most common complication associated with diabetes, and sensorimotor diabetic peripheral neuropathy (DPN) is the most common form of diabetic neuropathy [1, 2]. It is characterized by a progressive loss of nerve fibers that predisposes the sufferer to painful or numb extremities, ulceration, and amputation and results in a large disease burden in terms of inability to work, significantly reduced quality of life, and consumption of healthcare resources [3, 4]. In Taiwan, the prevalence of DPN in patients with diabetes mellitus is approximately 20–30% [5].

Many agents are currently utilized to manage diabetic neuropathic pain but only two, pregabalin and duloxetine, have been approved by the American FDA for this indication [6]. However, the significant side effects of these agents,

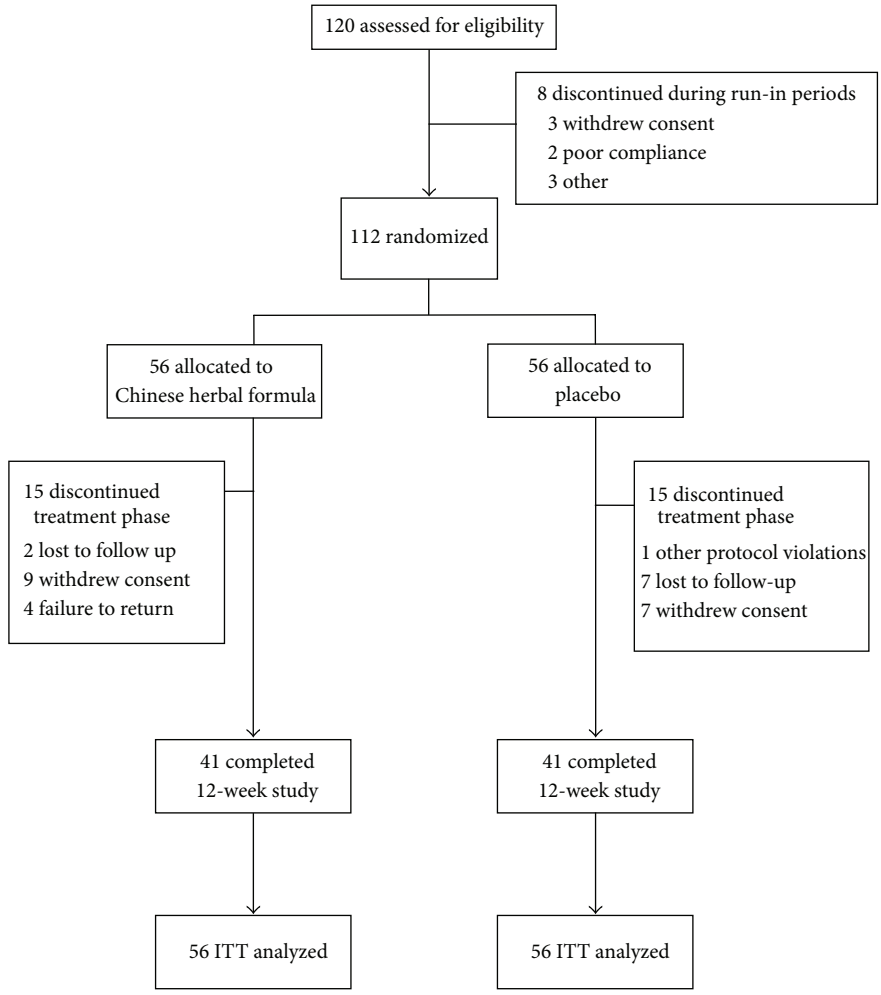

Figure 1: Participant flowchart depicting the randomization, treatment, and followup in MHGWT and placebo groups.

including dizziness, somnolence, infection, peripheral edema [7], hepatotoxicity, and related deterioration of blood cells, are not trivial [8]. Consequently, they must be used with caution [9]. For thousands of years, the Chinese herbal medicine *Hungqi Guizhi Wuwu Tang* (HGWT) has been prescribed to improve the circulation of the extremities [10]. Clinical investigations have suggested that it has therapeutic potential for DPN [11], with the added advantages of lower cost and fewer side effects compared with the aforementioned agents [12]. Several clinical trials have been conducted, but most of them were of low methodological quality. Thus, any positive findings concerning the efficacy of Chinese herbal medicines for treating DPN should be treated with caution [13]. In order to reliably evaluate the effectiveness of HGWT in treating DPN, high-quality clinical trials are required.

2. Materials and Methods

2.1. Participants. The participants in this study were men and women aged over 18 years with a Michigan Neuropathy screening score of at least 3. They were recruited between March 2008 and April 2009 from the outpatient clinic of the Taichung Veterans General Hospital, Taichung, Taiwan. Participants had to have been diagnosed with DM based on criteria consistent with those recommended by the American Diabetes Association (ADA) and were required to have fasting plasma ≥126 mg/dL, or two-hour plasma glucose ≥200 mg/dL as measured by the oral glucose tolerance test (OGTT) on two separate occasions, or random plasma glucose ≥200 mg/dL with symptoms (polyuria, polydipsia, and unexplained weight loss) [14]. Women who were pregnant or breastfeeding were excluded from the study, as were patients who satisfied any of the following conditions: renal dysfunction (serum creatinine (Cr) > 1.3 mg/dL); active liver disease or hepatic dysfunction (glutamic pyruvic transaminase (GPT) > double the upper limit of the normal range (ULN)); and cerebrovascular disease, previous cardiovascular surgery, myocardial infarction, or major trauma or operations up to six months prior to the study period, or participation in another clinical trial within 30 days before consideration for entry into this study. Patients who were taking analgesics (such as NSAIDs and tramadol), antidepressants (such as TCAs, SSRIs, and duloxetine), capsaicin topical cream, and

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重金屬成績表 Heavy Metal Test Report

Item code : G3220 Item name : Placebo Batch No. 413937

品號	G3220	品名	加味黃耆五物湯 安慰劑	批號	413937
Sampler/Date: Lai Hsin Qun		Sampling place : Finished product inspection pending area			
取樣者/日期	賴信榮 2008.06.28	取樣地點	成品待驗區	取樣量	1.0g
				Sampling Qty: 1.0g	
檢驗依據	參照中華藥典第六版附錄第26,27頁及廠規SOP-Q052				
Analysis Reference: Taiwanese Pharmacopeia Edition 6, p26-27. Internal specification SOP-052					

檢驗項目 Item of Analysis	規格（ppm） Spec (ppm)	檢驗結果（ppm） Result (ppm)	檢驗者／日期 Analyst / Date	備註 Remark
總重金屬 Total Heavy Metal	< 100 ppm	Pass	賴信榮 2008.06.30 賴信榮	中華藥典第六版附錄 第 26,27 頁 Taiwanese Pharmacopeia Edition 6, p26-27.
Analyst / Date	Approved by / Date			

檢驗者／日期	品管責任者 ／日期	判定 Result
賴信榮 2008.06.30	林淑貞 2008.06.30	合格

Figure 2: Report of test of Chinese medicinal herbs for heavy metals.

anticonvulsants (such as pregabalin) or Chinese medicinal herbs were considered for screening only after a four-week washout period. All the enrolled participants were able to communicate well enough to complete the questionnaires and were capable of responding to the nerve conduction study (NCS). The participants were excluded from the study if they had any history of psychiatric disorders, history of alcohol or drug abuse, and any condition associated with poor compliance with medical treatment. The study complied with the Declaration of Helsinki. The relevant institutional ethics review boards approved the protocol, and all participants provided informed consent. This trial was registered with ClinicalTrials.gov, number NCT00886655.

2.2. Randomization and Estimation of Sample Size. A permuted-block randomization was utilized to assign subjects randomly, based on the order of entry into two treatment groups. Each group received either MHGWT or a placebo. A sample size of 112 patients was used to evaluate and compare the efficacies and safeties of MHGWT and the placebo. The sample size was chosen according to the primary efficacy outcome. The change from the baseline mean Short-Form Brief Pain Inventory (SF-BPI) score to that after 12 weeks was determined for the two groups. The two-sided alpha (type I error) was set to 0.05 and the beta (type II error) was set to 0.10 (power of 90%). The effect size was 0.5 and the standard deviation (SD) of the BPI scores was 0.5. Based on these assumptions, a sample size of approximately 29 subjects per group was required for an assumed 30% loss of follow-up.

2.3. Interventions. The mixture of Chinese medicinal herbs that is known as modified *Hungqi Guizhi Wuwu Tang* (MHGWT) is composed of the following seven species: *Astragali Radix* (Leguminosae), radix; *Cinnamomum cassia* Presl., ramulus; *Paeonia lactiflora* Pall., radix; *Zingiber officinale* (Willd.) Rosc., radix; *Ziziphus jujuba* Mill.,fructus; *Spatholobus suberectus* Dunn., caulis; and *Pheretima aspergillum* (Perrier). The Chinese medicinal herb formula, MHGWT, given to the treatment group was powdered and packed in aluminum foil according to Good Manufacturing Practices (GMP) by Kaiser Pharmaceuticals Co., Ltd., Tainan, Taiwan. The placebo was composed of the same

Table 1: Patient's assignment, values at screening, and safety values.

Variables	Group, number		P value
	MHGWT group ($n = 56$)	Placebo group ($n = 56$)	
Screening values			
Male, n (%)	29 (51.79)	28 (50)	1.00
Female, n (%)	27 (48.21)	28 (50)	1.00
Age (years)	60.71 ± 10.20	60.46 ± 10.60	0.90
Height (cm)	162.14 ± 7.04	162.11 ± 8.07	0.98
Weight (kg)	67.00 ± 12.44	66.49 ± 10.28	0.81
Body-mass index	25.41 ± 3.87	25.24 ± 3.08	0.80
Duration of diabetes (years)	10.59 ± 6.49	9.91 ± 6.68	0.59
Duration of DPN (years)	2.49 ± 1.86	9.91 ± 6.68	0.35
Baseline safety values			
GPT (u/L)	25.82 ± 13.67	23.04 ± 8.24	0.20
Creatinine (mg/dL)	1.06 ± 0.25	1.06 ± 0.22	0.88
Fasting glucose (mmol/L)	155.84 ± 50.70	154.68 ± 47.82	0.90
HbA1c (%)	8.11 ± 1.71	7.86 ± 1.63	0.42
Triglycerides (mmol/L)	171.69 ± 195.91	200.76 ± 183.67	0.42
Total cholesterol (mmol/L)	189.65 ± 38.08	187.18 ± 49.22	0.77

Screening values and safety values were obtained at baseline.
Data were presented as mean ± SD.
P values are calculated for the comparison of difference between MHGWT and placebo groups by independent t-test and chi-square test.
GPT: glutamic pyruvic transaminase.
HbA1c: glycosylated hemoglobin.

Table 2: Changes of scores of Short-Form Brief Pain Inventory questionnaire after interventions.

Domains	Group, number		P value
	MHGWT group ($n = 56$)	Placebo group ($n = 56$)	
Sensory			
Baseline	25.43 ± 8.13	21.45 ± 8.37	0.01*
Time 2 − baseline	− 5.48 ± 6.70	0.20 ± 8.90	0.001***
Time 3 − baseline	− 12.62 ± 7.57	0.16 ± 8.19	<0.0001***
Time 4 − baseline	− 15.64 ± 8.78	0.53 ± 7.88	<0.0001***
Scheffe's test for time effect	a > b > c > d		
Daily life			
Baseline	27.43 ± 12.25	23.34 ± 13.00	0.10
Time 2 − baseline	− 9.77 ± 9.10	0.32 ± 11.25	<0.0001***
Time 3 − baseline	− 15.14 ± 10.63	− 3.47 ± 13.84	<0.0001***
Time 4 − baseline	− 18.36 ± 11.66	− 4.66 ± 14.49	<0.0001***
Scheffe's test for time effect	a > b > c, d	a > d	

Time 2 was in the end of 4 weeks, time 3 was in the end of 8 weeks, and time 4 was in the end of 12 weeks.
Data were presented as mean ± SD.
P values are calculated for the comparison of difference between MHGWT and placebo groups by independent t-test.
* $P < 0.05$; *** $P < 0.001$.
a: time 1; b: time 2; c: time 3; d: time 4.

medicine that was administered to the MHGWT group but at one-tenth the concentration. Identical packets of MHGWT and placebo powder, which were dispensed by the hospital pharmacy, were marked with codes that corresponded to the participants' names. Participants were asked to take one pack (4 g MHGWT or placebo) three times daily, 30 min after breakfast, lunch, and dinner, throughout the 84 days of the study and to return the remnants so that the packs could be counted at each visit to the clinic. High-performance liquid chromatography (HPLC) fingerprints were obtained to identify substances in the mixtures and to ensure that the product was of consistent quality. Contamination screening for heavy metals such as lead, arsenic, cadmium, and mercury was performed to ensure safety for human

Table 3: Changes of scores of Short-Form McGill Pain Questionnaire after interventions.

Domains	Group, number		P value
	MHGWT group ($n = 56$)	Placebo group ($n = 56$)	
Sensory			
Baseline	7.30 ± 3.42	5.91 ± 3.77	0.04*
Time 2 – baseline	−1.93 ± 2.58	0.04 ± 3.04	0.001**
Time 3 – baseline	−3.36 ± 3.01	0.13 ± 3.06	<0.001***
Time 4 – baseline	−3.85 ± 3.20	−0.07 ± 3.36	<0.001***
Scheffe's test for time effect	a > b > c, d		
Affective			
Baseline	2.22 ± 1.54	1.66 ± 1.75	0.08
Time 2 – baseline	−0.43 ± 1.52	0.43 ± 1.06	0.003**
Time 3 – baseline	−0.93 ± 1.60	0.26 ± 1.13	<0.001***
Time 4 – baseline	−1.07 ± 1.68	0.41 ± 1.26	<0.001***
Scheffe's test for time effect	a > b, c, d; b > d		
VAS			
Baseline	4.98 ± 1.39	4.07 ± 1.53	0.002**
Time 2 – baseline	−1.55 ± 1.16	−0.04 ± 1.38	0.002**
Time 3 – baseline	−2.50 ± 1.61	−0.10 ± 1.56	<0.001***
Time 4 – baseline	−3.10 ± 1.69	−0.31 ± 1.61	<0.001***
Scheffe's test for time effect	a > b > c > d		
PPI			
Baseline	2.52 ± 0.72	2.18 ± 0.69	0.01**
Time 2 – baseline	−0.45 ± 0.70	0.09 ± 0.75	<0.001***
Time 3 – baseline	−0.98 ± 0.72	−0.08 ± 0.78	<0.001***
Time 4 – baseline	−1.20 ± 0.75	−0.17 ± 0.89	<0.001***
Scheffe's test for time effect	a > b > c, d		
Total			
Baseline	9.52 ± 4.17	7.57 ± 5.09	0.03*
Time 2 – baseline	−2.36 ± 3.62	0.47 ± 3.65	<0.001***
Time 3 – baseline	−4.29 ± 3.80	0.39 ± 3.45	<0.001***
Time 4 – baseline	−4.93 ± 4.27	0.34 ± 4.08	<0.001***
Scheffe's test for time effect	a > b > c, d		

Time 2 was in the end of 4 weeks, time 3 was in the end of 8 weeks, and time 4 was in the end of 12 weeks.
Data were presented as mean ± SD.
P values are calculated for the comparison between MHGWT and placebo groups by independent t-test.
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.
a: time 1; b: time 2; c: time 3; d: time 4.

Table 4: Changes of scores of Modified Michigan Neuropathy Screening Instrument after interventions.

Domains	Group, number		P value
	MHGWT group ($n = 56$)	Placebo group ($n = 56$)	
Baseline	45.02 ± 17.07	32.95 ± 14.38	<0.001***
Time 2 – baseline	−11.75 ± 10.69	−1.02 ± 9.01	<0.001***
Time 3 – baseline	−23.05 ± 13.58	−2.97 ± 11.65	<0.001***
Time 4 – baseline	−28.05 ± 14.65	−1.03 ± 11.42	<0.001***
Scheffe's test for time effect	a > b > c, d		

Time 2 was in the end of 4 weeks, time 3 was in the end of 8 weeks, and time 4 was in the end of 12 weeks.
Data were presented as mean ± SD.
P values are calculated for the comparison between MHGWT and placebo groups by independent t-test.
***$P < 0.001$.
a: time 1; b: time 2; c: time 3; d: time 4.

Table 5: Changes of scores of short-form 36 questionnaire after interventions.

Domains	Group, number		P value
	MHGWT group (n = 56)	Placebo group (n = 56)	
Physical function			
Time 1[a]	68.16 ± 22.05	64.91 ± 21.38	0.43
Time 2[b]	73.80 ± 21.14	66.12 ± 24.39	0.11
Time 3[c]	81.70 ± 17.52	73.07 ± 20.32	0.04*
Time 4[d]	83.80 ± 14.38	69.38 ± 21.68	<0.001***
Scheffe's test for time effect	a, b < c, d	a, b < c	
Role limitation due to physical problems			
Time 1[a]	25.00 ± 33.74	26.82 ± 38.75	0.79
Time 2[b]	36.41 ± 31.93	21.94 ± 34.47	0.04*
Time 3[c]	61.36 ± 34.71	32.95 ± 38.04	<0.001***
Time 4[d]	73.91 ± 32.90	28.13 ± 37.05	<0.001***
Scheffe's test for time effect	a, b < c, d		
Bodily pain			
Time 1[a]	47.09 ± 10.95	50.13 ± 17.53	0.28
Time 2[b]	57.67 ± 11.61	54.02 ± 15.78	0.20
Time 3[c]	64.39 ± 14.47	56.05 ± 15.61	0.01*
Time 4[d]	66.07 ± 14.15	57.10 ± 15.19	0.004**
Scheffe's test for time effect	a < b < c, d	a < c, d	
General perception of health			
Time 1[a]	27.65 ± 17.18	31.89 ± 17.02	0.19
Time 2[b]	39.04 ± 17.37	33.14 ± 18.59	0.11
Time 3[c]	48.98 ± 18.70	34.64 ± 18.83	<0.001***
Time 4[d]	51.59 ± 19.67	33.15 ± 19.02	<0.001***
Scheffe's test for time effect	a < b < c, d		
Vitality			
Time 1[a]	46.23 ± 13.37	50.82 ± 18.25	0.13
Time 2[b]	51.20 ± 12.16	50.51 ± 13.63	0.80
Time 3[c]	56.02 ± 13.06	52.50 ± 13.91	0.22
Time 4[d]	58.26 ± 11.70	51.46 ± 14.25	0.01*
Scheffe's test for time effect	a, b < d; a < c		
Social functioning			
Time 1[a]	64.25 ± 17.11	65.68 ± 17.05	0.66
Time 2[b]	69.29 ± 16.61	66.07 ± 18.04	0.37
Time 3[c]	80.40 ± 17.13	70.17 ± 17.72	0.007**
Time 4[d]	80.16 ± 19.29	69.53 ± 16.08	0.005**
Scheffe's test for time effect	a, b < c, d		
Role limitation due to emotional problems			
Time 1[a]	49.12 ± 41.84	52.12 ± 42.92	0.71
Time 2[b]	61.48 ± 42.02	42.86 ± 41.39	0.03*
Time 3[c]	75.00 ± 34.57	51.52 ± 40.95	0.005**
Time 4[d]	82.61 ± 33.51	40.97 ± 37.81	<0.001***
Scheffe's test for time effect	a, b < d; a < c		
Mental health			
Time 1[a]	63.02 ± 14.53	64.95 ± 16.63	0.52
Time 2[b]	69.57 ± 13.19	66.20 ± 12.28	0.20
Time 3[c]	71.82 ± 15.14	67.36 ± 12.65	0.14
Time 4[d]	73.39 ± 13.18	66.17 ± 13.25	0.009**
Scheffe's test for time effect	a < b, c, d		

Table 5: Continued.

Domains	Group, number		P value
	MHGWT group ($n = 56$)	Placebo group ($n = 56$)	
Physical component scale			
Time 1[a]	34.97 ± 7.02	35.14 ± 7.63	0.90
Time 2[b]	38.41 ± 7.14	35.91 ± 9.03	0.14
Time 3[c]	43.72 ± 7.84	38.17 ± 8.54	0.002**
Time 4[d]	45.36 ± 7.05	37.69 ± 8.50	<0.001***
Scheffe's test for time effect	a < b < c, d	a < c, d	
Mental component scale			
Time 1[a]	43.80 ± 9.51	45.51 ± 9.66	0.35
Time 2[b]	47.11 ± 8.50	44.53 ± 7.96	0.13
Time 3[c]	49.05 ± 9.29	45.50 ± 7.81	0.06
Time 4[d]	49.94 ± 8.77	44.11 ± 7.26	<0.001***
Scheffe's test for time effect	a < c, d		

Time 2 was in the end of 4 weeks, time 3 was in the end of 8 weeks, and time 4 was in the end of 12 weeks.
Data were presented as mean ± SD.
P values are calculated for the comparison between MHGWT and placebo groups by independent t-test.
$P < 0.05^{*}$; $P < 0.01^{**}$; $P < 0.001^{***}$.
a: time 1; b: time 2; c: time 3; d: time 4.

consumption. The total concentration of heavy metals was less than 100 ppm. Please refer to Figure 2 for report of test of Chinese medicinal herbs for heavy metals.

2.4. Protocol. After a minimum run-in period of four weeks with stable plasma glucose, 112 patients were randomly assigned to 12 weeks of treatment with powdered placebo or MHGWT (Figure 1). The study was double-blind. Participants were reviewed every four weeks and blood samples were obtained after fasting overnight for 12 hours. The laboratory staff responsible for the analyses were blind to the treatment and received samples that were labeled with only name codes and dates.

2.5. Outcome Measures

2.5.1. Short-Form Brief Pain Inventory (SF-BPI). The SF-BPI questionnaire is self-administered by the patient and comprises 15 questions concerning various aspects of pain, which were adopted from the standard BPI. Questions 1 and 2 concern the type and location of the pain. Questions 3 to 6 concern the degree of the worst, mildest, and average pain during the preceding week on a 0–10 scale. Questions 7 and 8 are related to treatment of the pain and the percentage by which the treatment reduced the pain. Questions 9–15 assess the extent to which the pain affects general activity, mood, ability to walk, normal work, relationships, sleep, and enjoyment of life, on a scale of 0–10. A higher SF-BPI score indicates more severe neuropathic symptoms. Cronbach's α for this study ranged from 0.83 to 0.99.

2.5.2. Short-Form McGill Pain Questionnaire (SF-MPQ). The SF-MPQ comprises a series of questions regarding various aspects of the reported pain, with a total of 15 descriptors (11 sensory, four affective). It also includes the Present Pain Intensity (PPI) index (a simple verbal description of pain) and a Visual Analogue scale (VAS) (a quantitative measure of pain), both of which were adopted from the standard MPQ. A higher SF-MPQ score indicates more severe neuropathic symptoms. Cronbach's α ranged from 0.70 to 0.89.

2.5.3. Modified Michigan Neuropathy Screening Instrument (MMNSI). The MMNSI questionnaire comprises 13 self-administered questions concerning sensation in the foot, addressing positive (burning, tingling) and negative (numbness, temperature sensitivity) sensory symptoms, cramps, and muscle weakness. A higher MMNSI score indicates more severe neuropathic symptoms. For each question, answers are transformed to a scale from zero (no symptom) to 10 (the severest possible symptom). The instrument has been validated to evaluate symptoms of diabetic neuropathy. Cronbach's α coefficient ranged from 0.78 to 0.91.

2.5.4. SF-36. The SF-36 is a short questionnaire with 36 questions measuring eight multiitem variables: physical functioning (PF, 10 items), social functioning (SF, two items), role limitations due to physical problems (RP, four items), role limitations due to emotional problems (RE, three items), mental health (MH, five items), vitality (VT, four items), pain (BP, two items), and general perception of health (GH, five items). For each variable, scores are coded, summed, and transformed to a scale from 0 (the worst possible health state) to 100 (the best possible health state). The scores on the SF-36 Physical Component Summary (PCS) and the Mental Component Summary (MCS) scales are derived using the standard SF-36 scoring algorithms. A higher SF-36 score

Table 6: Summary of electrophysiology before and after interventions.

Variables	Group, number		P value
	MHGWT group ($n = 56$)	Placebo group ($n = 56$)	
Peroneal nerve			
Distal latency (ms)			
Pretherapy	4.44 ± 0.83	4.32 ± 0.95	0.48
Posttherapy	4.80 ± 0.99	4.43 ± 0.97	0.14
MNCV (m/s)			
Pretherapy	42.92 ± 6.64	42.27 ± 5.69	0.59
Posttherapy	40.35 ± 9.63	54.06 ± 71.27	0.27
Amplitude (μV)			
Pretherapy	3479.09 ± 2552.01	3783.02 ± 2122.90	0.50
Posttherapy	3436.67 ± 3021.24	3631.43 ± 2277.87	0.77
F-wave (ms)			
Pretherapy	47.74 ± 8.06	50.04 ± 5.42	0.11
Posttherapy	46.83 ± 8.60	51.25 ± 6.46	0.03*
Sural nerve			
Amplitude (μV)			
Pretherapy	10.09 ± 5.12	9.54 ± 5.43	0.66
Posttherapy	10.61 ± 6.61	10.99 ± 6.62	0.85
Distal latency (ms)			
Pretherapy	2.87 ± 0.41	2.84 ± 0.39	0.75
Posttherapy	2.91 ± 0.48	2.90 ± 0.27	0.94

Data were presented as mean ± SD.
P values are calculated for the comparison between MHGWT and placebo groups by independent t-test.
* $P < 0.05$.

Table 7: Comparison of adverse events.

Adverse events	Group, number				P value
	MHGWT group ($n = 56$)		Placebo group ($n = 56$)		
	No.	%	No.	%	
Dry mouth	24	21.43	15	13.39	1.00
Constipation	15	13.39	9	8.04	0.05
Bitter sensation of mouth	1	0.89	0	0	1.00

Data were presented as mean ± SD.
Fisher's exact test was performed to compare the numbers of subjects with adverse effects in the two groups.

indicates better health. Cronbach's α ranged from 0.68 to 0.90, except for SF and VT.

2.5.5. Nerve Conduction Study (NCS). The NCS provides an objective estimate of the functioning of the peripheral nerve. It uses a stimulator, grounding electrode, reference electrode, and recording electrode. The routine NCS in our laboratory included tibial and peroneal nerves (motor function) and sural nerve (sensory function). The differences between the pre- and posttherapy electrophysiologic measurements of distal motor and sensory latencies (DML, DSL), amplitudes of the compound muscle action potentials (CMAPs) and sensory nerve action potentials (SNAPs), motor nerve conduction velocities (MNCVs), and F wave latencies were recorded using an electromyelogram (Viking Select, Nicolet, USA).

2.5.6. Safety Analysis. Safety was evaluated by measuring serum GPT and Cr levels in all patients who had taken at least one dose of medication. All adverse effects that were observed during the clinical trial were recorded. The investigator studied the probability of their relationship to the study drug (definitely, probably, possibly, unlikely, and definitely not) and their intensity (mild, moderate, and severe). Physical examinations and clinical laboratory determinations were performed upon screening, randomization, and study termination.

2.6. Statistical Analysis. Descriptive statistics, including observations, means, standard deviations, and percentages, were used to summarize the baseline variables. All tests were two sided and were performed using the 0.05 level of significance. The demographic information about the

two groups was obtained using an independent t-test for continuous variables and a chi-square test for categorical variables. Changes from the baseline of scores in the questionnaire, safety parameters, the results of laboratory examinations, and the results of nerve conduction studies were analyzed based on the intention-to-treat principle, and the independent t-test was performed to determine between-group variation. The repeated ANOVA was assessed using Scheffe's test as a post hoc comparison. Fisher's exact test was performed to compare the numbers of subjects with adverse effects in the two groups.

3. Results

3.1. Study Population. Of the 120 patients screened, eight were ineligible. The main reasons for ineligibility included violation of selection criteria at entry ($n = 3$), withdrawal of consent ($n = 3$), and poor compliance ($n = 2$). A total of 82 (73%) of the 112 recruited subjects completed the 12-week study without any notable protocol violation. The reasons for the 30 withdrawals were withdrawal of consent ($n = 16$), absence during follow-up ($n = 9$), failure to return ($n = 4$), and deviation from protocol ($n = 1$) (Figure 1). The demographic and clinical characteristics of the study subjects did not differ significantly between the MHGWT and placebo groups (Table 1). The mean age of the patients that underwent the MHGWT treatment was 60.46 years (standard deviation, SD = 10.60 years) and was 60.71 years (SD = 10.20 years) in the placebo group. The baseline demographic and biomarker characteristics of the two groups were well balanced (Table 1).

3.2. Efficacy Analysis. The reductions in mean SF-BPI scores in the sensory and daily life domains in the MHGWT group exceeded those observed in the control group during all treatment phases, and the differences were the largest during weeks 1–12 (-15.64 ± 8.78 versus 0.53 ± 7.88, $P < 0.001$) (-15.64 ± 8.78 versus 0.53 ± 7.88, $P < 0.001$) (Table 2).

The total (sensory plus affective) SF-MPQ scores of the MHGWT group were significantly lower than those of the control group during weeks 1–4 (-2.36 ± 3.62 versus 0.47 ± 3.65, $P < 0.001$), weeks 1–8 (-4.29 ± 3.80 versus 0.39 ± 3.45, $P < 0.001$), and weeks 1–12 (-4.93 ± 4.27 versus 0.34 ± 4.08, $P < 0.001$). Significant differences from the baseline in the VAS and PPI domains during weeks 4, 8, and 12 were observed for both groups, as presented in Table 3 ($P < 0.001$).

Table 4 compares the baseline Modified Michigan Neuropathy Screening Instrument (MMNSI) scores of the MHGWT and placebo groups as well as the changes in scores compared with those at time points 2, 3, and 4. The mean decrease in the MMNSI score of the MHGWT group exceeded that of the placebo group during weeks 1–4 (-11.75 ± 10.69 versus -1.02 ± 9.01, $P < 0.001$), weeks 1–8 (-23.05 ± 13.58 versus -2.97 ± 11.65, $P < 0.001$), and weeks 1–12 (-28.05 ± 14.65 versus -1.03 ± 11.42, $P < 0.001$). The improvement in MMNSI scores differed significantly between the two groups after 4, 8, and 12 weeks of treatment, as presented in Table 4 ($P < 0.001$).

After the first eight weeks of treatment with MHGWT, the short-form 36 questionnaire scores in the two groups differed significantly for role limitation due to physical problems (RP), bodily pain (BP), general perception of health (GH), social functioning (SF), role limitation due to emotional problems (RE), and physical component scale (PCS), as shown in Table 5 ($P < 0.01$). After 12 weeks of treatment with MHGWT, the two groups' short-form 36 questionnaire scores differed significantly from their baseline values for all domains, as shown in Table 5 ($P < 0.01$).

Neither group exhibited significant changes on the NCS, except for the F-wave in the peroneal nerve (Table 6).

The repeated ANOVA was assessed using Scheffe's test and revealed that all domains of SF-BPI, Short-Form McGill Pain Questionnaire (SF-MPQ), and Modified Michigan Neuropathy Screening Instrument (MMNSI) differed significantly from their respective baseline values for the two groups, except for the affective domain of SF-MPQ after 4 and 8 weeks of treatment, as shown in Tables 2–4 ($P < 0.01$). All domains of SF-36 differed significantly from their baseline values in the two groups, except for the domains of vitality, role limitation due to emotional problems, mental health, and mental component scale after 8 weeks of treatment, as shown in Table 5 ($P < 0.01$).

3.3. Safety Issues. There were 39 events of dry mouth, 24 events of constipation, and one event of bitter sensation in the mouth that was judged to be probably related to the treatment, but the magnitudes of these effects were relatively small, and no major adverse event occurred (Table 7).

4. Discussion

In this randomized, double-blind, and placebo-controlled trial, a Chinese medicinal formula, modified *Hungqi Guizhi Wuwu Tang* (MHGWT), was found to be effective and well tolerated in diabetic patients with DPN. The MHGWT regimen reduced the pain and numbness of extremities and improved quality of life during the 12 weeks of treatment. Subjective and objective tests were performed and significant outcomes were detected using well-validated questionnaires and NCS during the study period.

With respect to the safety of MHGWT, no deterioration of hepatic and renal functions or major adverse event was detected throughout the period of treatment. This finding is consistent with our experience of practicing traditional Chinese medicine (TCM), as indicated in Table 1. Since pain is a subjective symptom, three pain questionnaires were utilized in this investigation to evaluate different aspects of the burden that was imposed by DPN. The well-validated SF-36 was also utilized to explore the impact on quality of life. NCSs were used to make an objective measurement. The significant reduction of MMNSI scores indicated that treatment with MHGWT had an effect within four weeks. The declines in the MMNSI scores increased with the period of treatment. The consistent declines in scores in the responses of the patients with DPN to the three pain questionnaires in

this study imply that the efficacy of MHGWT is reliable. This trend was also revealed by the SF-36 scores for quality of life.

In the current study, in addition to investigating various health benefits by subjective questionnaires, we attempted to show whether objective parameters, such as electrophysiological values, changed in response to treatment. Pain is transmitted through small or unmyelinated fibers, but routine NCS is used to investigate large myelinated fibers. Therefore, the absence of a significant improvement in NCS between pre- and posttherapy phases is reasonable. Although we failed to demonstrate objective evidence of improvement in pain in the experimental group, it is important to note the limitations of current techniques for reliable clinical assessment of pain and to note that self-reporting is widely considered to be a more effective measure of pain in clinical practice.

Hungqi Guizhi Wuwu Tang is a classic formula used in traditional Chinese medicine for improving microcirculation. To improve its efficacy, *Spatholobus suberectus* Dunn., caulis and *Pheretima aspergillum* (Perrier) were added. A number of studies have shown evidence of the efficacy of MHGWT in improving neuropathy [15–18]. However, very few clinical trials of high quality have been performed to investigate treatment of DPN with Chinese herbal medicine [19]. Such studies did not employ standardized treatment, well-validated questionnaires, adequate blindness or randomization, or the Good Clinical Practice (GCP) protocol [12]. The use of MHGWT warrants further study as it has fewer side effects and is safer than conventional drugs. Because the risk of developing neuropathic pain increases with age, it may provide an alternative therapy for the elderly patients [20].

The present study has some limitations. First, the success of blinding was not tested. Second, the poor ability of the subjects due to factors that often accompany old age, such as degeneration, use of multiple medications, and chronic illness, may have negatively affected the reliability of the responses to the self-administered questionnaires. Third, the main population in the study was older adults. Therefore, the effect of MHGWT on younger patients with DPN remains to be investigated.

Several studies on the possible mechanisms of ingredients of MHGWT that improve DPN have been conducted. The mechanism of endoneurial hypoxia resulting from arteriovenous shunting with the proliferation of new leaky neural vessels has been reported [21]. Previous studies suggest that the therapeutic mechanism of MHGWT may involve improvement of the circulation supplying peripheral nerves [22]. Inflammation contributes to the development of diabetes; *Astragalus membranaceus* (*Hungqi*) play an important role in lowering blood glucose and controlling inflammation through AMPK activity [23–27]. Recent studies have shown Astragaloside IV to be an aldose-reductase inhibitor and a free-radical scavenger and it exerts protective effects against the progression of peripheral neuropathy in STZ-induced diabetes in rats [28, 29]. Astragalus polysaccharides (APS) administration could also prevent the development of lipotoxicity through a mechanism dependent on the PPARα-mediated regulatory pathways [30]. *Paeonia lactiflora* (*Baishao*) helps to increase the antioxidant capacity of an organism and protect it against lipid peroxidation induced by oxidative stress [31, 32]. As TCM targets the underlying disturbed homeostasis, studies concerning the mechanisms related to the characteristics of TCM syndrome differentiation or body constitution that reflect the inner health status of the body should be conducted too [33].

5. Conclusions

MHGWT appears to be a well-tolerated and effective therapeutic alternative for treating painful sensation in patients, especially older patients, with DPN.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

Chia-I Tsai and Tsai-Chung Li denote equal contribution as cofirst authors.

Disclosure

The study sponsor did not participate in designing or conducting this study and was not involved in the collection, management, analysis, or interpretation of the data, or the preparation, review, or approval of the paper.

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Research Article

Proanthocyanidin Attenuation of Oxidative Stress and NF-κB Protects Apolipoprotein E-Deficient Mice against Diabetic Nephropathy

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Hyperlipidemia and hyperglycemia result in oxidative stress and play a major role in the development of diabetic nephropathy (DN). We explored the effects of proanthocyanidin (PA) on the induction and progression of DN in apolipoprotein E-deficient mice. Diabetes Mellitus was induced in ten-week-old male apoE$^{-/-}$mice using streptozotocin (STZ). Mice were fed with a high-fat diet in presence or absence of PA. PA treatment significantly reduced the high cholesterol levels, restored renal functions, and reduced albuminuria in the PA-treated diabetic mice compared with the diabetic untreated mice. In addition, the glomerular mesangial expansion in the diabetic mice was attenuated as a result of PA supplementation. Moreover, PA treatment restored the elevated levels of MDA and CML and the reduced activity of SOD and GSH in the diabetic mice. Furthermore, PA feeding reduced the activation and translocation of NF-κB to the nucleus compared with the diabetic untreated animals. Reduction of NF-κB activation resulted in the attenuation of the expression of IL-6, TGFβ, and RAGE which protected PA-treated mice against DN. The renoprotective effects of PA were found to be time independent regardless of whether the dietary feeding with PA was started pre-, co-, or post-STZ injection. In conclusion, part of the beneficial effects of PA includes the disruption of the detrimental AGE-RAGE-NFκB pathways.

1. Introduction

Diabetes mellitus (DM) is considered as the leading cause of end-stage renal disease. The relative risk for cardiovascular diseases is 10 folds higher for type-1 diabetic patients with nephropathy compared with those without diabetic nephropathy (DN) [1]. Proteinuria, which was considered an indicator of underlying DN, usually worsens with progression of diabetic kidney disease. This progression is characterized by declining glomerular filtration rate and kidney structural changes, including thickening of the basement membranes, mesangial sclerosis, and arteriolar hyalinosis.

Hyperglycemia is a major cause for the increased glycation of proteins and lipids that, in turn, enhances the generation of reactive oxygen species (ROS) [2–4]. Thus, diabetes mellitus is usually accompanied by an increased ROS production and impaired antioxidant defense mechanisms leading to increased oxidative stress. ROS interact with the free amino and sulfhydryl groups of proteins forming Amadori products which further modified to form advanced glycation endproducts (AGEs) specially carboxymethyl lysine (CML). The formed AGEs bind to their receptors (AGE-receptors) on the cell membrane resulting in the activation of the nuclear factor kaba B (NF-κB). NF-κB is found in the cytoplasm of the normal nonactivated cells binding to its inhibitor (I-κB). As a result of increased AGE production and interaction of AGE with RAGE, NF-κB dissociates from I-κB and migrates to the cell nucleus. Once in the nucleus, it stimulates the transcription of its controlled genes. The genes are inflammatory genes like interleukin-6, IL-1β, RAGE, TNFα, and others. Overexpression of these inflammatory genes plays a key role in the pathogenesis

of diabetic complications especially diabetic nephropathy [5–9].

In diabetic patients with suboptimal glycemic control, hyperlipidemia may develop. In addition, even in subjects with optimal glycemic control, the abnormal lipid profile that is potentially atherogenic may exist. In diabetes mellitus, hyperlipidemia is considered an independent and major determinant of progression of renal disease [10]. In this regard, an association between high consumption of saturated fat and albuminuria in type-1 diabetic patients has been reported [11].

Proanthocyanidin (PA) are polyphenolic compounds, which are derivatives of flavan-3-ol flavonoids. They are mainly composed of dimers, oligomers, and polymers of (+)-catechin, (−) epicatchin, and their gallic acid esters. Their degree of polymerization is generally distributed between 2 and 15. The wide presence of PAs in plants makes them an important part of the human diet [12].

PAs have been reported to show various beneficial properties. In addition to their free radical scavenging and antioxidant activity [13], PAs have been reported to have antibacterial, antiviral, anticancer, anti-inflammatory, anti-allergic, and vasodilator actions [14]. Moreover, PAs were found to attenuate diabetic nephropathy in rats [15] and cisplatin-induced nephrotoxicity [16]. Furthermore, PA was found to inhibit lipid peroxidation, platelet aggregation, capillary permeability, and fragility and to affect enzyme systems including phospholipase A_2, cyclooxygenase, and lipoxygenase [14]. The free radical scavenging activity of PAs has been well documented and commanded the most attention [14]. *In vivo* studies have shown that grape seed PA extract is a better free radical scavenger and inhibitor of oxidative tissue damage than vitamin C, vitamin E succinate, vitamin C and vitamin E succinate combined, and beta carotene [14].

The present study examined the effect of dietary PA supplementation on diabetes-mellitus-induced kidney damage in apoE−/mice, an animal model previously demonstrated to have a series of pathological conditions including dyslipidemia and atherosclerosis. Hyperlipidemia per se is associated with the development of early renal lesions in apoE−/mice [17]. Mice were rendered diabetic by intraperitoneal administration of low doses of STZ over 5 days, an established approach for inducing type-1 diabetes mellitus [18]. Given the previous features, STZ-induced diabetic apoE−/mice fed high-fat diet were used in our studies to accelerate DN and probe the role of dyslipidemia in DN development [19].

2. Materials and Methods

2.1. Experimental Design. All of the animal experiments were carried out under protocols approved by the Institutional Animal House of the University of King Abdulaziz at Jeddah, Saudi Arabia. Male apoE−/mice on C57BL/6J genetic background (14 weeks old) were divided into four groups, both control and diabetic groups, without or with PA supplementation. Diabetes mellitus was induced in the mice by intraperitoneal injections of STZ at a dose of 40 mg/kg bodyweight in citrate buffer, pH 4.5 (Sigma, St. Louis, MO, USA) for 5 days [18]. Animals of the control group received only citrate buffer. If the blood glucose concentration of a mouse is higher than 250 mg/dL 72 hours after the STZ injection, it is considered as a diabetic animal. Mice with blood glucose lower than 250 mg/dL were excluded from the experiment. Mice of all groups were housed in cages and received normal diet and tap water *ad libitum* in a constant environment (room temperature 25 ± 2°C, room humidity 50 ± 5%) with a 12 h light, 12 h dark cycle until being changed to a synthetic high-fat (HF) diet containing 21% (w/w, or 45% calories) fat, 0.05% cholesterol, 20% sucrose (Research Diets, Inc., New Brunswick, NJ, USA) [19] with or without 500 mg/kg PA purchased from Bioline Company, England [15]. Bodyweights and blood glucose levels were measured regularly. At the end of the experiment (12 weeks after induction of diabetes), animals were sacrificed using ether anaesthesia. Blood was collected and centrifuged, and serum was stored at −80°C and used for the assay of cytokines and biochemical and oxidative stress markers. Kidneys were removed and rinsed with cold PBS and then weighed.

Kidneys from each mouse were divided into several parts. The first part was used to prepare the kidney homogenate as discussed later. This homogenate was used for assaying the oxidant/antioxidant parameters as well as CML and IL-6 in the mice kidney. The second part was used for isolation of total RNA and DNA from the kidney cortex. The third part was used for preparation of the nuclear and cytoplasmic extracts for EMSA and Western blot analyses. The fourth part was used for histopathological examination.

2.2. Blood Sampling and Analysis. The diagnostic kits for determinations of levels of glucose, creatinine (Cr), blood urea nitrogen (BUN), sodium, potassium, cholesterol, and triglycerides were purchased from BioSystem (Barcelona, Spain). Serum cytokines and antioxidant markers as well as AGEs were assayed using commercially available kits. All analyses were performed in accordance with the manuals provided by the manufacturer.

2.3. Kidney Homogenate Preparation. Part of the kidney was cut into small pieces and washed by cold phosphate-buffered saline (PBS). Furthermore, it was ground in a homogenization buffer {0.05 M Tris-HCl pH 7.9, 25% glycerol, 0.1 mM EDTA, and 0.32 M $(NH_4)_2SO_4$} containing a protease inhibitor tablet (Roche, Germany). The solution was sonicated in an ice bath for 15 seconds followed by centrifugation at 12000 rpm, 4°C for 5 minutes. The supernatant was aliquoted, stored at −80°C, and assayed for protein concentration using BCA kit (Pierce, Rockford, IL, USA) using albumin diluted in a lysis buffer as standard [20]. The homogenate was used for the determination of reduced glutathione (GSH), level of lipid peroxidation (MDA), concentration of *Nε*-carboxymethyl lysine (CML), activity of superoxide dismutase (SOD), and level of IL-6.

2.4. Determining Enzymatic Activities. The activities of total SOD as well as the concentrations of MDA and GSH in the kidney homogenate were determined using commercially

available kits from BioVision Research Products (Linda Vista Avenue, Pasadena, CA, USA) according to the methods described by Nishikimi et al. [21]; Mekheimer et al., Ohkawa et al. [22, 23]; and Moron et al. [24], respectively.

2.5. Determination of IL-6. IL-6 concentration in the serum and in the kidney homogenate was determined by an ELISA kit. The ELISA for determination of IL-6 was performed using a commercially available kit from R&D (Mannheim, Germany) according to the instructions of the manufacturer.

2.6. Analysis of Urine Parameters. Urine samples were collected by placing the mice in individual metabolic cages for 24 h before diabetes mellitus had been induced and the day before the end of experiment. The urine albumin concentration was determined with an ELISA kit (Nephrat II, Exocell, Philadelphia, PA, USA), and the concentration of Cr in pooled urine samples was determined by the commercial assay kit. All analyses were performed in accordance with the manuals provided by the manufacturers.

2.7. Measurement of Urinary and Renal 8-Hydroxy-2′-deoxyguanosine. Urinary 8-hydroxy-2′-deoxyguanosine (8-OHdG) levels were determined using an ELISA kit from Genox Corporation (Baltimore, MD, USA) according to the method of Matsubasa et al. [25] and were corrected by using individual urine creatinine concentrations. Extraction of the renal DNA was performed using a DNA extraction kit (Promega, Germany) following the manufacturer's protocol. The genomic DNA samples from kidney tissues were also used for the determination of 8-OHdG using the competitive ELISA kit.

2.8. Assay of Serum and Renal CML. The supernatant of the kidney homogenate was tested for CML using the anti-CML mouse autoantibody ELISA kit which employs the semi-quantitative enzyme immunoassay technique. The ELISA kit for CML was provided by Roche Diagnostics (Mannheim, Germany). The absorbance of the resulting yellow product was measured at 450 nm [26–28].

2.9. Histopathological Examination. Renal tissues were collected after animal sacrifice, fixed in 10% formalin, processed routinely, and embedded in paraffin. 5 μm thick sections were prepared and stained with periodic acid-Schiff (PAS). Glomerular histopathological changes and mesangial lesions were scored in terms of the glomerular mesangial expansion (increase in the mesangial matrix). Mesangial matrix expansion (MME) was derived from the estimation of 100 glomeruli of 5 nondiabetic and 5 diabetic mice per dietary group [29].

2.10. Reverse Transcription Polymerase Chain Reaction. Total RNA of mice in different groups was isolated from kidney cortex by using TRIzol reagent (Invitrogen, Darmstadt, Germany) according to the manufacturer's protocol. Gene expressions were determined using real-time quantitative reverse transcription polymerase chain reaction. β-actin was considered as a reference gene. The sequences of the used primers were as follows.

Nephrin sens 5′-ACT ACG CCC TCT TCA AAT GCA-3′, antisens 5′-TCG AGG GCC TCA TAC CTG AT-3′; *TGFβ1* sens 5′-TGG AGC AAC ATG TGG AAC TC-3′, antisens 5′-GTC AGC AGC CGG TTA CCA-3′; *RAGE* sens 5′-CAC AGC CCG GAT TG-3′, antisens 5′-GCT GTA GCT GGT GGT CAG AAC A-3′; *SOD2* sens 5′-ACT GAA GTT CAA TGG TGG GG-3′, antisens 5′-GCT TGA TAG CCT CCA GCA AC-3′; *β-actin* sens 5′-GTG CTA TGT TGC TCT AGA CTT CG-3′, antisens 5′-ATG CCA CAG GAT TCC ATA CC-3′.

2.11. Western Blot Analysis. Cytoplasmic and nuclear fractions of the mice kidneys were separated as described [7]. In brief, cytoplasmic and nuclear extracts were separated by adding 200 μL buffer A (10 mM HEPES-KOH, pH 7.9 at 4°C, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, and 0.2 mM PMSF) to the kidney, mixed well for 30 seconds, incubated on ice for 15 minutes, mixed again, and centrifuged at 13000 rpm. for 5 minutes. The supernatant contained the cytoplasmic extract and was transferred to a fresh tube and kept at −80°C. To the pellet, 50 μL buffer C (20 mM HEPES-KOH, pH 7.9 at 4°C, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, and 0.2 mM EDTA) was added, vortexed for 30 seconds, incubated in ice for 40 minutes and vortexed every 10 minutes, and centrifuged at 13000 rpm for 5 minutes. The supernatant contains the nuclear extract and was transferred to a fresh tube and kept at −80°C.

Western blot was performed as described. Proteins were separated on a polyacrylamide gel and transferred to a nitrocellulose membrane (Amersham Pharmacia, Braunschweig, Germany). Membranes were blocked with nonfat milk, incubated with primary antibodies directed against NF-κB-p65 overnight at 4°C. After washing three times with PBS, the secondary antibody (horseradish peroxidase-coupled rabbit IgG) was added, and incubation was continued for 60 minutes. After washing 5 times, the immunoreactive proteins were detected with the ECL-Western blot system (Amersham Pharmacia, Braunschweig, Germany) and subsequent autoradiography for 2 minutes. All experiments were performed three times [7, 9].

2.12. Statistical Analysis. All of the biochemical assays were carried out in duplicates. Statistical analysis was performed with JMP software (SAS Institute, Cary, NC, USA). Diabetes mellitus effect, PA effect, and their interaction were analyzed using the two-way ANOVA, and Tukey-Kramer HSD test was applied for post hoc pairwise comparisons. Time effect was analyzed by MANOVA for repeated measurement.

3. Results

Results obtained showed thatthe diabetic untreated mice suffer from loss of bodyweight, but the diabetic mice treated with PA were able to keep their bodyweights ($P < 0.01$; Table 1). Moreover, PA treatment markedly slowed the gain of weight induced by HF diet in the nondiabetic mice, although

Table 1: Effects of STZ treatment and dietary PA feeding on the food intake, bodyweight, albuminuria, biochemical parameters, serum AGE, TNFα, IL-6, GSH, and 8-hydroxy-2′-deoxyguanosine of apolipoprotein E-deficient mice.

	Control	Control + PA	Diabetic	Diabetic + PA
Initial bodyweight, g	195.13 ± 9.5	196.33 ± 6.5	196.44 ± 7	197 ± 7.2
Final bodyweight, g	265.5 ± 8.2	234 ± 12.3^{a,b}	161.8 ± 7.2^{a}	215.52 ± 8.9^{a,b}
Glucose, mg/dL	92.78 ± 0.45	96.21 ± 0.57^{a,b}	265.35 ± 1.45^{a}	106.71 ± 1.23^{b}
HbA1c, %	5.24 ± 0.41	6.88 ± 0.52^{b}	9.42 ± 0.34^{a}	6.85 ± 0.38^{b}
Albumin, g/L	38.4 ± 5.7	38.9 ± 8.1	38.2 ± 7.7	38.7 ± 7.5
Total protein, g/L	76 ± 7	69.91 ± 7.5^{b}	62 ± 6.5^{a}	65.6 ± 7.1
GSH, mmol/L	0.281 ± 0.015	0.172 ± 0.02^{a,b}	0.105 ± 0.03^{a}	0.145 ± 0.021^{a,b}
Catalase, U/g Hb	94.54 ± 14	86.55 ± 12^{b}	50.98 ± 15^{a}	70.11 ± 11^{a,b}
Glutathione reductase, U/gHb	4.25 ± 0.09	3.21 ± 0.94^{b}	2.25 ± 0.62^{a}	2.54 ± 0.54^{a,b}
Glutathione peroxidase, U/gHb	57.1 ± 11	69.9 ± 9.55^{b}	145.5 ± 45^{a}	89.54 ± 10.2^{b}

Data shown represent mean ± SE of mice, 12 weeks after the treatment with STZ or with the buffer (control) in presence or absence of PA. ^{a,b}Significance difference between groups.

Table 2: Oxidant/antioxidant parameters as well as concentration of carboxymethyl lysine (CML) in rat retinas.

	Control	Control + PA	Diabetic	Diabetic + PA
MDA, nmol/mg protein	2.45 ± 0.16	2.95 ± 0.15^{b}	4.75 ± 0.17^{a}	2.95 ± 0.15^{b}
GST, nmol substrat·mg protein^{-1}·min^{-1}	176 ± 31	151 ± 9^{a,b}	82 ± 13^{a}	151 ± 9^{a,b}
GSH-Px, nmol substrat·mg protein^{-1}·min^{-1}	0.92 ± 0.17	0.82 ± .165^{b}	0.34 ± 0.09^{a}	0.82 ± .165^{b}
Catalase, IU·mg protein^{-1}	2.61 ± 0.032	1.45 ± 0.21^{a,b}	0.52 ± 0.03^{a}	1.45 ± 0.21^{a,b}
SOD, nmol substrat·mg protein^{-1}·min^{-1}	3.53 ± 0.45	2.95 ± 0.75^{b}	1.82 ± 0.35^{a}	2.95 ± 0.75^{b}
GSH, nmol/mg protein	16 ± 3	16 ± 2.3	16.11 ± 2	16 ± 2.3
CML, pg/mg protein	3.54 ± 0.22	3.2 ± 0.6^{b}	8.81 ± 0.34^{a}	3.2 ± 0.6^{b}

Oxidative stress markers in the kidney homogenate of apoE−/mice. Mice treated either with or without STZ in presence or absence of PA. PA treatment was continued for 12 weeks after the onset of diabetes. Data shown represent mean ± SEM. ^{a,b}Significance difference between groups.

the two groups received the daily dietary intake with or without PA (Table 1).

Although both bodyweight loss and hyperglycemia were reduced in mice within the pre-STZ group, the obtained data from mice of the three dietary groups were not markedly affected by the time of PA supplementation.

3.1. Markers of Oxidative Stress. Diabetes mellitus results in a significant increase of oxidative stress markers, as reflected by the reduction in serum and renal cortex GSH and increase of MDA and AGEs. Dietary PA administration ameliorated all of these changes (Tables 1 and 2). A similar effect on the urinary 8-isoprostane was obtained. The urinary 8-isoprostane was significantly increased as a result of diabetes, while dietary PA administration attenuated these elevated levels (Table 1). Total SOD activity in kidney homogenate was decreased as a result of diabetes mellitus. Treatment with dietary PA partially restored the reduced SOD activity (Table 2).

3.2. Activation of NF-κB in the Mice Kidney. As a result of oxidative stress and formation of AGE in the diabetic mice, we examined the activation of NF-κB in the mice kidneys using both EMSA and Western blot analysis. Data obtained from EMSA showed a marked increase in the activity of

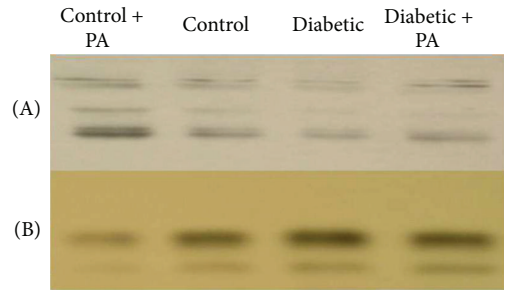


Figure 1: NF-κB activation in the rat kidney: Western blot of rat kidneys of control rats, control rats + PA, diabetic rats, and diabetic rats treated with PA. Cytoplasmic (A) and nuclear (B) extracts were obtained as described in the Materials and Methods section.

NF-κB-p65 compared with the control group. Treatment of diabetic mice with PA resulted in a significant reduction of NF-κB-p65 activity as shown in Figure 1. Using Western blot analysis, the translocation of NF-κB-p65 from the cytoplasm into the nucleus was observed. Feeding of the diabetic mice with PA normalizes these disorders.

3.3. Plasma Cytokines. Previous data [5, 7, 9] showed that activation of NF-κB results in the overexpression of cytokines like IL-6, TNFα, TGFβ1, and RAGE. In order to examine

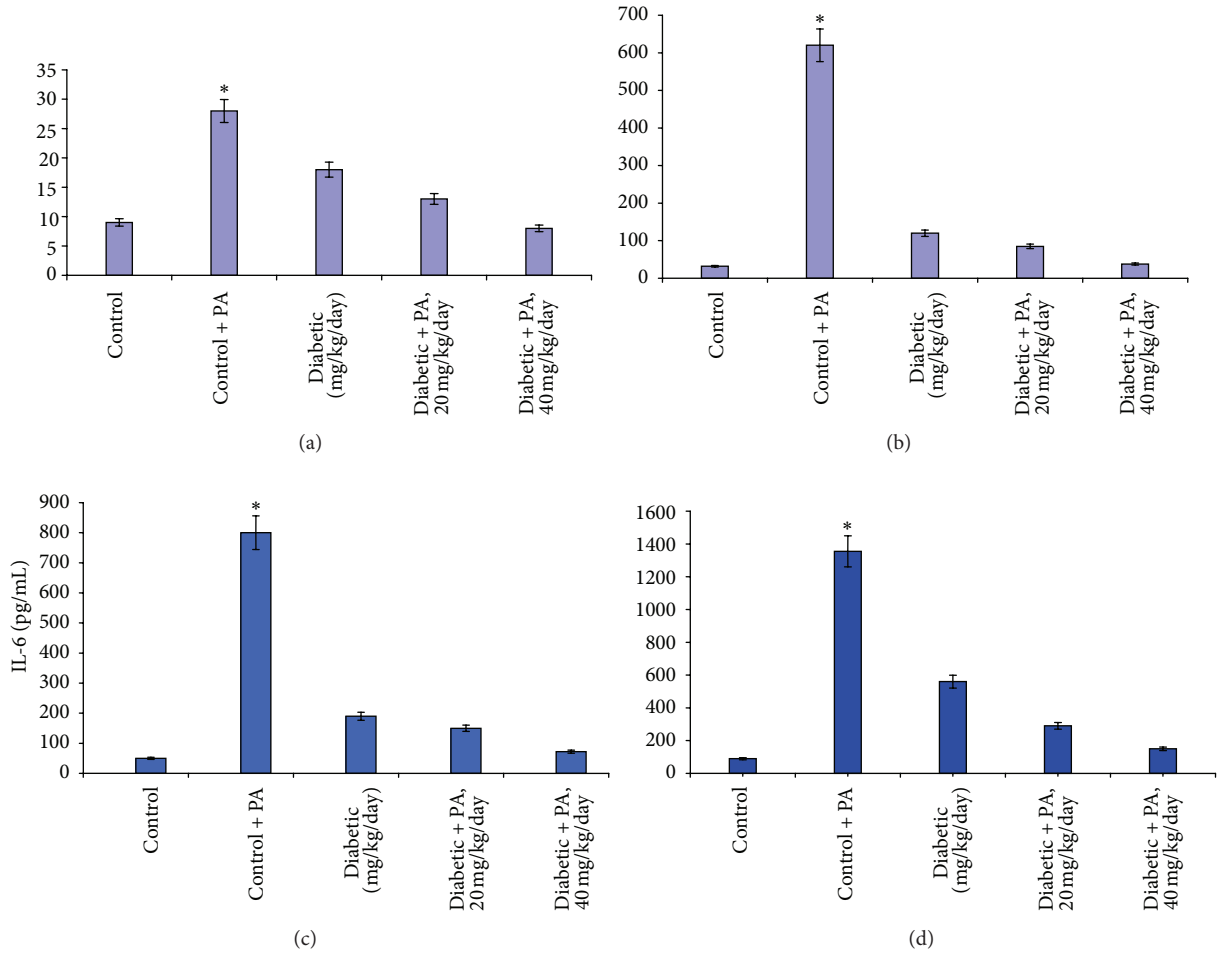


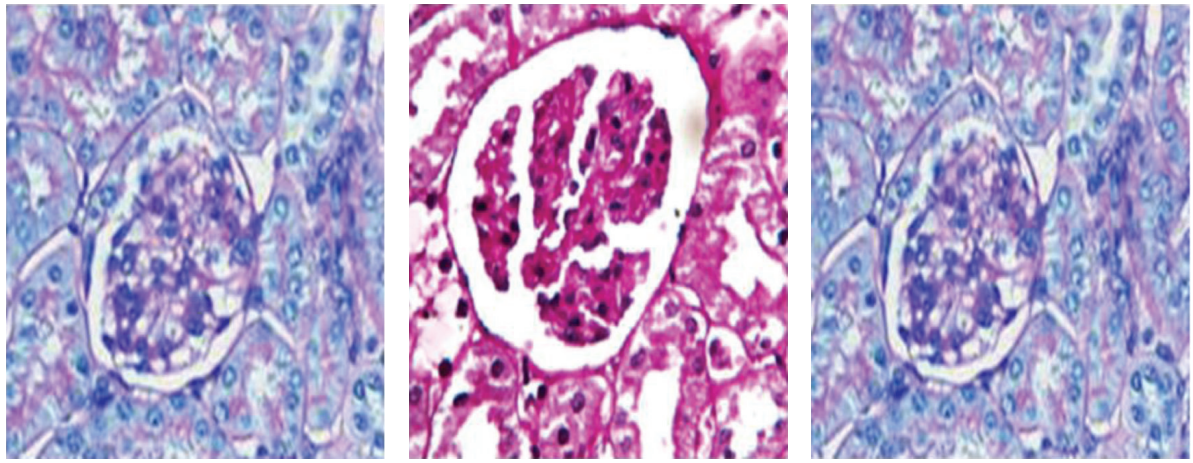
Figure 2: Nephrin mRNA expression (a), TGFβ1 (b), RAGE (c) and SOD2 (d) expressions were measured using ELISA kit. Kidneys and sera were obtained from all groups treated with saline and PA and mice pretreated with different concentrations of proanthocyanidin. Mean values ±SD are shown. The asterisk (*) values are different ($P < 0.05$) as compared with control group.

this hypothesis, we assayed the levels of IL-6 and TNFα in the mice serum. The levels of IL-6 in the diabetic mice were significantly increased compared with the control mice. Dietary feeding with PA significantly reduced the increase of IL-6 compared with the diabetic untreated mice. In contrast, there were no significant differences in the levels of TNFα in the diabetic and nondiabetic mice (Table 1).

3.4. Expression of Some Genes in the Kidney. Activation of NF-κB results in the overexpression of the inflammatory genes as previously described [7, 9]. The expression of nephrin and SOD2 genes in the kidney cortex of the diabetic untreated mice was downregulated. PA supplementation resulted in the upregulation of the expression of the two genes (Figures 2(a) and 2(d)). In contrast, the expression of the AGE receptor (RAGE) and the transforming growth factor β1 (TGFβ1) in the kidney cortex was upregulated due to STZ treatment and diabetes mellitus. Treatment of the diabetic mice with PA restored their expression near the control group as shown in Figures 2(b) and 2(c).

3.5. Renal Damage. All of the diabetic mice suffered from polyuria. PA treatment improved to some extent this disorder, although the effect was not statistically significant (Table 1). Kidney weight over body weight in the diabetic untreated mice was 40% higher than that of nondiabetic mice ($P < 0.05$); this resulted from the reduced body weight of the diabetic mice. As a result of dietary administration of PA, the reduction in the KW/BW ratio was reduced (Table 1). As a result of diabetes mellitus and the earlier forms of the diabetic kidney disease, the daily albuminuria excretion was clearly elevated in diabetic mice. PA supplementation significantly attenuated albuminuria (Table 1).

In the diabetic apoE−/mice, the kidneys appeared to be unaltered. Examinations with light microscope revealed mesangium hypercellularity, no dular or diffuse MME, and rare foci of mesangiolysis in diabetic mice without PA treatment. On the other hand, diabetic mice treated with PA had significantly reduced MME compared with the diabetic untreated mice (Figure 3). There were no significant morphological changes observed in the glomeruli of the control mice either with or without dietary PA supplementation.



(a) (b) (c)

Figure 3: Mesangial expansion and ultrastructural changes in diabetic apoE−/mice after 12 weeks after induction of diabetes mellitus. (a) Nondiabetic mice without PA. (b) Diabetic mice without PA. (c) Diabetic mice with PA. PAS staining, original magnification ×200.

4. Discussion

Our current findings are in line with the previous observations that PA treatment attenuated both hyperglycemia in diabetes mellitus and DN [15]. We also report the novel observation that, regardless of the time of PA treatment (i.e., 1 month before, at the same time, or 1 month after induction of diabetes mellitus), PA supplementation significantly reduced hyperglycemia, especially in the pre-STZ group where hyperglycemia was confirmed before PA treatment. Our observations of increased inflammatory cytokine suggested that inflammation played an important role in the development and progression of DN. The obtained data strongly indicated a therapeutic effect of PA on diabetes mellitus.

The effects of dietary PA supplementation on hyperglycemia and hyperglycemia-induced nephropathy are not fully understood [12, 14, 30]. The hypoglycemic effect of PA could be attributed to different mechanisms that include increasing of insulin sensitivity in type-2 diabetic patients [31] and/or direct binding site at the tyrosine kinase domain of the insulin receptor [32].

The obtained data indicated the increase of oxidative stress in the diabetic mice. The oxidative stress markers like the elevated urinary 8-isoprostane, which is considered a reliable parameter of *in vivo* lipid peroxidation [33], increased kidney MDA, and the reduced GSH are significantly attenuated as a result of PA treatment. The obtained data are in line with other previous data [34]. The mitochondria are considered the major organelles which produce superoxide radicals. As result of the absence of both of the DNA repair systems and histones, the mitochondria are a primary target for oxidative damage [35]. Accordingly, superoxide released from mitochondria could be proposed as a mechanism for the development of diabetic complications [36]. Our data showed that the expression of SOD and its total activity in the kidney of diabetic mice were markedly reduced, whereas PA treatment restored SOD activity. Accordingly, the PA protection of the kidney from damage induced by the production of superoxide radicals occurs via strengthening of the antioxidant defense mechanisms of apolipoprotein E-deficient mice such as SOD.

The role of dietary PA supplementation in the attenuation of AGEs formation *in vivo* is not fully understood. Our hypothesis is that PA inhibits AGEs production in two ways. PA improved glucose metabolism and reduced methylglyoxal formation. Methylglyoxal is a dicarbonyl that is generated in glycolysis from glyceraldehyde-3-phosphate. Methylglyoxal plays a key role in the AGEs formation [37]. Treatment with PA resulted in increasing the uptake of glyceraldehyde-3-phosphate in the Krebs cycle and enhanced the generation of ATP [38]. This resulted in decreasing the amount of glyceraldehyde-3-phosphate available for methylglyoxal formation.

It has been reported that AGEs trigger the activation of NF-κB by interaction with the receptor of AGE (RAGE), leading to the translocation of NF-κB to the nucleus where it induces transcription of its controlled inflammatory genes like IL-6, RAGE, TGFβ, and so forth. Therefore, AGEs accumulation in the kidney has been regarded as an index of progressive renal damage in DN. RAGE is an important receptor for mediating AGE effects. Binding of AGEs such as CML to RAGE enhances oxidative stress in renal tissues. RAGE signals via NF-κB activate target genes which have harmful potentials for the diabetic kidney. The higher levels of AGEs and IL-6, the overexpression of TGFβ and RAGE, and the marked NF-κB-p65 activation in the diabetic untreated animals showed a higher degree of oxidative stress and inflammation in the diabetic untreated animals. These elevated levels of AGEs, IL-6, RAGE, TGFβ, and the significant NF-κB-p65 activation were reduced as a result of PA administration. Thus, our data suggest that part of the beneficial effect of PA includes the disruption of the detrimental AGE-RAGE-NFκB pathways.

PA feeding can improve the general health of the diabetic animals, including prevention of severe weight loss, suggesting that glucose metabolism is responsible for this

reduction. PA is a naturally occurring antioxidant and could play an important role in the activity of several mitochondrial enzymes that are involved in the oxidation of glucose and ATP production. Therefore, the beneficial effects of PA on diabetes mellitus and DN could be attributed to the combined anti-inflammatory/antioxidant effects, the metabolic regulations that include increasing of the oxidation of glucose, and the attenuation of NF-κB activation rather than its antioxidative effects alone. The obtained data were in line with those of previous studies [5, 7, 9, 15, 28, 30, 31, 37, 38].

5. Conclusion

The present study demonstrated that dietary supplementation of PA significantly improved hyperglycemia and renal function in apolipoprotein E-deficient mice. In addition, it attenuated NF-κB activation and inflammation and improved kidney antioxidant status. PA is widely distributed in human diets, implying a lack of toxicity. PA, therefore, is a useful dietary adjunct for the treatment of diabetes mellitus and its complications. It is also considered as an important source required for the discovery of new active antidiabetic agent(s). Further preclinical experiments into the utility of PA supplementation are needed.

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Research Article

Hyperglycemia Induces Altered Expressions of Angiogenesis Associated Molecules in the Trophoblast

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We previously reported that the increased level of perlecan with altered glycosaminoglycan (GAG) substitution was present in the placenta with gestational diabetes mellitus (GDM) and in the trophoblasts cultured under hyperglycemic condition. Trophoblast is the first cell lineage to differentiate, invasive, and migrate into the vessel tissues of placenta and fetal membrane during pregnancy. Therefore, active matrix remodeling and vessel formation must occur during placentation. In this study, we further investigated whether hyperglycemia-induced alterations of perlecan in the extracellular matrix (ECM) affect the proliferation and the expressions of angiogenesis-related growth factors and cytokines in the trophoblasts. 3A-Sub-E trophoblastic cells cultured in high glucose medium were conducted to mimic the hyperglycemic condition. Results showed that the hyperglycemia-induced GAG alterations in the cell surface perlecan as well as in the ECM indeed upregulated the expressions of IL-6, IL-8, and MCP-1 and the activities of MMP-2 and MMP-9 and downregulated the expressions of TIMP-2. A regulatory molecular mechanism of hyperglycemia-induced alterations of the cell surface proteoglycans and the ECM remodeling on the expressions of angiogenesis-related cytokines and growth factors in trophoblasts was proposed. This mechanism may contribute to the aberrant placental structure and the maternal and fetal complications during development.

1. Introduction

Placental development is important for fetal health. Maternal diabetes or gestational diabetes mellitus (GDM) induced hyperglycemia could cause placental development abnormality that might result in maternal complications and poor fetal outcomes [1, 2]. Perlecan, a heparin sulfate proteoglycan, is a major component of basement membrane and is involved in blood vessel formation by regulation of cell proliferation, growth factors, and cytokines in the extracellular matrix [3–5]. In addition, perlecan can bind proangiogenic growth factors such as fibroblast growth factors (FGFs) and vascular endothelial growth factor (VEGF) and present them to their receptors on the cell surface [3, 4]. During embryonic development, perlecan is located in the apical surface of trophectoderm functioning in the initial blastocyst-uterine epithelium interaction for embryo preimplantation [6]. It appears that the trophoblast involved embryo implantation is mediated by heparin or heparin sulfate binding protein on uterine epithelium [7–9]. We previously have shown that perlecan is mainly expressed in the trophoblast and vessel basement membranes, and both the protein and mRNA levels of placental perlecan were significantly increased in the third trimester placentas with gestational diabetes mellitus (GDM) as well as in trophoblast cells cultured at high glucose (30 mM) condition [10]. We have also demonstrated that induced hyperglycemic condition increased chondroitin sulfate substitution on placental perlecan and in the cultured

trophoblasts [11], suggesting that induced hyperglycemia altered perlecan expression may contribute to the abnormality of placental development and the maternal and fetal complications.

Trophoblast is the first cell lineage to differentiate, invasive, and migrate into the vessel tissues of placenta and fetal membrane during pregnancy [12]. Growth factors, cytokines, and angiogenic molecules were found to regulate trophoblast motility [13]. In this study, the effect of hyperglycemia on growth factors, cytokines and angiogenic molecules that may regulate trophoblast migration was studied. In addition, whether any of the induced hyperglycemia altered expressions of cytokines and angiogenic molecules were mediated by the altered perlecan expression was also investigated.

2. Materials and Methods

2.1. Cell Culture. The trophoblast cell line 3A-Sub-E (ATCC CRL-1584) was cultured in MEM (Gibco), containing 10% FBS (Gibco), 100 unit/mL penicillin, and 100 μg/mL streptomycin (Gibco). For the hyperglycemia mimicking condition cell culture, the cells were cultured in the MEM medium with 1% FBS supplemented with 5.6 mmol/L D-glucose (the normal glycemic control), 30 mmol/L D-glucose (Merck) (the hyperglycemic group), or with 5.6 mmol/L D-glucose and 24.4 mmol/L mannitol (Sigma) (the osmotic matched hyperglycemic control). Trypan blue exclusion test was carried out to determine the number of viable cells.

2.2. Isolation of Proteoglycans in 3A-Sub-E Cells. The protocol for isolation of proteoglycans from cells is followed based on the report by Fischer et al. [14] with some modifications. 3A-Sub-E cells cultured in hyperglycemic, normal glycemic, and the osmotic control medium were harvested for the isolation of proteoglycans on 24 h, 48 h, and 72 h posttreatment. The conditioned medium was discarded, and the cells were washed with PBS. Subsequently, 4 M guanidine HCl in 50 mM sodium acetate, pH 5.8 with 0.1% CHAPS, and protease inhibitor cocktail for mammalian cells in appropriate amount (Sigma) were added into the cells and incubated on an orbital shaker at 4°C for overnight. The cell debris was scraped, and the extract solution was collected and dialyzed to 7 M Urea in 50 mM Tris, pH 6.8 with 150 mM NaCl, and protease inhibitor cocktail at 4°C for overnight. The supernatant containing proteoglycans was obtained by centrifugation at 12,000 rpm for 20 min and stored at −20°C for further analysis.

2.3. Immunoprecipitation of Perlecan. To reduce the nonspecific protein contamination, a two-cycle immunoprecipitation procedure was carried out for the study [15]. The cell extract (250–370 μg) was preincubated with the protein G resin (Sigma), 50% in 10 mM Tris, pH 8.0 (100 μL for the cell extract for each reaction) at 4°C for 1 h to remove any nonspecific protein binding to the protein G. Followed by centrifugation at 800 g for 20 s, the precleared protein supernatant was transferred to a clean tube for another immunoprecipitation by the addition of the monoclonal antibody against human perlecan, clone A7L6 (Chemicon), 0.5 μL together with new 50% protein G resin slurry in 10 mM Tris, pH 8.0 for end-over-end incubation at 4°C for overnight. The sample was then centrifuged at 800 g for 20 sec, and the supernatant was removed. Subsequently, the pellet was washed with 1 mL ice cold 10 mM Tris, pH 8. After three washes, the absorbed perlecan was eluted by the addition of SDS containing sample buffer and boiled for 5 min before subjected to SDS-6% polyacrylamide gel electrophoresis (PAGE) for analysis.

2.4. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis of Proteoglycans. The immunoprecipitated perlecan was analyzed by electrophoresis on a SDS-6% polyacrylamide gel. After fixation with 40% ethanol and 10% acetic acid, the gel was stained with alcian blue solution (0.5% alcian blue in 3% acetic acid) and Coomassie blue G25 for the observations of glycan chains and the core protein, respectively.

2.5. Antibody Arrays Analysis for Angiogenesis Associated Molecules. Both cell lysates and the conditioned media were collected from 3A-Sub-E cells cultured under hyperglycemic, the osmotic matched high glucose control, and normal glycemic conditions. Human angiogenesis array (RayBiotech) consisted of 20 different angiogenesis-related antibodies spotted in duplicates onto a membrane was used for the analysis. The membranes were incubated with 1X Blocking Buffer (10% bovine serum albumin in Tris-buffered saline) at room temperature for 1 h. One milliliter of conditioned medium or cell lysates was added to each membrane in separate wells of a 6-well plate. The membranes were then shaken at 110 rpm at room temperature for 2 h. Followed by the washes with Wash Buffer I and II in another new 6-well plate, 1 mL of a 1 : 250 dilution of the biotin-conjugated antibodies was added to each membrane, respectively, and the mixture was incubated on a shaker for 2 h at room temperature. Following by the washes, the membranes were incubated with horseradish peroxidase (HRP-) conjugated streptavidin (1 : 1,000 dilution) for 1 h at room temperature. After a thorough wash, the membranes were exposed to a peroxidase substrate for 5 min in the dark before imaging. Two or four individual membranes were placed side by side in a plastic protective folder and sealed. Imaging was done using an imaging system. Exposure times ranged from 3 min to overnight. All target signals from antibody array were quantified by Scion Image software. Horseradish peroxidase (HRP-) conjugated antibody at 6 spots served as a positive control and was also used to identify the membrane orientation. For each spot, the net density of gray level was determined by subtracting the background from the total raw density of gray levels. The experiment was repeated to confirm the reproducibility.

2.6. GAG Degradation Treatment. To release heparin/hepa rin sulfate, appropriate amount of heparinase III (HIII) from *Flavobacterium heparinum* (Sigma) in 10 mM Tris (pH 8.0) containing 0.1 mg/mL BSA and 4 mM $CaCl_2$ was added at 25°C for 3 h. For chondroitin sulfate degradation, chondroitinase ABC (Chabc) from *Proteus vulgaris* (Sigma) in 10 mM

Table 1: Primer pairs for real-time quantitative polymerase chain reaction.

Target gene	Primer sequences
IL-6	F-AAATGCCAGCCTGCTGACGAAC
	R-AACAACAATCTGAGGTGCCCATGCTAC
IL-8	F-AACTTCTCCACAACCCTCTG
	R-TTGGCAGCCTTCCTGATTTC
MCP-1 (CCL-2)	F-CAGCCAGATGCAATCAATGCC
	R-TGGAATCCTGAACCCACTTCT
RANTES (CCL-5)	F-CCTCATTGCTACTGCCCTC
	R-CACTGGTGTAGAAATACTCC
TIMP-2	F-GCACATCACCCTCTGTGA
	R-CTCTGTGACCCAGTCCATCC
bFGF	F-GGCGTGTACATGTGGTCTCAGA
	R-TTATGGCTCACTGCAACCTTGA
b-actin	F-CATGTACGTTGCTATCCAGGC
	R-CTCCTTAATGTCACGCACGAT

Tris (pH 8.0), 60 mM sodium acetate, and 0.02% BSA was used for the incubation at 37°C for 1 h. For degradation of both heparin/heparin sulfate and chondroitin sulfate, the samples were incubated with heparanase III prior to chondroitinase ABC.

2.7. Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR) Analysis. Total RNA was extracted using TRIzol reagent (Ambion Life Technologies). One microgram of total RNA was used to perform reversed transcriptase-polymerase chain reaction (RT-PCR) using QuantiTect Reverse Transcription kit (Qiagen). 100 ng of reverse-transcribed cDNA per sample with desired primers for the targeted gene (Table 1) was used to perform real-time PCR using a Rotor-Gene Q (Qiagen). The quantitation was performed as absolute number of DNA copies per sample using QuantiFast SYBR Green PCR Kit (Qiagen) and its software (Rotor-Gene Q Series Softwares version 2.1.0). The amount of transcripts was normalized to that of β-actin. The results are presented as relative values as ratio of the number of copies for the targeted gene and β-actin. Each gene was analyzed in duplicates. Three independent experiments were carried out for data validation.

2.8. Zymography Analysis for MMP Activity. Equal amount of the total protein in the cultured conditional medium from 3A-Sub-E cells with high glucose (30 mM) and normal glucose (5.8 mM) followed by the treatment with the indicated GAG degradation enzyme was collected, respectively, and separated on a 10% gelatin zymography gel (Life Science). The enzyme activity was performed based upon the instruction by the manufacture. The gel image was developed by ChemiDoc imager (BioRad).

3. Results and Discussions

3.1. Hyperglycemic Condition Has No Effect on the Cell Proliferation of Trophoblast 3A-Sub-E Cells in Short-Term Culture. Hyperglycemia may affect the cell proliferation. The number of viable trophoblast 3A-Sub-E cells cultured under hyperglycemic (30 mmol/L D-glucose), normal glycemic control (5.6 mmol/L D-glucose), or the osmotic control (5.6 mmol/L D-glucose with 24.4 mmol/L mannitol) conditions were followed for 3 days. No significant difference on the proliferation of the cells cultured under hyperglycemic, normal, or the osmotic control for 48 h (Figure 1). This finding was similar to a first trimester trophoblast-derived cell line, ACH-3P under hyperglycemic treatment (25 mmol/L D(+)-glucose); there was no effect on the cell proliferation up to 3 days of the culture [16]. However, hyperglycemic condition might impair the proliferation of endothelial cells for short term culture (1–3 days) [17] and enhanced the proliferation for long-term culture [18]. In addition, glucose also stimulated smooth muscle cell proliferation [19]. These results suggest that hyperglycemic condition has less effect on trophoblast proliferation. Glucose consumption may vary depending on the cell type and the cultured conditions [20].

3.2. The Effect of Hyperglycemia on the Expression of Cell-Associated Perlecan in Trophoblast 3A-Sub-E Cells. Our previous studies reported that the expression of perlecan was increased in the third trimester placenta with gestational diabetes mellitus (GDM), and histology studies revealed that perlecan was mainly expressed around trophoblasts; in addition, the GDM placental perlecan had increased chondroitin sulfate content on its GAG chains [10]. Since perlecan plays important roles on cytotrophoblast and uterin cell interface interaction, in this study, we further investigated the cell-association activity of perlecan in trophoblasts upon treatment with high-glucose condition. A time course study for monitoring the cell associated perlecan by immunoprecipitation was conducted in 3A-Sub-E cell lysates. Figure 2 showed that the cell-associated perlecan was gradually decreased upon treatment with high-glucose from 24 h to 72 h whereas the cell-associated perlecan was gradually increased in normal glycemic control cells (Figure 3). It suggested that hyperglycemic condition induced more perlecan deposited

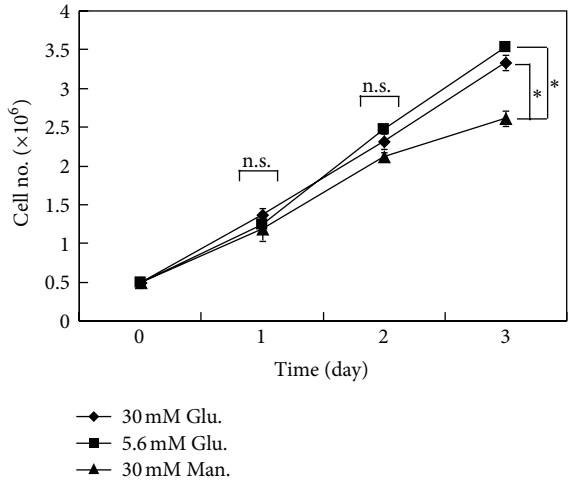

Figure 1: The effect of hyperglycemic condition on the proliferation of trophoblasts. Trypan blue exclusion test was carried out to determine the number of viable cells in the suspension harvested from 30 mmol/L D-glucose (30 mM Glu., the hyperglycemic group), 5.6 mmol/L D-glucose (5.6 mM Glu., the normal glycemic control), or 5.6 mmol/L D-glucose with 24.4 mmol/L mannitol (30 mM Man., the osmotic control) culture for 24 h, 48 h, and 72 h (1–3 days). Data are presented as mean ± SD ($n = 3$). $^{*}P < 0.05$. n.s., not significant.

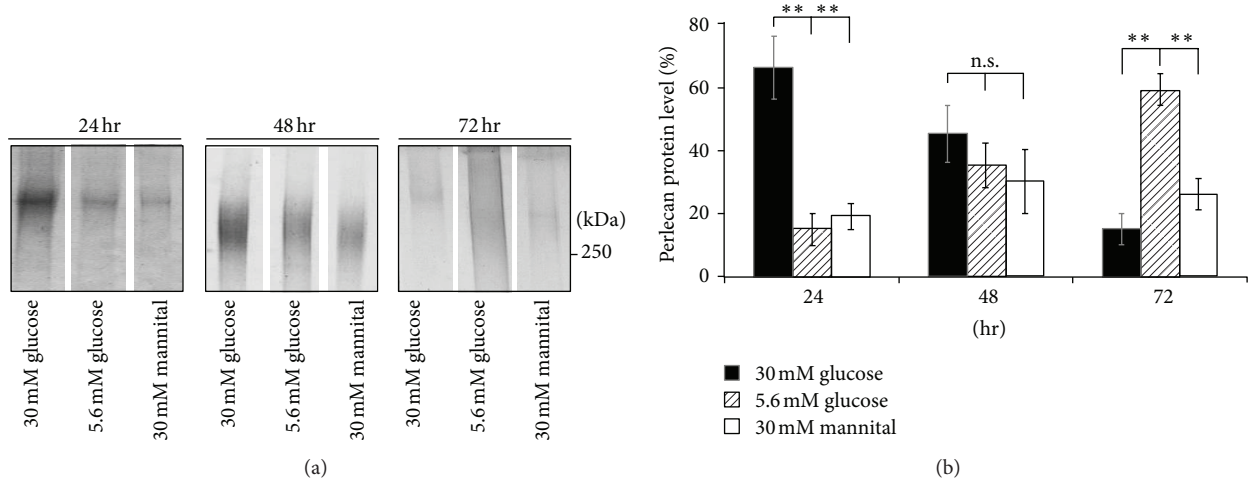

Figure 2: The expression of cell-associated perlecan in trophoblasts upon treatment with high-glucose condition. To characterize perlecan expression profile upon treatment with high-glucose condition, time course analysis of perlecan immunoprecipitation was conducted in 3A-Sub-E cell lysates with pretreatment of 30 mM D-glucose for 24, 48, and 72 h. (a) Perlecan expression was electrophoresis and shown on a 6% SDS polyacrylamide gel stained with alcian blue and Coomassie blue G25 solutions. (b) Quantitative results of perlecan protein from cell lysates under different stimulations. The expression of perlecan was performed as mean ± SD by quantification of the relative intensity of the scanned smear bands from double stains with Coomassie blue and alcian blue ($n = 3$). The total intensity (30 mM Glu. + 5.6 mM Glu. + 30 mM Man.) from each time point was considered as 100. $^{**}P < 0.01$. n.s., not significant.

on the cell surface and osmotic effect might contribute at least in part on the increased density of perlecan present on the cell surface.

3.3. The Effect of Hyperglycemia on the Expression of Angiogenesis Associated Proinflammatory Cytokines, C-C Motif Chemokines, and Matrix Metalloproteinase Inhibitors. Subsequently, we used high glucose treatment between 24 h and 48 h in 3A-Sub-E cells to study the effect of hyperglycemia on perlecan, cytokines, and the angiogenesis associated molecule expressions. Antibody array analysis showed that the proinflammatory cytokine, growth-regulated oncogene (GRO), interleukin-8 (IL-8), and interleukin-6 (IL-6) were expressed in 3A-Sub-E cells (Figure 4(a)). Hyperglycemic condition did not affect the expression of IL6 whereas significant expression of IL-8 was present in the cell lysates at high glucose condition (Figure 4(b)). IL-8 is one of the key inflammatory cytokines during wound healing process, and it is a potent autocrine proangiogenic molecule [21]. IL-8 is chronically upregulated in diabetes but its expression is impaired in the acute inflammatory phase following injury in diabetic patients [22]. Another study reported that hyperglycemic condition did not affect IL-8 expression in dermal microvascular endothelial cells [17].

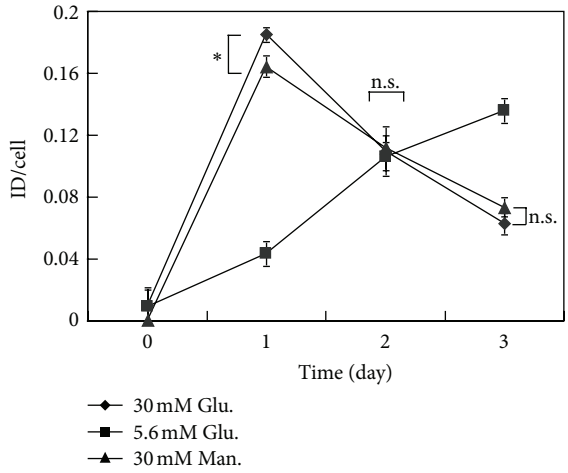

Figure 3: Altered cell-associated perlecan expression upon stimulation with high glucose and high osmotic condition in proliferated trophoblasts. The cell-associated perlecan expression according to the proliferation of 3A-Sub-E cells under high and normal glucose treatment was analyzed. It appeared that the cell-associated perlecan expression is increased 24 h posttreatment with either hyperglycemic condition (30 mM Glu.) or the osmotic matched control (30 mM Man.) and gradually decreased 24–72 h posttreatment. The hyperglycemia mimicking condition (30 mM Glu.) indeed upregulated the level of perlecan expression on 24 h posttreatment compared to that in the osmotic control (30 mM Man.). The cell-associated perlecan expression under normal glycemic condition (5.6 mM Glu.) was gradually increased as the cell proliferated. Data are presented as mean ± SD ($n = 3$). $^{*}P < 0.05$. $^{**}P < 0.01$. n.s., not significant.

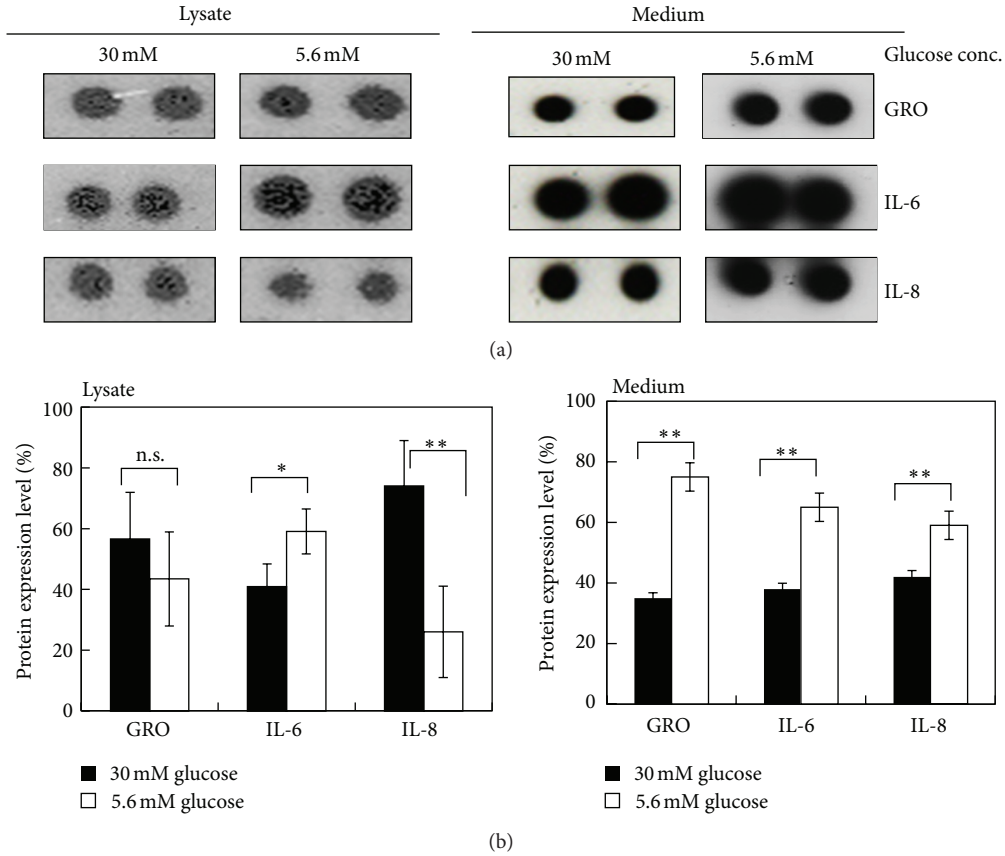

Figure 4: The effect of hyperglycemia on the expression of proinflammatory cytokines in 3A-Sub-E trophoblast cells. 3A-Sub-E cells were exposed to 30 mM D-glucose or to 5.6 mM of D-glucose (control) for 48 h. Cell lysates (250–370 μg) and the cultured conditioned media were collected for the antibody array analysis, respectively. (a) Representative dot image of the indicated proinflammatory cytokines. (b) Average intensity for each pair of cytokine spots was quantitatively measured by Scion Image software. Data are presented as mean ± SD ($n = 4$). Each cytokine is represented by duplicate spots as GRO (growth-regulated oncogene), IL-6 (interleukin-6), IL-8 (interleukin-8), and control (HRP-conjugated antibody as a positive control). $^{*}P < 0.05$. $^{**}P < 0.01$. n.s., not significant.

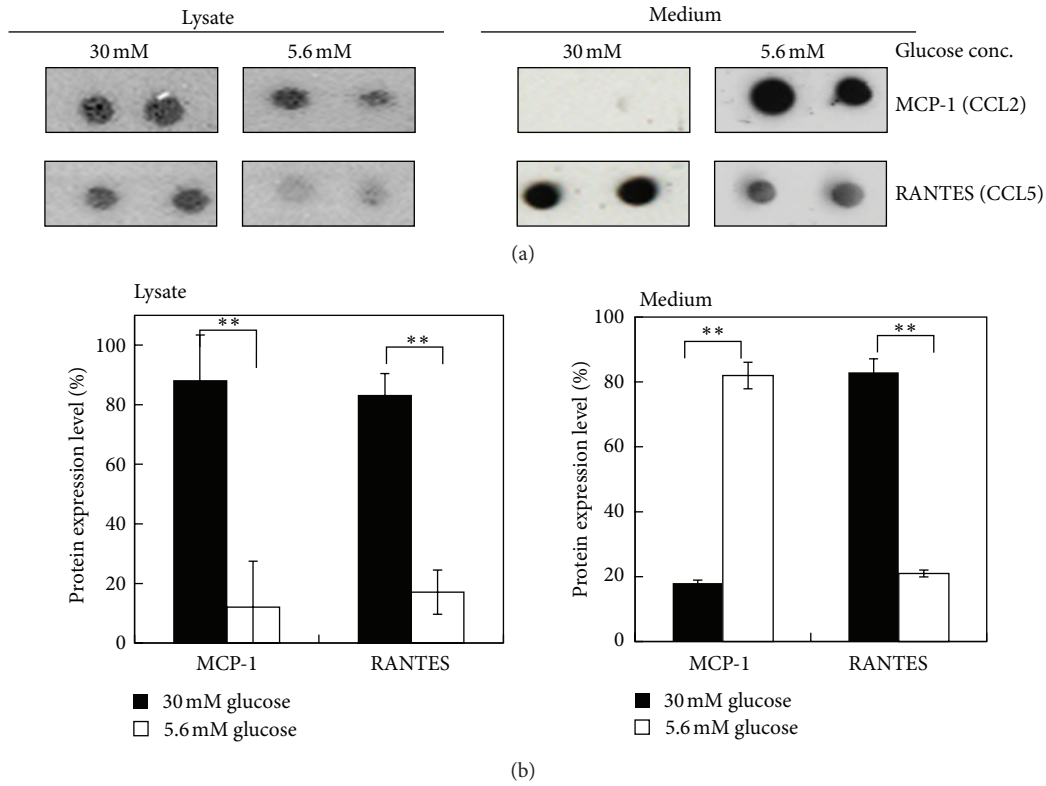

Figure 5: The effect of hyperglycemia on the expression of C-C motif chemokines in 3A-Sub-E trophoblast cells. 3A-Sub-E cells were exposed to 30 mM D-glucose or to 5.6 mM of D-glucose (control) for 48 h. Cell lysates (250–370 μg) and the cultured conditioned media were collected for the antibody array analysis, respectively. (a) A representative dot image of the indicated C-C motif chemokines. (b) Average intensity for each pair of cytokine spots was quantitatively measured by Scion Image software. Each cytokine is represented by duplicate spots: MCP-1 (monocyte chemoattractant protein-1, CCL-2), RANTES (regulated upon activation, normal T-cell expression and secreted, CCL-5), and control (HRP-conjugated antibody as a positive control). Data are presented as mean ± SD ($n = 4$). $^{**}P < 0.01$.

We also investigated C-C motif chemokine expressions. Figure 5 showed that hyperglycemia induced significant expressions of regulated upon activation, normal T-cell expression and secreted (RANTES, CCL5). Monocyte chemo-attractant protein (MCP-1, CCL-2) was mainly expressed in the cell lysates under hyperglycemic condition and was present in the medium under normal glucose condition (Figure 5). MCP-1 plays a critical role in the recruitment and activation of leukocytes during acute inflammation. A study by deleting the expression of MCP-1 caused a significant upregulation of decorin, a small leucine rich proteoglycan (SLRPs) in acute wounds [23]. CCL-5 could upregulate decorin expression and its posttranslational modification [24]. Hyperglycemia elevated level of RANTES/CCL5 in patients with type 2 diabetes [25]. Inhibition of CCL2 and CCL5 may attenuate hyperglycemia and inflammation [26]. We previously have reported increased chondroitin sulfate proteoglycan, decorin, and biglycan expressions, and more chondroitin sulfate than heparin sulfate was substituted on perlecan core protein in the placenta with gestational diabetes mellitus (GDM) as well as in the trophoblasts cultured under hyperglycemic condition [11]. Heparin or heparan sulfate rather than chondroitin sulfate is involved in trophoblast implantation on uterine epithelium [27]. The present study suggested that our previous findings on hyperglycemia induced increased expressions of decorin and biglycan may be mediated by increased CCL-2 and CCL-5 expressions. In addition, C-C motif chemokines might also regulate GAG composition on perlecan. Hyperglycemia results in impaired placental structure, vascular abnormality, and embryo implantation defect during pregnancy.

Placental development requires proper trophoblast invasion and tissue remodeling that involves the balanced matrix metalloproteinase (MMP) and their inhibitor, tissue inhibitor for MMP (TIMP) expressions. Figures 6(a) and 6(b) revealed that TIMP-2 is the major inhibitor expressed by trophoblasts. However, less TIMP-2 was present in the medium of 3A-Sub-E cultured under high glucose culture condition. Zymography analyses revealed that the expressions and activity of MMP2 and MMP9 were indeed increased in the cells with high glucose treatment (Figure 6(c)). Interestingly, the hyperglycemia induced MMP2 and MMP9 activities were diminished while 3A-Sub-E cells were treated with chondroitinase ABC. Our previous studies identified hyperglycemia-induced chondroitin sulfate proteoglycan expressions, such as decorin and biglycan, and more chondroitin sulfate content than heparin sulfate GAGs was substituted on perlecan core protein in 3A-Sub-E cells [11]. The present studies suggest that hyperglycemia resulting matrix remodeling also affects the secretion and activation of MMP and TIMP expressions

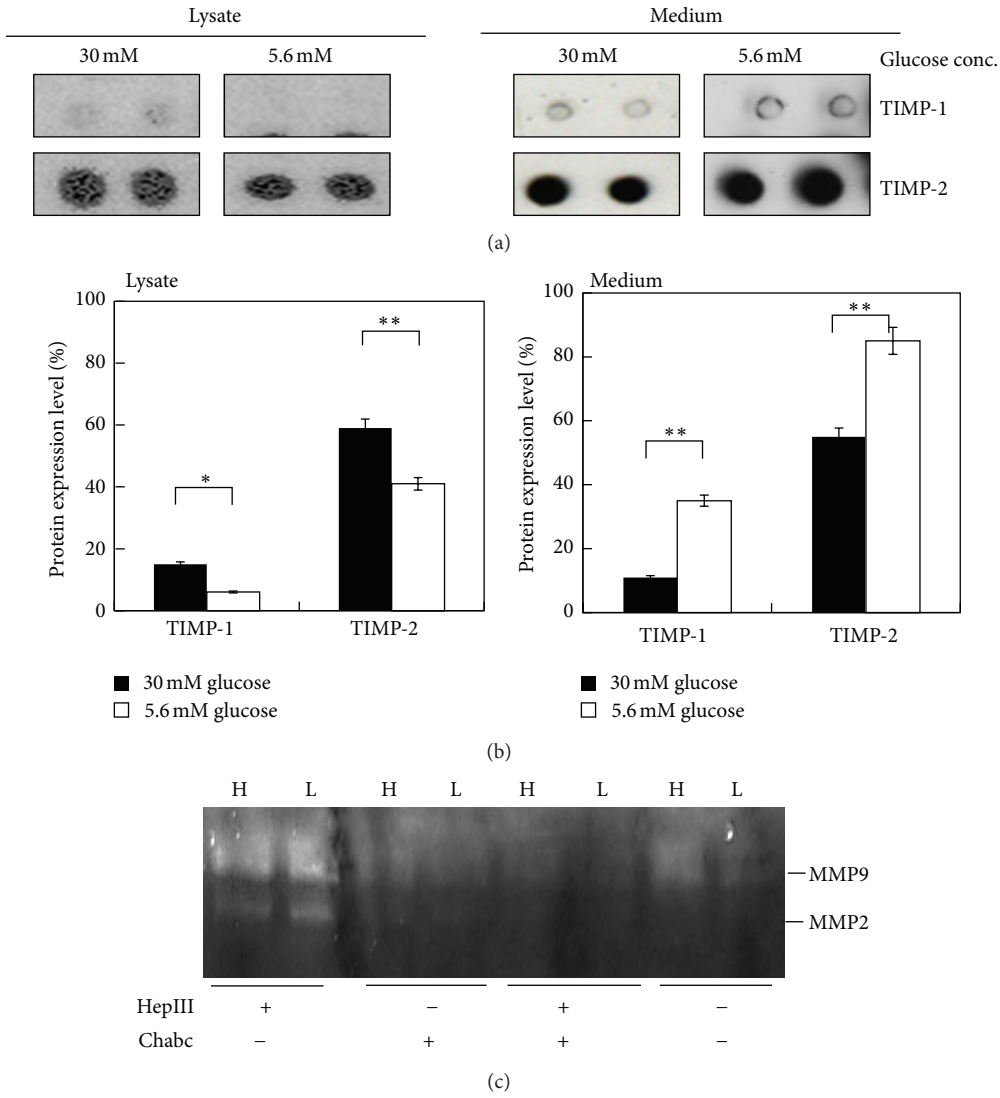

Figure 6: The effect of hyperglycemia on the expression of MMP inhibitors in 3A-Sub-E trophoblast cells. 3A-Sub-E cells were exposed to 30 mM D-glucose or to 5.6 mM of D-glucose (control) for 48 h. Cell lysates (250–370 μg) and the cultured conditioned media were collected for the antibody array analysis, respectively. (a) Representative dot image of the indicated MMP inhibitor. (b) Average intensity for each pair of cytokine spots was quantitatively measured by Scion Image software. Data are presented as mean ± SD ($n = 4$). (c) A gelatin zymography gel image for MMP2 and MMP 9 activity. $^{*}P < 0.05$. $^{**}P < 0.01$. Each cytokine is represented by duplicate spots as indicated, TIMP-1 (tissue inhibitors of metalloproteinase-1), TIMP-2 (tissue inhibitors of metalloproteinase-2), and control (HRP-conjugated antibody as a positive control).

by trophoblasts. Our findings are consistent with the other reports that induced hyperglycemic condition or diabetes increased MMP and decreased TIMP expressions [28, 29]. Trophoblasts are sensitive to the local microenvironment in responses to hyperglycemic condition and the extracellular matrix (ECM) composition and structure.

3.4. The Effect of Hyperglycemia on the Expression of Perlecan Binding Growth Factors in Trophoblasts. Perlecan can bind to proangiogenic growth factors, such as fibroblast growth factors (FGFs) and vascular endothelial growth factor (VEGF), and present them to their receptors on the cell surface [3, 4]. Figure 7(a) showed that basic fibroblast growth factor (bFGF) was present in cell lysates but not in the media, and VEGF, VEGF-D, or PDGF-bb was at nondetectable level in 3A-Sub-E cells. A recent report has confirmed that PDGF-bb is not derived from trophoblast [30]. Although an immunohistochemistry study revealed strong staining for VEGF and VEGFR-2 in vascular and trophoblastic cells from women with mild hyperglycemia and the staining for VEGFR-1 was discrete and limited to the trophoblast [31], we did not detect VEGF signal in the cultured 3A-sub-E trophoblasts. In addition, at normal glucose condition, the expression of bFGF is regulated by GAG composition in the extracellular matrix. Hyperglycemia-induced alterations of proteoglycan expressions and GAG compositions may impair the regulatory activity of HSPG such as perlecan on the regulations of bFGF (Figure 7(b)). Perlecan positively

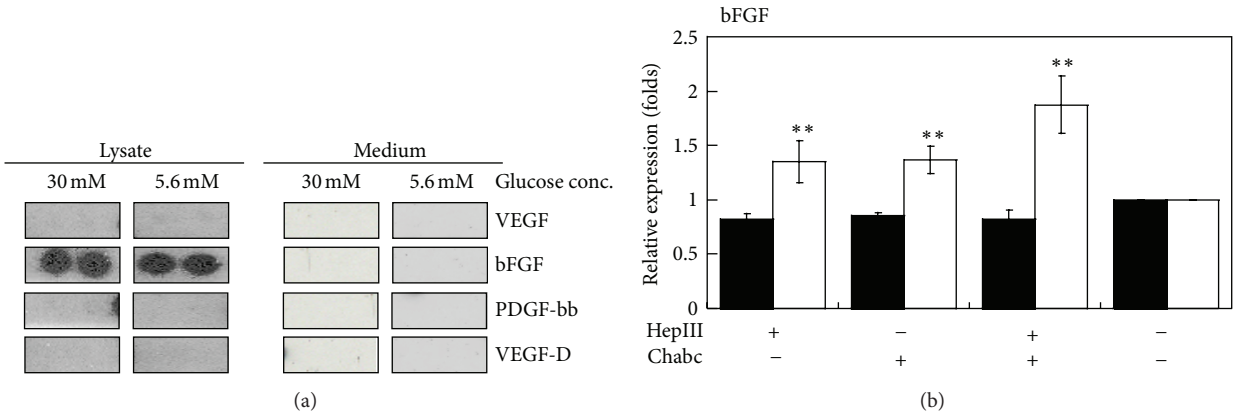

Figure 7: The effect of hyperglycemia on the expression of perlecan binding growth factors in 3A-Sub-E trophoblast cells. 3A-Sub-E cells were exposed to 30 mM D-glucose or to 5.6 mM of D-glucose (control) for 48 h. Cell lysates (250–370 μg) and the cultured conditioned media were collected for the antibody array analysis, respectively. (a) A representative dot image of the indicated growth factors was shown. Compared to the control, only bFGF was expressed in the cell lysates. bFGF, basic fibroblast growth factor; VEGF, vascular endothelial growth factor; PDGF-bb, platelet derived growth factor-bb; VEGF-D, vascular endothelial growth factor D. (b) The mRNA expressions of bFGF in responses to heparinase (HepIII), chondroitinase ABC (Chabc), or both treatment in 3A-Sub-E cells under high (black bar) or normal glucose (white bar) culture condition. Compared to the control cells without any treatment at normal glucose condition, the mRNA level of bFGF was significantly increased in 3A-Sub-E cells cultured with HepIII, Chabc, or both treatments.

regulates angiogenesis by promoting bFGF binding to bFGF receptor which is heparin sulfate-dependent [4, 32–34].

3.5. Glycosaminoglycan Composition Substituted on the Proteoglycans Mediate Hyperglycemia-Induced Proinflammatory Cytokine and the Chemokine Expressions. Previous studies have demonstrated that hyperglycemic condition indeed regulated the expressions of angiogenesis associated cytokines, MMPs, and growth factors at protein level. Real-time PCR studies confirmed the expressions of hyperglycemia sensitive cytokines at the mRNA level in 3A-Sub-E trophoblasts (Figure 8(a)). To further investigate whether the GAG composition may mediate hyperglycemic effect on the expressions of cytokines, GAG degradation enzyme treatment using heparanase III (HepIII), chondroitinase ABC (Chabc), or both in 3A-Sub-E cells cultured at high glucose condition was carried out to investigating the hyperglycemia sensitive cytokines at the mRNA level. Results indicated that treatment for eliminating heparin sulfate or/and chondroitin sulfate significantly increased mRNA expressions of IL-6, IL-8, and MCP-1 in the trophoblasts (Figure 8(b)) but did not affect the expressions of RANTES, TIMP-2 (data not shown), and bFGF (Figure 7(b)). It suggests that the hyperglycemia-induced expressions of IL-6, IL-8, and MCP-1 maybe mediated by the GAG composition and structure on the cell surface and the ECM. The regulatory mechanisms of heparin sulfate and chondroitin sulfate on the inflammatory cytokines maybe different. Heparan sulfate is essential for inflammatory response [35, 36]. The heparin binding interaction may retain IL-6, IL-8, and MCP-1 close to its sites of secretion that may favor a paracrine mode of activity [37, 38]. However, the soluble form of heparin sulfate may diminish the expression and production of inflammatory cytokines [39]. Chondroitin sulfate proteoglycans mainly present in the extracellular matrix may retain the cytokines such as IL-6 in the extracellular matrix and protect for further inflammation [40]. In addition, chondroitin sulfate as well as heparin sulfate function in regulation of inflammation may depen on their structure, size, and sequence [38]. Data in the present studies revealed that IL-8 and MCP-1 were mainly cell associated in trophoblasts at hyperglycemic condition and were present in the medium at normal glucose condition (Figures 4 and 5). It was known that induced hyperglycemia altered GAG substitution with more CS than HS on cell surface was not favorable for the binding with cytokines or growth factors. The increased cell-associated IL-8 and MCP-1 under hyperglycemic condition may be mediated by differ mechanism. These cytokines may play important roles on the regulation of inflammation and proliferation of 3A-Sub-E cells. The altered proteoglycan expression may result in the release of cytokines from the cell surface that may associate with increased levels of circulated inflammation related and angiogenesis associated cytokines in serum of women with GDM [41] or in patients with DM [42]. A diagram summarizing the possible regulatory mechanism of the altered glycosylation of proteoglycan on the expression of angiogenesis associated cytokines in trophoblast under hyperglycemic condition was shown in Figure 8(c).

4. Conclusions

This study identified hyperglycemia-induced angiogenesis associated cytokine expressions by trophoblasts 3A-Sub-E. In addition, the altered proteoglycan expression and their glycosaminoglycan structure may regulate the activity of the angiogenesis associated cytokines and the matrix composition. Hyperglycemia-induced alterations in the GAG structure of proteoglycans and the angiogenesis cytokines may affect the growth and migration of trophoblasts that may contribute to the aberrant placental structure and the maternal and fetal complications during development.

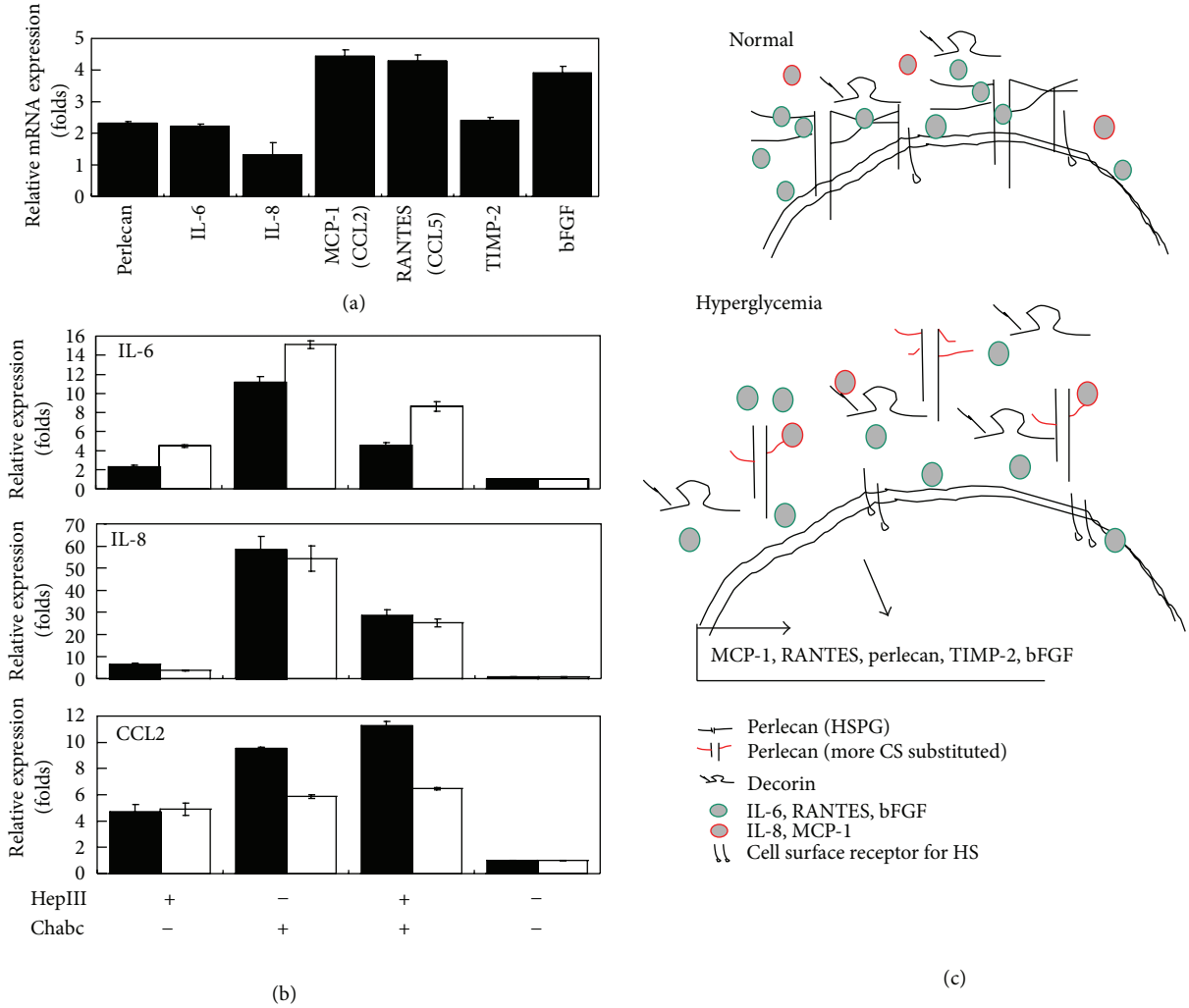

Figure 8: The effect of hyperglycemia induced altered glycosylation on the expressions of proinflammatory and chemokines in 3A-Sub-E trophoblast cells. Real-time quantitative polymerase chain reaction (RT-PCR) was performed to quantitatively measure. (a) The mRNA level of the indicated genes in responses to high glucose culture condition was increased at least 2 folds except IL-8 expression in 3A-Sub-E cells. (b) The mRNA expressions of IL-6, IL-8, and MCP-1 (CCL2) in 3A-Sub-E cells, were exposed to 30 mM D-glucose (black bar) or to 5.6 mM of D-glucose (normal control, grey bar) for 24 h followed by GAG degradation enzyme treatment with heparanase III (HepIII), chondroitinase ABC (Chabc), or both. Significantly increased expressions of IL-6, IL-8, and MCP-1 were occurred while 3A-Sub-E cells with Chondroitinase ABC treatment. (c) A diagram describes hyperglycemia-induced alterations of GAG substitution on perlecan (shorter and more chondroitin sulfate (CS) substituted than heparan sulfate (HS)) and matrix degradation by decreased TIMP-2 and increased activities of MMP2 and MMP9 resulting in the release of IL-6, RANTES, and bFGF into the extracellular matrix (soluble form) and enhance deposition of IL-8 and MCP-1 on the cell surfaces. The altered extracellular environment and the cell-associated and the soluble cytokine may contribute to the expressions of the indicated cytokines and chondroitin sulfate proteoglycans such as decorin at transcriptional and translational levels.

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Research Article

The Action of Antidiabetic Plants of the Canadian James Bay Cree Traditional Pharmacopeia on Key Enzymes of Hepatic Glucose Homeostasis

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We determined the capacity of putative antidiabetic plants used by the Eastern James Bay Cree (Canada) to modulate key enzymes of gluconeogenesis and glycogen synthesis and key regulating kinases. Glucose-6-phosphatase (G6Pase) and glycogen synthase (GS) activities were assessed in cultured hepatocytes treated with crude extracts of seventeen plant species. Phosphorylation of AMP-dependent protein kinase (AMPK), Akt, and Glycogen synthase kinase-3 (GSK-3) were probed by Western blot. Seven of the seventeen plant extracts significantly decreased G6Pase activity, *Abies balsamea* and *Picea glauca*, exerting an effect similar to insulin. This action involved both Akt and AMPK phosphorylation. On the other hand, several plant extracts activated GS, *Larix laricina* and *A. balsamea*, far exceeding the action of insulin. We also found a significant correlation between GS stimulation and GSK-3 phosphorylation induced by plant extract treatments. In summary, three Cree plants stand out for marked effects on hepatic glucose homeostasis. *P. glauca* affects glucose production whereas *L. laricina* rather acts on glucose storage. However, *A. balsamea* has the most promising profile, simultaneously and powerfully reducing G6Pase and stimulating GS. Our studies thus confirm that the reduction of hepatic glucose production likely contributes to the therapeutic potential of several antidiabetic Cree traditional medicines.

1. Introduction

Diabetes is a chronic disease that occurs in two forms, namely, Type 1 and Type 2 diabetes. Type 1 diabetes affects mostly young people and is due to an autoimmune destruction of islet cells which secrete insulin [1]. Type 2 diabetes is characterized by decreased insulin sensitivity in major target organs such as liver, muscle, and adipose tissues, in addition to a decreased insulin secretion by the beta pancreatic cells [2].

These defects in patients with Type 2 diabetes cause an increase in fasting and postprandial glucose, which increases the risk of microvascular (e.g., retinopathy, neuropathy, and nephropathy) and macrovascular (mainly stroke and peripheral vascular dysfunction) complications of diabetes [3].

Approximately 370 million people in the world are affected by diabetes. In Canada more than 9 million people are diabetic or prediabetic. The risk to develop Type 2 diabetes in Aboriginal population is three to five times higher than the general Canadian population [4, 5]. Since 2003, our team has been examining the antidiabetic potential of selected plants used in the traditional medicine of several Cree communities in the James Bay region of Northeastern Canada. Previous studies from our group have concentrated on a group of 17

promising plant species identified through ethnobotanical surveys [6, 7]. These plants have notably been screened in bioassays of skeletal muscle and adipose tissue to identify the plants' potential to improve glycemic control [8, 9]. In continuity with these studies, the aim of this project is to evaluate the effect of these seventeen medicinal plants on hepatic glucose homeostasis.

Glucose homeostasis is the result of a balance between glucose production by the liver (gluconeogenesis), its storage as glycogen (liver and muscle), and its uptake by peripheral tissues, notably insulin-responsive skeletal muscle and adipose tissue. Indeed, insulin, a hormone secreted by the beta pancreatic cells, works by decreasing glucose production in the liver, stimulating its uptake by skeletal muscle and peripheral tissues and enhancing its storage as glycogen [10]. Therefore, in Type 2 diabetes, beta pancreatic cell insulin deficiency is combined with insulin resistance, thus contributing to a state of hyperglycemia through an increased hepatic glucose production and a reduced peripheral glucose disposition [11].

Gluconeogenesis is the major metabolic pathway through which the liver produces glucose from precursors such as amino acids, lactate, glycerol, and pyruvate. This process includes several linked enzymatic reactions and is mainly activated after a fast and in diabetic patients [12]. Hepatic gluconeogenesis is controlled at three major points, namely, the reactions catalyzed by phosphoenolpyruvate carboxykinase (PEPCK), by fructose-1,6-biphosphatase, and by Glucose-6-phosphatase (G6Pase). Insulin normally reduces the activity of these enzymes to help normalize blood glucose. It does so through the activation of the signaling kinase Akt and the subsequent phosphorylation of transcription factors controlling the expression of PEPCK and G6Pase. The phosphorylated transcription factors are then expelled from the nucleus, the expression of enzymes is inhibited, and the production of glucose in the liver is eventually reduced [13, 14]. In diabetic patients, hepatic insulin resistance interferes with these events, resulting in an increased hepatic glucose, a major contributor to fasting hyperglycemia [15, 16]. G6Pase is an endoplasmic reticulum enzyme responsible of the final release of glucose into the circulation [17] and is considered to represent the rate-limiting step of gluconeogenesis [18]. Moreover, an inactivating mutation in the gene of this enzyme leads to hypoglycemia, but an increase in its expression is followed by hyperglycemia and the onset of diabetes [19]. We have thus selected a G6Pase assay to probe the Cree plants for additional antidiabetic potential targeting the liver.

On the other hand, glycogen synthase (GS) catalyzes the rate limiting step of glycogenogenesis and is thus responsible for the storage of glucose as in both the liver and skeletal muscle. This enzyme is regulated by several transcriptional factors and kinases, the most important one being glycogen synthase kinase-3 (GSK-3). GSK-3 is a serine/threonine kinase implicated in many diseases such as diabetes, cancer, inflammation, and Alzheimer [20]. GSK-3 phosphorylates and inhibits GS thereby decreasing glycogen synthesis in liver and muscles [21]. Insulin phosphorylates and inactivates GSK-3, thus leading to the activation of the GS and the storage of glucose as glycogen [22, 23]. Therefore, we have also chosen to measure GS activity to determine the action of putative antidiabetic Cree plants on hepatic glucose homeostasis.

Aside from the hormonal regulation involving insulin, hepatic glucose homeostasis is also regulated by factors implicated in the control of energetic balance. A key kinase in this context is called AMP-activated protein kinase (AMPK), which is activated after metabolic stress (increase of the ratio AMP/ATP in the cell) [24]. Once phosphorylated, active AMPK inhibits anabolic pathways (decrease of the fatty acids synthesis, decrease of the gluconeogenesis, and increase of the storage of glucose as glycogen) and simultaneously increases catabolic pathways (increase of muscle glucose uptake, increase of the fatty acid oxidation, and increase of the glycolysis). Moreover, AMPK is activated by metformin, one of the most common oral hypoglycemic drugs used worldwide for glycemic control in diabetes and metabolic syndromes [25]. We therefore probed liver cell cultures for a potential action of Cree plants on AMPK, in relation to their effects on liver glucose metabolic enzymes.

The present study thus reports the effect of seventeen plants used in the traditional pharmacopeia of the Eastern James Bay Cree of Northern Quebec (Canada) on the activity of key enzymes of gluconeogenesis and glycogen synthesis as well as key kinases regulating these enzymes.

2. Material and Methods

2.1. Cell Culture. The cell lines H4IIE (rat hepatoma) and HepG2 (human hepatoma) were provided by American Type Culture Collection (ATCC). H4IIE cells were grown in high glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS) and 0.5% antibiotics (PS: Penicillin 100 U/mL, Streptomycin 100 μg/mL). The HepG2 cells were grown in DMEM/F12 (50/50) medium supplemented with 10% FBS and 0.5% PS. The cells were incubated at 37°C, 5% CO_2 until reaching 90% confluence.

2.2. Plant Preparation. As mentioned, plant species were selected on the basis of ethnobotanical surveys previously reported [6, 7]. A taxonomist, Dr. Alain Cuerrier, identified these plants and voucher specimens have been deposited at the herbarium of the Montreal Botanical Garden. They were harvested in the Easter James Bay area in respect of Aboriginal guidelines and extracted with 80% ethanol, as previously described [8, 9].

2.3. Cytotoxicity Assay (LDH). As was done previously for skeletal muscle and adipocyte cell lines [8, 9], maximal nontoxic concentrations of extracts were determined with the help of a cytotoxicity test, namely, that of lactate dehydrogenase (LDH) release (LDH Colorimetric kit; Roche, Mannheim, Germany). After an overnight treatment (16–18 hours) with different extracts at different concentrations, the cell culture media for each condition were collected separately (representing released LDH). The cells were then lysed with culture medium containing 1% triton X-100, for 10 minutes at 37°C, 5% CO_2 (representing cellular LDH).

All samples were collected in eppendorf tubes and centrifuged at 250 ×g at 4°C for 10 minutes. Results were expressed as the ratio of released LDH to total LDH (total LDH = released LDH + cellular LDH), normalized to values obtained from cells treated with the vehicle control (0.1% DMSO) and used to determine the optimal nontoxic concentration for each extract.

2.4. Hepatic Glucose Production. Glucose-6-phosphatase activity was assessed in the H4IIE cell line. Briefly, cells (90% confluent in 12-well plates) were treated for 18 h with negative control (0.1% DMSO vehicle), positive control (Insulin, 100 nM), and seventeen plant extracts at their respective optimal nontoxic concentrations. After 18 h, cells were washed then lysed in 15 mM phosphate buffer containing 0.05% triton X-100 and 1.3 mM phenol (pH = 6.5). Cell lysates were incubated in glucose-6-phosphate-containing buffer (200 mM) for 40 min at 37°C where the G-6-P serves as a substrate for endogenous glucose-6-phosphatase to yield glucose. Quantification of the glucose generated in this reaction was measured using Wako AutoKit Glucose colorimetric assay (Wako Chemicals USA Inc., Richmond, VA, USA), according to manufacturers' instructions. Protein content was determined using the BCA method. Results are presented relative to vehicle control (0.1% DMSO).

2.5. Glycogen Synthase Activity. HepG2 cells were grown to confluency in DMEM/F12 (50/50) medium containing 10% FBS (Fetal Bovine Serum) and 0.5% PS at 37°C, 5% CO_2. Cells were plated in 6-well plates for 4 days, then treated overnight (16–18 h) with negative control (0.1% DMSO vehicle) or each of the seventeen plant extracts at their respective optimal concentration. Positive controls were insulin (100 nM for 15 min) and 5-Aminoimdazole-4-carboxamide-1-β-D-ribofuranoside (AICAR, 2 mM for 120 min). After treatment, cells were suspended in buffer solution (50 mM glycylglycine, 100 mM sodium floride, 20 mM EDTA, 0.5% glycogen, pH 7.4 + complete protease inhibitor cocktail). The lysates were centrifuged at 1000 ×g for 20 minutes at 4°C. 30 μL of supernatant was added to 100 μL of buffer solution for active GS (25 mM glycylglycine, 0.275 mM UDP-glucose, 0.12 μCi/mL U-^{14}C UDP-glucose, 1% glycogen, 1 mM EDTA, 10 mM sodium sulfate, pH 7.5) and another 30 μL of supernatant was added to 100 μL of buffer solution for total GS (25 mM Tris, 5 mM UDP-glucose, 0.12 μCi/mL U-^{14}C UDP-glucose, 1% glycogen, 3 mM EDTA, 5 mM glucose-6-phosphate, pH 7.9). All the tubes were incubated at 30°C for 120 minutes. After incubation 90 μL of the mix was transferred on Watman paper (Watman 31 ET chr 2 cm^2). The papers were rinsed with cold ethanol (4°C) for 30 minutes then 2 times with ethanol 66% at room temperature for 30 minutes. The papers were covered with acetone for 2-3 minutes then transferred into scintillation vials. The resulted radioactivity was counted with a specific protocol for ^{14}C using a β-counter (LKB Wallac 1219; Perkin-Elmer, Woodbridge, ON, Canada).

2.6. Western Blot. Cells were lysed in RIPA buffer (RadioImmun Precipitation Assay) (0.1 M Hepes, 0.3 M NaCl, 10 mM EGTA, 4 mM $MgCl_2 \cdot 6H_2O$, 10% glycerol, 2% Triton X-100, 0.2% SDS, 2 mM PMSF, 10 mM NaF, 100 μM Na-orthovanadate, 1 mM Na-pyrophosphate). After centrifugation at 12000 ×g, 4°C, for 12 minutes, the supernatants were collected and used for western blot analysis. 40 μg of proteins was loaded onto an electrophoresis gel then transferred on nitrocellulose membrane (Millipore, Bedford, MA, USA). The membranes were blocked for 2 hours in TBST (20 mM Tris, pH 7.6, 137 mM NaCl, 0.1% Tween-20) + 5% milk. Antibodies used were the following: p-GSK-3 (Ser 9), GSK-3 (1 : 1000, 5% milk, Millipore, Bedford, MA, USA) and secondary antibody : anti-rabbit (1 : 50000, 5% milk, Jackson ImmunoResearch Laboratories, West Grove, PA, USA); p-AMPKα (Thr 172) (1 : 350, 5% BSA, Cell Signaling Technology, Danvers, MA, USA), AMPK (1 : 500, 5% BSA, Cell Signaling Technology, Danvers, MA, USA) and secondary antibody : anti-rabbit (1 : 4000, 5% BSA, Jackson ImmunoResearch Laboratories, West Grove, PA, USA); p-Akt (Thr 308) (1 : 1000, 5% BSA, Cell signaling Technology, Danvers, MA, USA), Akt (1 : 1000, 5% BSA, Cell Signaling Technology Danvers, MA, USA) and secondary antibody : anti-rabbit (1 : 10000, 5% BSA, Jackson ImmunoResearch Laboratories, West Grove, PA, USA).

The membranes were incubated with primary antibodies overnight at 4°C then with secondary antibodies 1 hour at room temperature. Finally the bands were detected with the ECL Plus western blot detection system (Perkin Elmer, Woodbridge, Canada).

2.7. Statistical Analysis. All data are reported as the mean ± SEM of 3 different experiments with triplicate for each sample. Results were analyzed by one-way analysis of variance (ANOVA) using StatView software (SAS Institute Inc., Cary, NC, USA). A P value below 0.05 was considered statistically significant.

3. Results

3.1. LDH Test (Cytotoxicity). Hepatic cells (H4IIE and HepG2) were treated overnight (16–18 h) with plant extracts at different concentrations and LDH release measured. Table 1 presents the maximal nontoxic concentrations determined for each plant extract. At the selected concentrations, none of the extracts induced more than 9% release of LDH. These concentrations were subsequently used for all experiments.

3.2. Seven of the Seventeen Plant Extracts Have a Potential to Decrease Hepatic Glucose Production. We used the inhibition of rate-limiting glucose-6-phosphatase activity in H4IIE hepatocytes as a measure of the antidiabetic potential of Cree plant extracts at the level of the liver. As expected, insulin (positive control) decreased G6Pase activity by approximately 60%. Out of the seventeen plant extracts tested, eight had a statistically significant effect on G6Pase activity when compared to respective DMSO vehicle controls run in parallel (Figure 1). Seven of the eight plants reduced activity by 50 to 30% on average, whereas *Salix planifolia* increased activity by 43%. The inhibitory activity of extracts of *Picea glauca* and *Abies balsamea* was almost as important as the insulin

Table 1: Optimal concentrations of plant extracts used to treat H4IIE and HepG2 cells were determined by the LDH cytotoxicity.

Plant extracts	Abbreviation used	Concentrations in μg/mL
Abies balsamea	Abies	50
Kalmia augustifolia	Kalmia	50
Larix laricina	Larix	25
Sarracenia purpurea	Sarra	25
Sorbus decora	Sorbus	15
Juniperus communis	Juniper	3.75
Rhododendron tomentosum	Rhod.T	50
Rhododendron groenlandicum	Rhod.G	50
Gaultheria hispidula	Gault	25
Picea mariana	Picea.M	10
Picea glauca	Picea.G	125
Salix planifolia	Salix	15
Alnus incana	Alnus	50
Populus balsamifera	Populus	100
Lycopodium clavatum	Lyco	100
Pinus banksiana	Pinus	10
Vaccinium vitis	Vitis	200

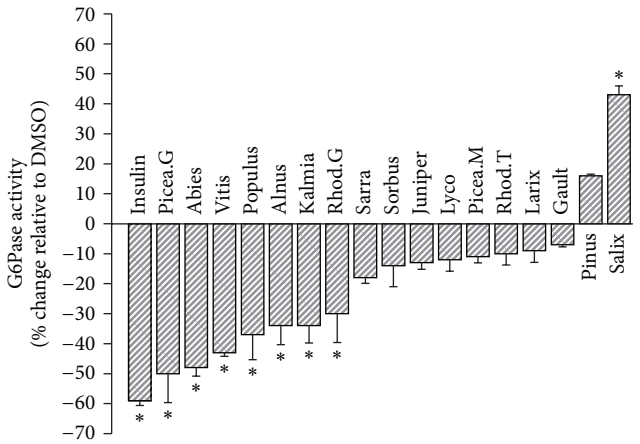

Figure 1: Effect of 17 plant extracts on the activity of G6Pase. Results shown represent the change in G6Pase activity observed after overnight treatment of H4IIE cells with optimal nontoxic concentrations of indicated plant extracts. They are expressed relative to DMSO (0.1%) vehicle controls (0% inhibition). Assays were carried out in triplicate on three different cell cultures. Insulin (100 nM) was used as a positive control. $^{*}P < 0.05$ significantly different from DMSO vehicle control.

positive control (reductions of 50% and 48%, respectively, compared to DMSO).

3.3. Absence of Additive Effect of either P. glauca or A. balsamea Extracts with Insulin. Since the effects of *P. glauca* and *A. balsamea* were in the same order of magnitude as the insulin control, we sought to examine whether the plant extracts and

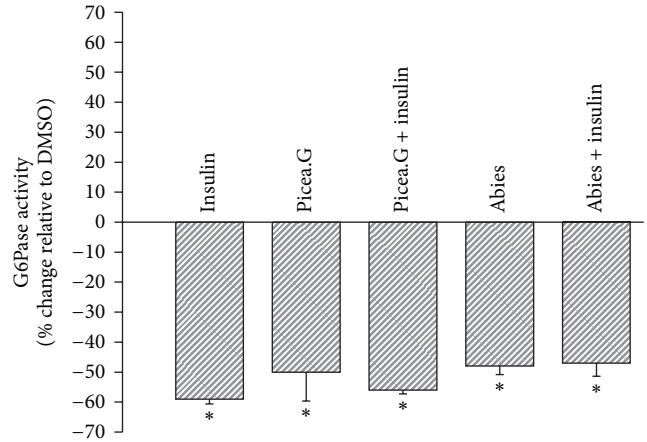

Figure 2: Effect of insulin and two plant extracts on G6Pase activity. Results shown represent the change in G6Pase activity observed after overnight treatment of H4IIE cells with *Picea glauca* or *Abies balsamea* plant extracts in the presence or absence of insulin (100 nM). They are expressed relative to DMSO (0.1%) vehicle controls (0% inhibition). Assays were carried out in triplicate on three different cell cultures. $^{*}P < 0.05$ significantly different from DMSO vehicle control.

the hormone showed any interaction in biological activity. Figure 2 presents the results of experiments where each plant extract was administered individually with or without insulin. As can be easily appreciated, insulin did not influence the effect of each plant extract and vice versa.

3.4. Differential Modulation of Insulin-Dependent and -Independent Signaling by Cree Plant Extracts in H4IIE Hepatocytes. We next sought to determine if insulin-dependent and/or -independent signaling pathways were involved in the observed effects of plant extracts on G6Pase activity. H4IIE hepatocytes were thus incubated as above with each of the seventeen plant extracts and probed for phosphorylated Akt (key signaling kinase of the insulin-dependent metabolic pathway) and for phosphorylated AMPK (key metabolic switch kinase responsible for insulin-independent metabolic pathway). As shown in Figure 3(a), six of the seventeen plants significantly enhanced the phosphorylation of Akt. Two of these six, namely, *A. balsamea* and *P. glauca*, were plant extracts also observed to significantly decrease G6Pase activity in H4IIE hepatocytes. However, we found no correlation between Akt phosphorylation and modulation of G6Pase activity (data not illustrated).

Unlike Akt, all but two of the seventeen plant extracts tested were found to induce a significant increase in the phosphorylation of AMPK when compared to vehicle control (Figure 3(b)). A number of plant extracts, notably *A. balsamea*, *Rhododendron groenlandicum*, *Larix laricina*, *Pinus banksiana*, *Picea mariana,* and *Gaultheria hispidula* exerted effects that were 2-4-fold greater than the positive control AICAR. However, as with Akt, no correlation was found between the ability of a plant's extract to activate AMPK and to inhibit G6Pase (data not illustrated).

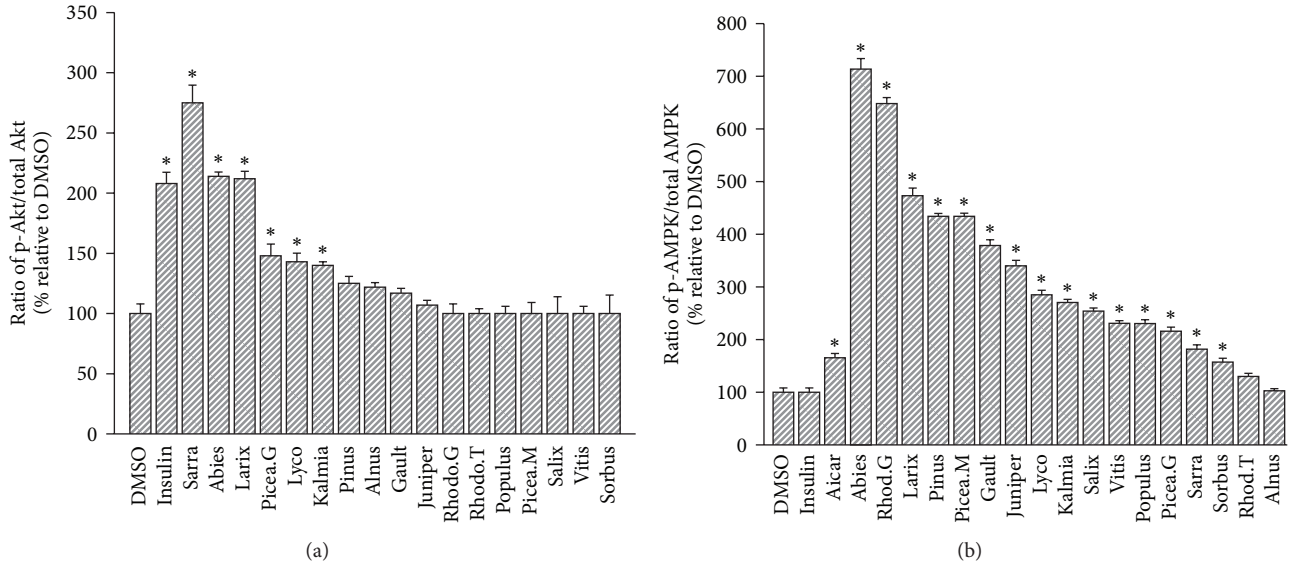

FIGURE 3: Effect of 17 plant extracts on kinases regulating G6Pase activity. (a) Phosphorylation of Akt. Phosphorylated (p-Akt) and total Akt were measured by Western blot in H4IIE cells treated with optimal nontoxic concentrations of indicated plant extracts. The ratio of p-Akt/total Akt is expressed as a percentage relative to values obtained for DMSO (0.1%) vehicle controls. Insulin (100 nM) was used as a positive control. $^*P < 0.05$ significantly different from DMSO vehicle control. (b) Phosphorylation of AMPK. Phosphorylated (p-AMPK) and total AMPK were measured by Western blot in H4IIE cells treated with optimal nontoxic concentrations of indicated plant extracts. The ratio of p-AMPK/total AMPK is expressed as a percentage relative to values obtained for DMSO (0.1%) vehicle controls. AICAR (2 mM) was used as a positive control. $^*P < 0.05$ significantly different from DMSO vehicle control.

3.5. Two Cree Plants Stand out for Very Potent Stimulation of Glycogen Synthase Activity. Glycogen synthase (GS) is the second rate-limiting enzyme involved in hepatic glucose homeostasis that we used to probe for the antidiabetic potential of Cree plant extracts. As expected, the hormone insulin, used as a positive control activated GS by 2-fold when compared to the DMSO vehicle control in HepG2 cells (Figure 4(a)). AICAR, an activator of AMPK, also induced a similar 2-fold increase in GS activity. Several plants also significantly stimulated GS activity as much or greater than the insulin and AICAR controls (between 3- and 2-fold). However, two plants clearly stood out of the lot. Indeed, *A. balsamea* and *L. laricina* activated the enzyme by 11- and 9-folds, respectively, compared to the DMSO vehicle control (Figure 4(a)).

3.6. Implication of GSK-3 in the Action of Cree Plants on Glycogen Synthase. As expected, and compared to the DMSO vehicle control, insulin treatment lead to a significant (over 2-fold) increase in the phosphorylation of GSK-3, which is known to inactivate the kinase, hence releasing its inhibitory action on GS and favoring glucose storage as glycogen. Nine of the seventeen plant extracts tested also significantly increased GSK-3 phosphorylation (Figure 4(b)). Consistent with the results of the GS activity assay, *L. laricina* and *A. balsamea* were among the most active species to enhance GSK-3 phosphorylation. In fact, when the ability of a given plant extract to activate GS was compared to the same plant's capacity to induce GSK-3 phosphorylation, a modest yet significant positive correlation was obtained ($r^2 = 0.36$, $P < 0.05$, Figure 4(b) inset).

4. Discussion

As mentioned in the introduction, Canadian Aboriginals suffer from a higher incidence of Type 2 diabetes [4, 5] as well as diabetic complications, a situation that has been linked to poor treatment adherence due, in part, to the cultural inappropriateness of modern drug based approaches [26, 27]. To provide culturally adapted complementary and alternative treatment options for these populations, we have put together a comprehensive platform of *in vitro* (mostly cell based) bioassays and *in vivo* animal models to rigorously assess the action of Boreal forest plants on tissues/organs involved in metabolic control [28]. Plant species were first identified through a novel ethnobotanical survey methods based on a set of clinically relevant diabetes symptoms [6]. A set of seventeen plants with promising antidiabetic potential was initially screened in pancreatic, skeletal muscle, adipocyte and, preneuronal cell lines [8, 9]. We notably established that some species exhibited the potential to accelerate adipogenesis and to stimulate glucose uptake in muscle cells and adipocytes [8, 9].

Along with peripheral glucose disposition, hepatic glucose production plays a major role in the pathogenesis of the obesity-diabetes metabolic disease continuum [19]. We thus sought to further assess the antidiabetic potential of the Cree plants in liver cell lines. Two key enzymes involved in hepatic glucose storage and release were selected. We also examined major metabolic control/signaling kinases that modulate these key enzymes.

Firstly, we selected G6Pase, which is a key enzyme implicated in hepatic gluconeogenesis, being the rate-limiting step

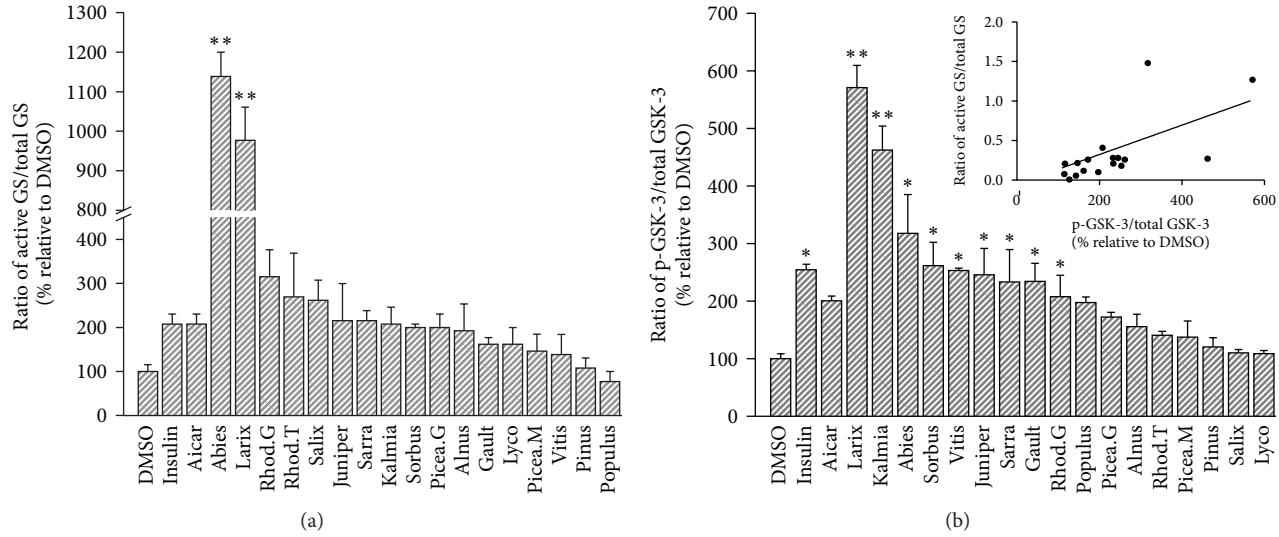

Figure 4: Effect of 17 plant extracts on components involved in hepatic glucose storage. (a) Activation of GS. Results shown represent the glycogen synthase (GS) activity observed after overnight treatment of HepG2 cells with optimal nontoxic concentrations of indicated plant extracts. They are expressed relative to DMSO (0.1%) vehicle controls (100% activity). Assays were carried out in triplicate on three different cell cultures. Insulin (100 nM) and AICAR (2 mM) were used as positive controls. $^{**}P < 0.01$ significantly different from DMSO vehicle control. (b) Phosphorylation of GSK-3. Phosphorylated (p-GSK-3) and total GSK-3 were measured by Western blot in HepG2 cells treated with optimal nontoxic concentrations of indicated plant extracts. The ratio of p-GSK-3/total GSK-3 is expressed as a percentage relative to values obtained for DMSO (0.1%) vehicle controls. Insulin (100 nM) and AICAR (2 mM) were used as positive controls. $^{*}P < 0.05$ and $^{**}P < 0.01$ significantly different from DMSO vehicle control. Inset: Correlation between GSK-3 phosphorylation and activation of GS induced by the 17 plant extracts ($r^2 = 0.36$, $P < 0.05$).

for glucose release from hepatocytes [18]. The expression of this enzyme is enhanced by several transcription factors such as forkhead transcription factor O1 (FoxO1), hepatic nuclear factor 4 (HNF4), and peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) [29, 30]. Through its receptor signaling pathways, insulin controls the expression of G6Pase. It does so by inhibiting the aforementioned transcriptional factors as well as their binding to the promoter of the G6Pase gene and to that of the PEPCK gene, another key enzyme involved in the gluconeogenesis pathway [31]. The signaling kinase Akt plays a crucial role in insulin's metabolic action in the liver and was thus also assessed in H4IIE hepatocytes to gain insights on the potential of Cree plants to modulate insulin-dependent pathways. On the other hand, AMPK is an insulin-independent serine/threonine kinase implicated in energy balance [24]. It is involved in hepatic metabolic homeostasis [32], which includes the ability to decrease G6Pase activity through the phosphorylation of CREB (c-AMP-regulator element-binding protein) [33]. We thus also probed H4IIE hepatocytes for AMPK activation after treatment with plant extracts in order to assess insulin-independent pathways.

As expected, we found that an optimal supraphysiological concentration of insulin (100 nM) reduced G6Pase activity in H4IIE hepatocytes to approximately half of that seen in DMSO vehicle controls. Interestingly, a number of the Cree plant species also exhibited the capacity to lower G6Pase activity. Most prominent were *P. glauca* and *A. balsamea*, whose action was equivalent to that of the insulin positive control. When the extracts of these two plants were combined with insulin, no additive effect was observed. One plausible interpretation of this result is that *P. glauca* and *A. balsamea* may act through pathways similar to insulin, these being already maximally stimulated by the supraphysiological dose of the hormone. This was confirmed for *A. balsamea*, whose extract stimulated the phosphorylation of Akt as prominently as insulin did. This was also true for *P. glauca*, albeit to a lesser extent. In fact, no correlation could be found between the plant extracts effects on G6Pase inhibition and Akt phosphorylation. In skeletal muscle cells, several Cree plants had no effect on Akt [34] suggesting that plant extracts may have differential effects on insulin signaling pathways in different tissues. *A. balsamea* extract also strongly stimulated the insulin-independent AMPK pathway. In fact, its action was over threefold greater than the AICAR positive control. In contrast, *P. glauca* stimulated the AMPK pathway to a slightly greater extent than AICAR, yet decreased G6Pase activity to the same extent as *A. balsamea*. These observations combined with the clear lack of correlation between either Akt or AMPK activation, and G6Pase inhibition highlights the fact that other pathways modulating G6Pase activity may be influenced by the Cree plants. Future studies will have to take this into consideration.

On the other hand, HepG2 cells were selected to study the potential action of Cree plants on the second key enzyme of hepatic glucose homeostasis, namely, glycogen synthase, because they exhibit a better expression of this enzyme [35]. Insulin increases the activity of glycogen synthase by inhibiting the GSK-3 (phosphorylation mechanism) thus increasing the glucose storage as glycogen [36, 37]. Again,

we observed that several Cree Boreal forest plants exerted a positive action on glycogen synthase, a majority of which acting at least as much as the insulin positive control, which doubled GS activity over baseline (DMSO control). However, extracts of *A. balsamea* and *L. laricina* enhanced GS activity 9–11-fold over baseline (corresponding to 4-5-fold above insulin). This skewed the statistical analysis such that only these two plants appeared to have a statistically significant impact on GS activity.

We also assessed the impact of Cree plant extracts on GSK-3 phosphorylation (leading to its inactivation [21]) since this kinase plays a central role in the control of GS activity [22]. Unlike G6Pase, we found that the phosphorylation of GSK-3 induced by respective Cree plant extracts could explain approximately 40% ($r^2 = 0.36$) of their capacity to increase GS activity. This is a respectable proportion that indicates that GSK-3 might be a common target of several Boreal forest plants. However, such a result also implies that other signaling/metabolic control pathways may also be involved, and additional studies will be necessary to identify these.

Our study thus clearly supports the notion that part of the therapeutic potential of several putative antidiabetic Boreal forest plants can involve the reduction of hepatic glucose output. The results more specifically highlight the favorable profile of three plants. Firstly, *P. glauca* also stood out as the most potent Cree plant capable of reducing G6Pase activity as much as a supraphysiological concentration of insulin. This action is clearly associated with the phosphorylation of the key kinases Akt and AMPK in H4IIE hepatocytes. Previous *in vitro* studies from our group demonstrated that *P. glauca* crude extract did not affect muscle glucose uptake or adipogenesis [9], yet exhibited potent effects for diabetes complications (neuroprotective action) [38]. The present results add an additional therapeutic potential by demonstrating the ability of *P. glauca* to reduce the activity of the enzyme limiting hepatic glucose output.

Secondly, *L. laricina* extract was found to be a potent modulator of the glycogen storage pathway. Indeed, it increased GS activity severalfold more than a supraphysiological concentration of our insulin reference positive control. Likewise, the plant's capacity to enhance GSK-3 phosphorylation was the greatest of all Cree plants and, again, more than twofold that of insulin. Moreover, although *L. laricina* did not significantly alter G6Pase activity in H4IIE hepatocytes, it was among the top Cree plants stimulating the phosphorylation of Akt and AMPK, two important kinases involved in hepatic metabolic control [14, 25]. Such an apparently important antidiabetic potential is consistent with recent *in vitro* and *in vivo* studies from our group with the same crude extract of *L. laricina* used in the present work. Indeed, *in vitro*, it stimulated glucose uptake [8] and AMPK [34] in C2C12 myocytes while enhancing adipogenesis in 3T3-L1 cells [8]. In the mouse diet-induced obesity model, *L. laricina* demonstrated a clear capacity to reduce glycemia and improve insulin resistance [39]. The current study thus further validates the antidiabetic potential of *L. laricina* crude extracts and adds the liver as a putative target organ for the plant.

Finally, the results presented herein highlight the very strong action of *A. balsamea* on key enzymes and modulating kinases controlling hepatic glucose production. Indeed, the plant's crude extract reduced G6Pase and increased Akt phosphorylation to the same extent as insulin in H4IIE hepatocytes. It was also the most potent of all Cree plant extracts to enhance AMPK phosphorylation in the same cell line. Likewise, *A. balsamea* was the most potent activator of glycogen synthase in HepG2 cells when compared to other Boreal forest plant extracts, exerting an effect severalfold higher than insulin. This action was also associated with an enhanced phosphorylation of the key kinase GSK-3. Hence, *A. balsamea* crude extract was the only Cree plant to simultaneously and potently affect the two major pathways involved in hepatic glucose homeostasis. Together with the prior *in vitro* demonstration of the ability of this plant to enhance glucose uptake in muscle and adipose cells [8], this encourages more detailed study of the antidiabetic potential of the plant, notably through the determination of active principles and validation using *in vivo* animal models.

5. Conclusion

In summary, this study confirmed that Boreal forest plants of the Cree traditional pharmacopeia have the potential to reduce hepatic glucose output and hence provide beneficial therapeutic action in the context of the obesity-diabetes continuum of metabolic diseases. Our results also lend further support to the soundness of considering Cree traditional medicine and its associated medicinal plants as worthy complementary and alternative approaches to improve diabetes care and management, doing so in a culturally mindful and respectful manner.

Acknowledgments

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Research Article

Prescription Pattern of Chinese Herbal Products for Diabetes Mellitus in Taiwan: A Population-Based Study

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Background. Traditional Chinese medicine (TCM), when given as a therapy for symptom relief, has gained widespread popularity among diabetic patients. The aim of this study is to analyze the utilization of TCM among type 2 diabetic patients in Taiwan. *Methods.* The use of TCM for type 2 diabetic patients were evaluated using a randomly sampled cohort of 1,000,000 beneficiaries recruited from the National Health Insurance Research Database. *Results.* Overall, 77.9% (n = 31,289) of type 2 diabetic patients utilized TCM and 13.9% (n = 4,351) of them used TCM for the treatment of type 2 diabetes. Among the top ten most frequently prescribed herbal formulae, four remedies, *Zhi-Bo-Di-Huang-Wan, Qi-Ju-Di-Huang-Wan, Ji-Sheng-Shen-Qi-Wan* and *Ba-Wei-Di-Huang-Wan* are derivative formulae of *Liu-Wei-Di-Huang-Wan.* In other words, *Liu-Wei-Di-Huang-Wan* and its derivatives were found to be the most common herbal formulae prescribed by TCM doctors for the treatment of diabetes in Taiwan. *Conclusion.* Although some evidence does support the use TCM to treat diabetes, the results from the current study may have been confounded by placebo effect, which emphasize the need for well conducted, double-blind, randomized, placebo-controlled studies in order to further evaluate the efficacy of *Liu-Wei-Di-Huang-Wan* on patients with type 2 diabetes.

1. Introduction

Type 2 diabetes is becoming a pandemic disorder, and the related alarming increase in the prevalence of both microvascular and macrovascular disease has raised significant concerns [1–7]. Importantly, clinically significant morbidity is present at diagnosis, but the development of diabetes-related microvascular and macrovascular diseases may occur much earlier and well before diagnosis. Due to a lack of appropriate care for preclinical diabetes, diabetes expenditures have grown dramatically annually due to increased medical care required by patients with diabetes-related complications. Even worse, although many drugs improve glycemic control, they do not necessarily provide real-world benefits. Previous reports have indicated that combination therapy with metformin and glyburide and the use of thiazolidinediones increase the risk of a composite end point involving cardiovascular events and mortality [8, 9]. In addition, some diabetes medication unfortunately results in a number of common side effects such as nausea or upset stomach; these unwanted conditions drive patients to seek alternative advice [10]. Therefore, despite recent advances in intensive glycemic control, diabetes mellitus continues to be an important public health concern because it causes substantial morbidity and mortality as well as long-term complications [11, 12]. Not surprisingly, alternative therapies have become increasingly popular and are quickly approaching conventional therapy in their frequency of use as a treatment for diabetes and/or diabetes-related complications [13–15].

Previous studies of traditional Chinese medicines have found that *tianhuafen* (Radix Trichosanthis) [16], *gegen* (Radix Puerariae) [17], *maimendong* (Radix Ophiopogonis) [18],

rougui (Cortex Cinnamomi) [19, 20], *huangbai* (Cortex Phellodendri) [21] and *aconitum* (Aconitum carmichaeli) [22] may have antidiabetic activity, and *dihuang* (Radix Rehmanniae) [23], *shanzhuyu* (Fructus Corni) [24], *renshen* (Radix Ginseng) [25], *gancao* (Radix Glycyrrhizae Praeparata) [26], and *bitter orange* (Aurantii Fructus) [27] have been suggested to increase insulin secretion. Unfortunately, evidence obtained in human studies is limited regarding patterns of use of classical traditional Chinese medicine (TCM) in relation to type 2 diabetes, which seem to be an area in which complementary and alternative medicines have recently grown in popularity. Furthermore, TCMs now seems to be marketed without established efficacy or safety in many Western countries [28, 29]. Owing to the previously and to a lack of knowledge about the prescription profile of TCMs, researchers and conventional doctors have found it difficult to explore the potential mechanisms of TCM therapies targeting type 2 diabetes. Furthermore, it also has proved difficult to assess the cost effectiveness of using TCM therapy and to observe the interaction between Chinese herbs and conventional diabetes drugs.

TCM, which includes acupuncture, traumatology manipulative therapies, and Chinese herbal products, has been an important part of health care in Taiwan for hundreds of years and is fully reimbursed under the current National Health Insurance (NHI) system. Previous studies using the NHI research database have reported that female [30] and individuals aged in their 30s [31] are more likely to use TCM among the general population in Taiwan. Chinese herbal remedies are the most common TCM modality used by this population, followed by acupuncture and traumatology manipulative therapies. The unique approach to TCM diagnosis involves gathering clinical symptoms and signs, and then a treatment principle is put forward in accordance with the aforementioned diagnostic process. In this situation, researchers in Taiwan have found that symptoms, signs, and ill-defined conditions are one of the most common reasons for TCM visits across various different patient populations [32–34]. Accordingly, the claims that database, part of the Taiwan National Health Insurance Research Database, provides are a platform for understanding the utilization of TCM therapies by licensed TCM doctors [30, 31, 35]. The aim of our study is to analyze a random sample from this comprehensive database and to determine the TCM utilization patterns of newly diagnosed type 2 diabetes patients in Taiwan. The results of this study should provide valuable information that will enable physicians to respond to patient use of TCM in an informed way, which will in turn strengthen further the patient-physician relationship when treating diabetes and diabetes-related complications.

2. Materials and Methods

2.1. Data Resources. This study was designed as a population-based study analyzing a sample of one million subjects selected at random from the 22 million beneficiaries of the National Health Insurance scheme of Taiwan and aimed to determine the prevalence of using prescribed Chinese herbal prescriptions (CHP) among diabetes patients between January 1, 1998 and December 31, 2008. All data were obtained from the National Health Insurance Research Database (NHIRD), which includes all the reimbursement data of the NHI with the identification numbers of all individuals being encrypted and transformed; this database is maintained by the National Health Research Institutes of Taiwan [36]. The NHIRD database contains patient's gender and date of birth, all records of clinical visits and hospitalization, prescribed drugs and dosages, including CHP, and three major diagnoses coded in the *International Classification of Diseases, Ninth Revision,* and *Clinical Modification* (ICD-9-CM) formats [37].

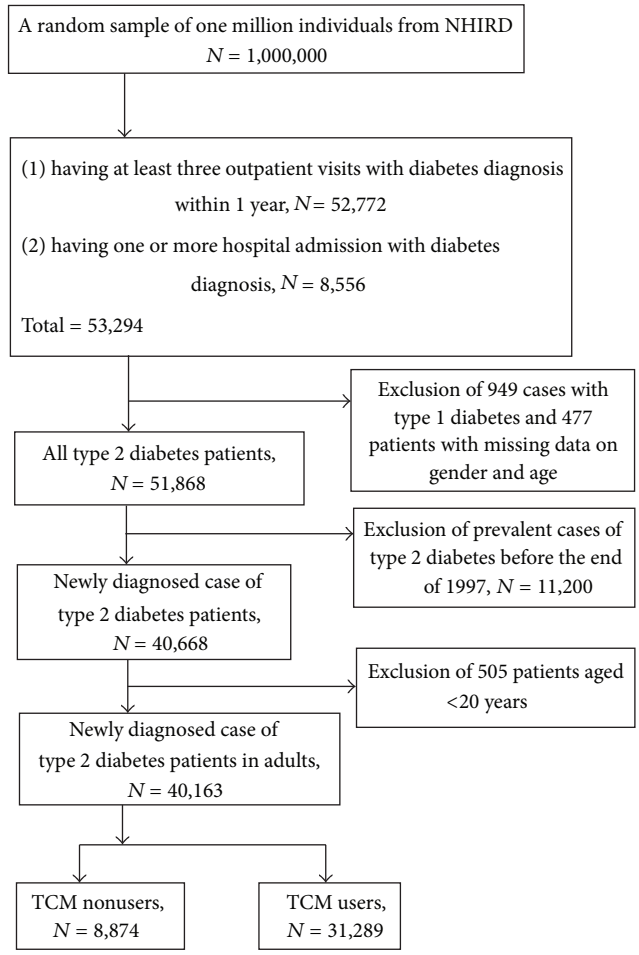

Figure 1: Flow recruitment chart of subjects from the one million random samples obtained from the National Health Insurance Research Database (NHIRD), 1997 to 2008, in Taiwan.

2.2. Study Subjects. The selection of study subjects from the random sample of one million individuals was performed as follows (Figure 1). First, we included all patients that (1) had at least three outpatient visits with a diabetes diagnosis within 1 year (n = 52,772) or (2) having one or more hospital admission with diabetes diagnosis (n = 8,556) [38]. A total of 53,294 subjects were obtained. Second, we excluded all patients with type 1 diabetes (n = 949) or with missing information on gender and age (n = 477). Third, cases of diabetes

($n = 11,200$) that had been diagnosed before the end of 1997 were also excluded to ensure that all the subjects included were newly diagnosed with type 2 diabetes in the time period 1998–2008. Fourth, subjects under 20 years of age ($n = 505$) were also excluded to limit the study sample to adults. Finally, 40,163 study subjects were included in the study cohort.

2.3. Study Variables. To determine the key independent variables for utilization of TCM among diabetes patients, we selected a series of demographic factors based on previous studies [3, 38–41]. The subjects were categorized into four groups according to age: 20–39, 40–59, 60–79, and ≥80 years. Taiwan was divided into seven geographic regions: Taipei city, Kaohsiung city, Northern region, Central region, Southern region, Eastern region and Outlying islands. We split the monthly wage of individuals into four levels: new Taiwan dollars (NT$) 0, 1–19,999, 20,000–39,999, and ≥40,000. We also searched the NHIRD database for clinical complications and treatment records related to diabetes as independent variables. The complications associated with diabetes included nephropathy (ICD-9 code, 581.81, 583.81, 585.1–585.9), retinopathy (362.01–362.07, 365.44, 366.41, 369.00–369.9), neuropathy (353.5, 536.3, 354.0–355.9, 713.5, 337.1, 357.2), peripheral circulatory disorders (250.70), and other specified manifestations (250.80) [42]. The reimbursement database contains all details related to the prescription of conventional medicines for treating diabetes mellitus. Then, for the final analysis, we categorized the types of preparations into the following categories: sulfonylureas, biguanides, α-glucosidase, meglitinide, thiazolidinediones, guar gum, and insulin.

2.4. Statistical Analysis. Data analysis consisted of descriptive statistics, including the prescription rates of TCM users stratified by patient's demographic characteristics, indications for the prescription of TCM, and the most frequently prescribed herbal formulae used when treating diabetes. Primary indications were classified according to their ICD-9 code. The diagnoses were coded according to the ICD-9 and grouped into a series of distinct broad disease categories. The potential effects of Chinese herbs contained in the ten most commonly prescribed CHPs were grouped according to previous *in vivo* and *in vitro* studies and are summarized in Table 4 [16–27]. Multiple logistic regression was conducted to evaluate the factors that correlated with TCM use. A significance level of $\alpha = 0.05$ was selected. The statistical software package SAS 9.13 was used for data management and analysis.

3. Results

The database of outpatient claims contained information on 40,163 patients with type 2 diabetes from 1998 to 2008. Among them, 31,289 (77.9%) patients used TCM outpatient services at least once. Most TCM users (91.2%) also received diabetes treatment. Among all TCM users, 13.9% ($n = 4,351$) used TCM for the treatment of type 2 diabetes. Details of the demographic distribution of TCM users and nonusers are presented in Table 1. The mean age of TCM nonusers was slightly higher than that of TCM users. There were more TCM users than TCM nonusers with an income level of NT$ 20,000–39,999 or residing in Central Taiwan.

The adjusted odds ratios (aORs) and 95% confidence intervals (95% CIs) obtained by multiple logistic regression are also presented in Table 1. Compared with the age group 40–59 years (aOR = 1.00), those aged 20–39 years were more likely to be TCM users. There was also a significant difference between TCM users and nonusers with there being more of the former in the income group of NT$20,000–39,999. After adjusting for other factors, patients with more type 2 diabetes chronic complications (one complication: OR = 1.10, 95% CI: 1.04–1.16; two complications: OR = 1.28, 95% CI: 1.19–1.38; more than three complications: OR = 1.25, 95% CI: 1.13–1.38) were more likely to seek TCM treatment than those with no chronic complication (aOR = 1.00). There was no significant difference in the diabetes treatment modalities received (monotherapy (aOR = 1.00) or combination therapy) between TCM users and TCM nonusers, except among those who took more than five types of antidiabetic drugs (OR = 1.13, 95% CI: 1.01–1.26) for the control of blood sugar or HbA1c.

Among the diabetes patients visiting TCM doctors, 3,627,622 (92.6%) visits involved the prescription of TCM, while the rest were prescribed acupuncture and traumatology manipulative therapies. Analysis of the major disease categories for all TCM visits made by 31,289 TCM users are summarized in Table 2. The findings show that "symptoms, signs, and ill-defined conditions" were the most common reason for using Chinese herbal prescriptions (CHP) (16.8%, $n = 608,535$), followed by "endocrine, nutritional and metabolic diseases, and immunity disorders" (12.1%, $n = 439,612$), and "diseases of digestive system" (11.5%, $n = 417,611$). Details of the most frequently prescribed CHP for treating type 2 diabetes by TCM doctors are provided in Table 3. *Liu-Wei-Di-Huang-Wan* (Rehmannia six pill) was the most frequently prescribed CHP, followed by *Bai-Hu-Jia-Ren-Shen-Tang* (white tiger plus ginseng combination), *Zhi-Bo-Di-Huang-Wan* (zhibai Rehmannia six pill), *Qi-Ju-Di-Huang-Wan* (chichu Rehmannia pill), *Yu-Quan-Wan* (jade spring pill), *Ji-Sheng-Shen-Qi-Wan* (economic health shenqi pill), *Xue-Fu-Zhu-Yu-Tang* (persica and achyranthes combination), *Ba-Wei-Di-Huang-Wan* (eight-flavour Rehmannia pill), *Bai-Hu-Tang* (white tiger combination), and *Gan-Lu-Yin* (sweet combination drink). Among the top ten most frequently prescribed herbal formulae, *Zhi-Bo-Di-Huang-Wan, Qi-Ju-Di-Huang-Wan, Ji-Sheng-Shen-Qi-Wan*, and *Ba-Wei-Di-Huang-Wan* are four derivative formulae of *Liu-Wei-Di-Huang-Wan,* which all contain Rhizoma Rehmanniae Preparata, Fructus Corni, Rhizoma Dioscoreae, Rhizoma Alismatis, Cortex Moutan Radicis, and Poria. In other words, *Liu-Wei-Di-Huang-Wan* and its various derivatives are the most common herbal formulae prescribed by TCM doctors for the treatment of diabetes in Taiwan. The ten most frequently prescribed CHPs include Chinese herbs that have been historically used to lower serum glucose. The potential effects of these Chinese herbs when used to treat type 2 diabetes are summarized in Table 4 and include increasing insulin secretion, enhancing glucose uptake by adipose and muscle

Table 1: Demographic characteristics and results of multiple logistic regressions showing the adjusted odds ratio (aOR) and 95% CI (confidence interval) for diabetes from 1998 to 2008 in Taiwan.

Characteristic	All patients	TCM[a] nonusers (%)	TCM users (%)	aOR[b] (95% CI[c])
Number of cases	40,163	8,874	31,289	
Gender				
Male	20,971 (52.2)	5,718 (64.4)	15,253 (48.7)	1.00
Female	19,192 (47.8)	3,156 (35.6)	16,036 (51.3)	1.98 (1.88–2.08)
Age at diagnosis (years)				
Mean ± SD	56.7 ± 12.4	58.3 ± 12.9	56.3 ± 12.2	
20~39	3,175 (7.9)	609 (6.9)	2,566 (8.2)	1.11 (1.01–1.22)
40~59	20,523 (51.1)	4,184 (47.1)	16,339 (52.2)	1.00
60~79	15,244 (38.0)	3,653 (41.2)	11,591 (37.0)	0.79 (0.75–0.83)
≥80	1,221 (3.0)	428 (4.8)	793 (2.5)	0.46 (0.41–0.52)
Insured salaries (NT$[d]/month)				
0	9,981 (24.9)	2,207 (24.9)	7,774 (24.9)	1.00
1–19,999	21,256 (52.9)	4,801 (54.1)	16,455 (52.6)	1.01 (0.95–1.08)
20,000–39,999	5,414 (13.5)	982 (11.1)	4,432 (14.2)	1.33 (1.21–1.46)
≥40,000	3,512 (8.7)	884 (9.9)	2,628 (8.4)	1.02 (0.92–1.12)
Insured region				
Taipei city	6,975 (17.4)	1,746 (19.7)	5,229 (16.7)	1.00
Kaohsiung city	2,802 (7.0)	606 (6.8)	2,196 (7.0)	1.21 (1.09–1.35)
Northern Taiwan	11,316 (28.2)	2,652 (29.9)	8,664 (27.7)	1.10 (1.02–1.18)
Central Taiwan	7,172 (17.8)	1,070 (12.1)	6,102 (19.5)	1.92 (1.76–2.09)
Southern Taiwan	10,447 (26.0)	2,436 (27.4)	8,011 (25.6)	1.12 (1.04–1.20)
Eastern Taiwan	1,130 (2.8)	288 (3.3)	842 (2.7)	0.97 (0.83–1.12)
Outlying islands	319 (0.8)	75 (0.8)	244 (0.8)	1.11 (0.85–1.45)
Number of diabetic complications				
0	17,913 (44.6)	4,213 (47.5)	13,700 (43.8)	1.00
1	12,833 (32.0)	2,823 (31.8)	10,010 (32.0)	1.10 (1.04–1.16)
Nephropathy	2,990 (7.4)	724 (8.1)	2,266 (7.2)	
Retinopathy	2,764 (6.9)	674 (7.6)	2,090 (6.7)	
Neuropathy	5,339 (13.3)	993 (11.2)	4,346 (13.9)	
Peripheral circulatory disorders (PCD)	844 (2.1)	213 (2.4)	631 (2.0)	
Other specified manifestations	896 (2.2)	219 (2.5)	677 (2.2)	
2	6,296 (15.7)	1,226 (13.8)	5,070 (16.2)	1.28 (1.19–1.38)
Nephropathy + retinopathy	1,039 (2.6)	241 (2.7)	798 (2.5)	
Nephropathy + neuropathy	1,588 (4.0)	300 (3.4)	1,288 (4.1)	
Nephropathy + PCD	256 (0.6)	64 (0.7)	192 (0.6)	
Nephropathy + others	273 (0.7)	61 (0.7)	212 (0.7)	
Retinopathy + neuropathy	1,579 (3.9)	250 (2.8)	1,329 (4.3)	
Retinopathy + PCD	234 (0.6)	45 (0.5)	189 (0.6)	
Retinopathy + others	206 (0.5)	46 (0.5)	160 (0.5)	
Neuropathy + PCD	582 (1.4)	107 (1.2)	475 (1.5)	
Neuropathy l + others	429 (1.1)	81 (0.9)	348 (1.1)	
PCD + others	110 (0.3)	31 (0.4)	79 (0.3)	
≥3	3,121 (7.7)	612 (6.9)	2,509 (8.0)	1.25 (1.13–1.38)

Table 1: Continued.

Characteristic	All patients	TCM[a] nonusers (%)	TCM users (%)	aOR[b] (95% CI[c])
Number of medical treatments for diabetes				
None	3,370 (8.4)	627 (7.1)	2,743 (8.8)	
Monotherapy[e]	6,048 (15.1)	1,443 (16.3)	4,605 (14.7)	1.00
Two-drug combination[f]	13,609 (33.9)	3,127 (35.2)	10,482 (33.5)	1.01 (0.94–1.09)
Three-drug combination	8,675 (21.6)	1,937 (21.8)	6,738 (21.5)	1.00 (0.93–1.09)
Four-drug combination	5,026 (12.5)	1,093 (12.3)	3,933 (12.6)	1.01 (0.92–1.10)
Over five-drug combination	3,435 (8.5)	647 (7.3)	2,788 (8.9)	1.13 (1.01–1.26)

[a]TCM refers to traditional Chinese medicine; [b]OR refer to odds ratio; [c]CI refers to confidence interval; [d]NT$ refers to new Taiwan dollars, of which US$ 1 = NT$30 approximately.
[e]Monotherapy is the use of a single antidiabetic drug (sulfonylureas, biguanides, α-glucosidase, meglitinide, thiazolidinediones, guar gum, or insulin).
[f]combination therapy is the use of more than one antidiabetic drug.

tissues, inhibiting glucose absorption from intestine, inhibiting glucose production from hepatocytes, and decreasing insulin resistance or enhancing insulin sensitivity.

4. Discussion

The prevalence of type 2 diabetes in Taiwan over the 11 years in the study was 4.0%, which is in line with the estimates given by previous surveys [1, 43]. Worthy of note, the utilization of TCM among adults with type 2 diabetes in Taiwan during the study period was 77.9%, which appears to be high compared with previous findings [14, 15]. TCM is a unique traditional therapy approach for various ailments that has been used in Taiwan for over hundreds of years, and this long period of use may contribute significantly to the high prevalence of TCM usage among type 2 diabetic subjects. In addition, it should be noted that TCM treatment is covered by the NHI system. Therefore, unsurprisingly, the prevalence of CHP for treating type 2 diabetes among adults is comparatively higher in Taiwan than in other countries [15, 44]. The present study includes all patients who were newly diagnosed with type 2 diabetes by qualified conventional doctors between 1998 and 2008 from a random sample of one million subjects among the insured general population; importantly the rate of insured individuals has been consistently above 96% since 1997, and therefore we can rule out the possibility of selection bias.

The present results show that, although 91% of type 2 diabetic patients in Taiwan have received antidiabetic treatment, over half of them still have suffered from one or more diabetes complications during the 11-year follow-up. Nephropathy and neuropathy were the two most common diabetes complications. One possibility is that type 2 diabetes has a long asymptomatic preclinical phase that is likely to go undetected [45–49], and the injurious effects of asymptomatic hyperglycemia, therefore, have resulted in a high incidence of microvascular and macrovascular complications [4, 42]. The present study found that patients with type 2 diabetes who developed more than one site of involvement re-diabetes complications were more likely to seek advice from a TCM doctor. However, regardless of their experience in receiving more than one type of antidiabetic drug with the aim of improving their poor control of serum blood sugar, the choice of any of the major medical options available to patients with type 2 diabetes was not associated with the use of TCM. Hence, we suggest that when TCM is used to treat type 2 diabetes in Taiwan, this is generally an adjunct to diabetes treatment rather than a replacement for it.

The present findings show that, among diabetes patients, females and those aged 20–39 years were more likely to be TCM users than males and other age groups as shown in Table 1. As shown in Table 2, "symptoms, signs, and ill-defined conditions" were the most common reasons for using CHP (16.8%, $n = 608,535$), followed by "endocrine, nutritional and metabolic diseases, and immunity disorders" (12.1%, $n = 439,612$) and "diseases of digestive system" (11.5%, $n = 417,611$). Further analysis found that TCM doctors tended to use Chinese herbal remedies targeting diabetes as well as gastrointestinal disorders that might be the uncomfortable side effects of diabetes drugs. Although previous studies have demonstrated that acupuncture might be related to an alternative therapy for treating hyperglycemia and diabetes complications, the present study indicated that acupuncture in Taiwan is used by this study population mainly for diseases of the musculoskeletal system and connective tissue.

Liu-Wei-Di-Huang-Wan was the most frequently prescribed formula for treating type 2 diabetes in Taiwan during the study period, as shown in Table 3. *Liu-Wei-Di-Huang-Wan* is among the most highly regarded ancient Chinese herbal formulae and was first documented in the classical Chinese text *Xiao Er Yao Zheng Zhi Jue* (Key to Therapeutics of Children's Diseases) circa 1119 A.D. In the classical literature, *Liu-Wei-Di-Huang-Wan* is said to nourish yin and to invigorate the kidney, which might indicate it as a potentially efficacious therapy for reducing hyperglycemia and relieving neuropathic and nephropathic complications in diabetes mellitus [50–53]. Among the top ten most frequently prescribed formulae for treating type 2 diabetes, *Zhi-Bo-Di-Huang-Wan*, *Qi-Ju-Di-Huang-Wan*, *Ji-Sheng-Shen-Qi-Wan*, and *Ba-Wei-Di-Huang-Wan*, which are all derivatives of *Liu-Wei-Di-Huang-Wan*, are prescribed to alleviate various common symptoms of type 2 diabetes, namely, unusual thirst, blurred vision, frequent urination, and cold feeling in the

Table 2: Frequency distribution of traditional Chinese medicine (TCM) visits by major disease categories (according to 9th ICD codes) among diabetes patients from 1998 to 2008 in Taiwan.

Major disease category	ICD-9-CM codes	No. of visits (no. of patients)		
		Chinese herbal remedies	Acupuncture or manipulative therapies	Total of TCM
Infectious and parasitic diseases	001–139	21,083 (761)	69 (13)	21,152 (772)
Neoplasms	140–239	33,132 (366)	613 (20)	33,745 (381)
Endocrine, nutritional and metabolic diseases, and immunity disorders	240–279	439,612 (5,565)	1,995 (127)	441,607 (5,620)
Diabetes	250	377,621 (4,328)	1,505 (65)	379,126 (4,350)
Others		61,991 (1,703)	490 (62)	62,481 (1,742)
Mental disorders	290–319	24,834 (858)	536 (28)	25,370 (876)
Diseases of nervous system and sense organs	320–389	104,033 (3,962)	4,593 (802)	108,626 (4,513)
Diseases of circulatory system	390–459	180,821 (3,647)	5,857 (444)	186,678 (3,892)
Diseases of respiratory system	460–519	377,262 (10,505)	1,423 (126)	378,685 (10,537)
Diseases of digestive system	520–579	417,611 (9,387)	1,332 (129)	418,943 (9,432)
Diseases of genitourinary system	580–629	171,262 (4,145)	1,294 (63)	172,556 (4,170)
Diseases of skin and subcutaneous tissue	680–709	61,387 (2,784)	280 (41)	61,667 (2,810)
Diseases of musculoskeletal system and connective tissue	710–739	318,920 (8,808)	82,936 (12,682)	401,856 (16,932)
Symptoms, signs, and ill-defined conditions	780–799	608,535 (14,216)	3,839 (458)	612,374 (14,345)
Injury and poisoning	800–999	17,994 (1,389)	90,542 (14,141)	108,536 (14,625)
Supplementary classification[+]	V01–V82,	115 (9)	0 (0)	115 (9)
	E800–E999	0 (0)	0 (0)	0 (0)
Others[*]		851,021 (15,034)	96,311 (11,164)	947,332 (18,954)
Total		3,627,622 (27,135)	291,620 (22,891)	3,919,242 (31,289)

[*] Others include ICD-9-CM codes 280–289, *630–677, 740–759,* 760–779 and missing/error data; [+] Supplementary classification of factors influencing health status and contact with health service, external causes of injury and poisoning.

Table 3: Ten most common herbal formulae prescribed by TCM doctors for the treatment of type 2 diabetes among 31,289 patients from 1998 to 2008 in Taiwan.

Herbal formulae	English name	Number of person-days $N = 775,447$ (%)	Average daily dose (g)	Average duration for prescription (days)
Liu-Wei-Di-Huang-Wan	Rehmannia six pill	62,249 (8.0)	8.0	47.9
Bai-Hu-Jia-Ren-Shen-Tang	White tiger plus ginseng combination	42,676 (5.5)	7.5	47.4
Zhi-Bo-Di-Huang-Wan	Zhibai Rehmannia six pill	37,918 (4.9)	5.7	45.4
Qi-Ju-Di-Huang-Wan	Chichu Rehmannia pill	37,796 (4.9)	5.7	64.4
Yu-Quan-Wan	Jade spring pill	35,878 (4.6)	5.6	54.8
Ji-Sheng-Shen-Qi-Wan	Economic health shenqi pill, life-saving renal Chi pill	27,347 (3.5)	7.1	43.6
Xue-Fu-Zhu-Yu-Tang	Persica and achyranthes combination	22,708 (2.9)	5.3	45.9
Ba-Wei-Di-Huang-Wan	Eight-flavour Rehmannia pill	19,247 (2.5)	9.1	41.5
Bai-Hu-Tang	White tiger combination	18,801 (2.4)	7.0	42.1
Gan-Lu-Yin	Sweet combination drink	18,502 (2.4)	5.1	38.1

Table 4: Potential effects of herbs present in the ten most common herbal formulae prescribed by TCM doctors for treating type 2 diabetes.

Herbal formulae	Number of herbs	Ingredient herbs
Liu-Wei-Di-Huang-Wan	6	Rhizoma Rehmanniae Praeparata[A,B,D,E], Fructus Corni[A,D], Rhizoma Dioscoreae[B,E], Rhizoma Alismatis[B], Cortex Moutan Radicis, Poria[B,E].
Bai-Hu-Jia-Ren-Shen-Tang	5	Gypsum Fibrosum, Rhizoma Anemarrhenae[E], Radix Glycyrrhizae Praeparata[A], Semen Oryzae Sativae, Radix Ginseng[A,B,C,D].
Zhi-Bo-Di-Huang-Wan	8	Rhizoma Anemarrhenae[E], Cortex Phellodendri, Rhizoma Rehmanniae Praeparata[A,B,D,E], Fructus Corni[A,D], Rhizoma Dioscoreae[B,E], Rhizoma Alismatis[B], Cortex Moutan Radicis, Poria[B,E].
Qi-Ju-Di-Huang-Wan	8	Flos Chrysanthemi, Fructus Lycii[B,E], Rhizoma Rehmanniae Praeparata[A,B,D,E], Fructus Corni[A,D], Rhizoma Dioscoreae, Rhizoma Alismatis[B], Cortex Moutan Radicis, Poria[B,E].
Yu-Quan-Wan	9	Radix Trichosanthis, Radix Puerariae[A,B], Radix Ophiopogonis[A,C,D], Radix Ginseng, Poria[B,E], Radix Astragali[B,E], Radix Glycyrrhizae Praeparata, Fructus Mume, Radix Astragali Praeparata.
Ji-Sheng-Shen-Qi-Wan	10	Semen Plantaginis, Radix Achyranthis Bidentatae, Ramulus Cinnamomi, Radix Aconiti, Rhizoma Rehmanniae Praeparata, Fructus Corni, Rhizoma Dioscoreae[B,E], Rhizoma Alismatis[B], Cortex Moutan Radicis, Poria[B,E].
Xue-Fu-Zhu-Yu-Tang	11	Chinese Angelia Root, Rhizoma Rehmanniae Praeparata, Peach Kernel, Safflower, Bitter Orange[A], Red Peony Root, Bupleurum Root, Glycyrrhiza, Platycodon Root, Chuanxiong Rhizome, Cyathula Root.
Ba-Wei-Di-Huang-Wan	8	Ramulus Cinnamomi[E], Radix Aconiti[B], Rhizoma Rehmanniae Praeparata, Fructus Corni, Rhizoma Dioscoreae, Rhizoma Alismatis, Cortex Moutan Radicis, Poria[B,E].
Bai-Hu-Tang	4	Gypsum Fibrosum, Rhizoma Anemarrhenae, Radix Glycyrrhizae Praeparata, Semen Oryzae Sativae.
Gan-Lu-Yin	10	Rhizoma Rehmanniae, Radix Ophiopogonis[A,C,D], Radix Glycyrrhizae Praeparata, Herba Dendrobii, Radix Asparagi, Eriobotryae Folium, Bitter Orange[A], Scutellariae radix, Wormwood Herb, Rhizoma Rehmanniae Praeparata.

[A]Increase in insulin secretion, [B]enhancement of glucose uptake by adipose and muscle tissues, [C]inhibition of glucose absorption by the intestine, [D]inhibition of glucose production by hepatocytes, and [E]decrease in insulin resistance or enhancement of insulin sensitivity.

limbs, respectively. Other frequently prescribed formulae are associated with severe dysphoric thirst and lassitude (*Bai-Hu-Jia-Ren-Shen-Tang* or white tiger plus ginseng decoction, *Bai-Hu-Tang* or white tiger decoction, *Yu-Quan-Wan* or jade spring combination, and *Gan-Lu-Yin* or sweet dew decoction or sweet combination drink) and with peripheral neuropathy due to blood stasis (*Xue-Fu-Zhu-Yu-Tang* or Persica and Carthamus Combination). Although, previous *in vitro* studies have found that some Chinese herbs are able to decrease serum levels of glucose, glycosylated proteins, and hemoglobin A1C, possibly by blocking intestinal absorption and/or inhibiting hepatic glucose-6-phosphatase [50, 54], there have not yet been any clinical trials that have demonstrated the efficacy and safety of *Liu-Wei-Di-Huang-Wan* and its derivatives when treating diabetes type 2. In general, TCM doctors treated diabetes patients' complaints according to the syndrome differentiation theory rather than by making a specific diagnosis; this is based on holistic consideration of diabetes patients who are suffering from symptoms and complications at various sites. In this context and in line with previous results [51], the present study found that TCM doctors in Taiwan prescribed herbal therapies mainly to optimize the body's ability to function normally, rather than as a cure for diabetes. Moreover, despite inadequate data on the clinical safety and efficacy of CHP when treating diabetic patients, a large number of patients use them. Thus, based on the present trend in TCM utilization, herbal remedies for treating diabetes and/or diabetic complications will continue to be used. Although we respect the patients' choice of medical care, we recommend that TCM practitioners and physicians should carefully monitor patient blood glucose levels and the potential side effects of CHP when they are being used alongside or in lieu of diabetes drugs. Further studies are warranted to assess the formulae generally used by TCM doctors in this study in order to determine whether they are really useful as add-on treatments for patients receiving antidiabetic treatment.

The present study has three limitations. First, this study did not include Chinese herbal remedies or decoctions that were purchased directly from TCM herbal pharmacies, nor did we include health foods containing herbs. Thus, the frequency of CHP utilization might have been underestimated. However, because the NHI system covers TCM prescriptions, which generally cost less than the herbs sold in Taiwan's markets, the likelihood that subjects purchased a lot of other herbs outside the NHI database is not high. Second, we are unable to draw any conclusion about the relationship of blood glucose and haemoglobin A1C levels with respect to TCM utilization owing to the lack of actual clinical data. Third, this was a retrospective study and thus does not include a

randomized placebo group. Thus, great caution is necessary when interpreting the results of the most commonly prescribed Chinese formulae obtained in the present study due to the possibility of a placebo effect.

5. Conclusions

Our results suggest that, based on the coexistence of both conventional and traditional Chinese medical treatments, of most the diabetes patients consume herbal therapies with the intention of relieving their diabetes-related symptoms, rather than because they have rejected standard diabetes treatments. *Liu-Wei-Di-Huang-Wan* and its derivatives are the most frequently prescribed formulae by TCM doctors in Taiwan for diabetes patients. Having recognized the use of TCM, exploring any potential interactions and adverse effects, and integrating both technologies into a holistic treatment system may be beneficial to the overall health, presence of comorbidities, and quality of life, of patients with type 2 diabetes. It is worth noting that although some evidence does support the use of TCM to treat diabetes, the results from the current study may have been confounded by the placebo effect. This emphasizes the need for well-conducted, double-blind, randomized, placebo-control studies to further evaluate efficacy when *Liu-Wei-Di-Huang-Wan* is given to patients with type 2 diabetes.

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Review Article

Adjunct Methods of the Standard Diabetic Foot Ulceration Therapy

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The outcome of management of diabetic foot ulceration (DFU) is poor and insufficient. DFU therapy includes the standard management as debridement of the wound, revascularization procedures, off-loading of the ulcer and antibacterial actions, and supplementation of growth factors and cytokines, leading to stimulation of granulation, epidermization, and angiogenesis. The aim of the present review is to summarize the adjunct methods of the standard DFU therapy as hyperbaric oxygen therapy (HBOT), maggot therapy (MT), and platelet-rich plasma therapy (PRPT). The results of preclinical and clinical trials indicated that the methods may reduce time of therapy, short-term morbidity, and the risk of major amputation.

1. Introduction

Diabetes mellitus (DM) is one of the most deceitful diseases that affect more than 371 million people all over the world in 2012; by 2030 this will rise to 552 million [1]. The disease often leads to the development of serious, health threatening complications [2]. Of all diabetic complications, diabetic foot syndrome (DFS) is one of the most devastating and costly [2, 3]. According to the WHO, DFS are an infection, ulceration, and/or destruction of deep tissue, that comes along with neurologic abnormalities and/or different stages of arterial closure disease in the lower limbs. The International Consensus on the Diabetic Foot defined a diabetic foot ulcer as a full thickness wound below the ankle in a person with diabetes, irrespective of duration [4].

Diabetic foot ulceration (DFU) develops in 15–25% of DM patients. Approximately 15–25% of those cases require amputation [4, 5]. Some estimates have stated that the likelihood of amputation is 25–30 times higher among patients with diabetes than in the general population. Various studies have shown that the rates of major amputation of the diabetic foot are now decreasing at the regional level with rates declining from around 550 to 160–360 per 100,000 patients with diabetes, but the rates of minor amputation (toe/forefoot) have not changed [6].

Ethiopathogenetic factors of DFU involve neuropathic, ischaemic, mechanic, metabolic and systemic risk factors, and infection (superinfection). Peripheral neuropathy is the most important causal pathway leading to foot ulceration and often leads to sensory deficit with the loss of protective pain sensation. Ischemia, on the other hand, results from atherosclerotic peripheral vascular disease, which usually affects the distal vessels of the lower limb [7, 8]. Infection can complicate any type of diabetic foot ulcer and is one of the most common causes of hospital admission among people with diabetes. Pathogenetic factors include the increased collagen deposition and network by advanced glycosylation end products, the loss of adipose tissue, and the occurrence of edema, which destroy the compensating balance between preventive and damaging factors.

Neuropathic changes occur in approximately 85% [6] and ischaemic in 10–60% of cases [9, 10]. DFU is often associated with a secondary bacterial infection causing inflammation of the skin, subcutaneous tissue, muscles, tendons, and bones leading to necrosis of those tissues. Chronic ulceration or limb amputation, besides mutilation of a patient, is associated also with reduction of his/her dexterity and quality of life and other numerous threats for the patient's life and health [11, 12].

Therapy of DFU complications constitutes also a social and economic problem [13]. Treatment requires the know-how of a specialized center in collaboration with different medical disciplines, for example, a diabetologist, surgeon, vascular surgeon, orthopaedist, radiologist, educator, shoemaker, and kinesiotherapist [4, 14]. The main purpose of that multidisciplinary foot care team is the prevention of DFU and its prompt therapy if the condition develops. The basic principles of prevention and treatment described in these guidelines are based on the International Consensus of the Diabetic Foot [4]. The standard of care for treating DFU includes optimization of glycemic control levels, appropriate nutrition, extensive debridement, infection elimination and dressings, and pressure relief in the areas of the foot. DFS therapy, following a possible vascular reconstruction, may consider a preventive surgery aimed at reduction of ulceration risk [14, 15]. However, even the best preventive management cannot exclude DM complications, and even the best DFU management based on standardised procedures gives no guarantee of a cure [10–15].

Knowledge regarding wound healing in DM patients has been rapidly expanding lately. It is a result of the development of new research on advanced therapeutic products, including stem cells, growth factors, skin substitutes, and gene therapy. Despite some promising results their efficacy remains unsatisfactory, and their combinations with the standard therapy often fails.

Interest is aroused by relatively efficient adjunct DFU treatment methods, including hyperbaric oxygen therapy (HBOT), maggot therapy (MT), and platelet-rich plasma therapy (PRPT). Those methods have been developing rapidly since the 1980s. The main reason for interest in those methods was the observation of rapidly increasing bacterial antibiotic resistance consequently leading to resignation of topical antibiotic application.

Wound healing is a complex and dynamic process in which the following factors play significant roles:

(1) inflammation and associated immunological processes,

(2) granulation and epidermization and associated cytokines and growth factors,

(3) bioregulator-stimulated neoangiogenesis process.

Therefore, the main aims of DFU therapy include antibacterial actions and supplementation of growth factors and cytokines, leading to stimulation of granulation, epidermization, and angiogenesis [16].

2. Hyperbaric Oxygen Therapy (HBOT)

For over 50 years, HBOT has been a method applied to selected, serious cases of nonhealing, and infection-complicated DFU resistant to other therapeutic methods. Application of the method was initially based on theoretical assumptions and then on experimental research [17–20].

HBOT comprises patient inhalation with pure oxygen at the pressure of 2-3 absolute atmospheres ATAs (1 ATA = 14.7 psi, 1 kg per square centimeter, 101.3 kPa, 760 torr, or 760 mm Hg) provided by appropriate single- and multipatient pressure chambers. A single session lasts for 70–120 minutes, usually 90 minutes, and the number of sessions usually exceeds 20. HBOT-related complications are rare and involve claustrophobia, ear, sinus, or lung damage due to the pressure, temporary worsening of short sightedness, and oxygen poisoning [21]. Besides the commonly known relative and absolute contraindications, transcutaneous oximetry (TcPO2) is considered an additional criterion of classification for HBOT, treated as a valuable prognostic factor for ulceration treated with the method [22]. In DFU patients, the TcPO2 method-measured oxygen pressure over 400 mm Hg at 2.5 ATA or over 50 mm Hg in pure oxygen environment at normal atmospheric pressure should be perceived as a good prognostic index [23–25].

Precise mechanism of action of HBOT in DFU healing has not been uncovered yet. Increased oxygen levels in wound environment instigate healing by a mechanism of angiogenesis. The process involves physical dissolution of oxygen in plasma, leading to increased supply of oxygen to hypoxia-affected tissues. In DFU pathogenesis, local and systemic metabolic disorders lead to abnormal oxygen supply to affected tissues, affecting locally the immunological system and favouring wound infection. Reduced activity of phagocytic macrophages, reduced chemotaxis, and adhesion of neutrophils are observed in DFU. Reduced immunity of tissues favours development of pathogenic bacterial flora, including anaerobic microorganisms. They release toxins causing hypoxia and oedema of tissues [26–29].

Hyperbaric chamber has a bactericidal and bacteriostatic effect. Oxygen administered under increased ambient pressure enhances *in vitro* phagocytosis in regions of limited perfusion by increasing local oxygen tension to levels consistent with normal phagocytic function [21]. At the pressure of 2.5 ATA and respiration with 100% oxygen, its tension in the plasma may be as high as 2000 mm Hg, causing a 10–15-fold increase in oxygen transport, a 4-fold increase in oxygen diffusion to tissues on the arterial side, and a double increase on the venous side of the capillary circulation [20, 21].

Oxygen is an important cellular signal regulating intracellular and intratissue transformations. Increased oxygen level in chronically hypoxic or ischaemic wounds stimulates proliferation and differentiation of epithelial cells and fibroblasts and collagen synthesis in fibroblasts. Oxygen is a potent proangiogene. The element increases neovascularisation by angiogenic stimulation leading to new blood vessel formation from local endothelial cells and by the stimulation of the systemic stem/progenitor cells to differentiate in the form of blood vessels [18, 20–23, 27, 28]. It was demonstrated

that HBOT stimulates vasculogenic stem cell mobilisation from bone marrow and recruits them to skin wound [29]. Increased tissue oxygenation during HBOT improves also tolerance to ischemia and reduces metabolic abnormalities in those tissues [30, 31].

The first broadly commented results of a nonrandomised clinical trial were published in 1987 by Baroni et al. [17]. The authors reported that 89% of patients with DFU endangered by amputation because of necrotic changes (16 of 18 patients) healed in the HBO group, whereas only 60% (6 of 10 patients) healed in the control group (only treated with standard therapy). The control group included patients who did not consent to additional application of HBOT (Table 1).

Case reports, case series, case control studies, and randomised controlled trials presented in the end of the 20th century encouraged the application of HBOT [37–41] (Table 1). However, those studies were accused of methodology differences, including, among others, lack of inclusion and exclusion criteria.

From the methodological point of view, the strongest evidence of HBOT efficacy is offered by the randomized, double-blinded, and placebo-controlled clinical trial by Abidia et al. [35] (Table 1), but the study was small and included only patients with Wagner grade 1 and 2 ulcers [15] (Table 2). The authors demonstrated a significant decrease of the wound areas in the treatment group in comparison to the control group. Moreover, the cost-effectiveness analysis has shown a potential saving in the total cost of treatment with HBOT for each patient during the study.

The study by Kalani et al. included 38 patients with ischemic ulcers without full-thickness gangrene. After three years, 76% of the 17 patients receiving HBOT had healed their ulcers to intact skin compared with 48% of those given conventional treatment [36] (Table 1). In the randomized trial by Kessler et al. [68], the effect of two daily 90 min sessions of HBOT five days a week for two weeks was compared with regular treatment in 28 hospitalized patients with neuropathic Wagner grade 1 to 3 ulcers. After two weeks of treatment, the reduction in ulcer area was doubled in the HBOT group. However, this improvement disappeared during the next two weeks of followup.

In the unblinded, randomized study by Duzgun et al. [34], the effect of HBOT was compared with standard therapy in 100 patients with a foot ulcer duration of at least four weeks (Table 1). During a mean follow-up period of 92 weeks, primary healing was achieved in 66% of patients receiving HBOT compared with 0% following standard therapy. A review of 6 studies prepared by Roeckl-Wiedmann et al. [69] demonstrated that additional application of HBOT reduced the risk of amputation in 118 patients. Other double-blinded, randomized, and placebo-controlled clinical trials presented by Löndahl et al. [33, 70, 71] proved applicability of HBOT in DFU adjunct therapy. Complete healing of the index ulcer (acc. Wagner scales) was achieved in 37 patients at 1 year of followup in 25/48 (52%) in the HBOT group and 12/42 (29%) in the placebo group (Table 1).

The Cochrane database systemic review, based on 8 studies of DFU (455 participants), demonstrated a favourable effect of HBOT on wound healing on the early stage of healing (6 weeks), but a longer observation (one year) failed to demonstrate any long-standing positive effects of the therapy. Additional application of HBOT had no significant effect on reduction of the number of major amputations [32].

Liu et al. [72] summarize thirteen trials (a total of 624 patients), including 7 prospective randomized trials, performed between January 1, 1966, and April 20, 2012. Pooling analysis revealed that, compared with treatment without HBO, adjunctive treatment with HBO resulted in a significantly higher proportion of healed diabetic ulcers (relative risk, 2.33; 95% CI, 1.51–3.60). The analysis also revealed that treatment with HBO was associated with a significant reduction in the risk of major amputations (relative risk, 0.29; 95% CI, 0.19–0.44); however, the rate of minor amputations was not affected. Adverse events associated with HBO treatment were rare and reversible and not more frequent than those occurring without HBO treatment.

DFU infections are often asymptomatic and for that reason systemic antibiotic therapy is used earlier in their case than it is for other wounds. That long-standing therapy favours bacterial antibiotic resistance. Early introduction of HBOT reduces the risk of infection and the risk of amputation [73].

HBOT does not substitute the antibiotic therapy, local humid therapy, or surgical wound debridement. It may only support the complex DFU therapy. Maybe soon HBOT will become a routine procedure in standard DFU therapy, but still standardised procedures and therapy cost estimates are lacking. Some authors believe that HBOT should be introduced to standard therapy as early as possible, without months of delay spent on ineffective therapy.

3. Maggot Therapy (MT)

Successful DFU therapy largely depends on regular wound debridement and creation of favourable humid conditions free from bacterial infection. Physical-mechanic means are the simplest ones to be used for wound debridement. During a surgical procedure, necrotic tissue, fibrin, and pathological granulation are removed with a scalpel, scissors, and a scraper (surgical debridement). However, the method is associated with a risk of intense bleeding; it is painful and imprecise. Debridement often extends beyond the necessary boundary, as it is difficult to separate and differentiate necrotic tissue, granulation, or poorly perfused tissue from a healthy one. That is particularly important in case of DFU, where debridement is a common procedure and the wound's area is small [74]. Therefore, other, superior and more efficient methods of local therapy are sought. Among them, there are alternative physical methods of wound debridement: sonotherapy (with use of ultrasounds) and hydrosurgical (with use of water jets). They seem less efficient than MT—a method known for centuries.

Therapeutic effect of MT is based in three mechanisms:

(1) removal of necrotic tissue from the wound,

(2) antibacterial effect and destruction of bacterial biofilm,

(3) stimulation of healing processes.

Table 1: Therapeutic protocols used in the intervention and control groups in included studies focusing on the use of HBOT in DFU [5, 8, 32].

First author and year of publication	Study group (no. of patients)	Control group (no. of patients)
Löndahl et al., 2010 [33]	$N = 49$ Evaluation of wether adjunctive treatment with HBOT compared with treatment with hyperbaric air (placebo) would have any therapeutic effect HBOT a treatment period at 2.5 ATA for 85 min daily (session duration 95 min), five days a week for 8 weeks (40 sessions) Complete healing of the index ulcer was achieved in 25/48 (52%), 3 major amputations, 4 minor amputations	$N = 45$ Complete healing of the index ulcer was achieved in 12/42 (29%), 1 major amputation, 4 minor amputations
Duzgan, 2008 [34]	$N = 50$ Standard therapy (ST) + HBOT HBOT 2-3 ATA for 90 min/2 sessions per day, followed by 1 session on the following day; 33% were healed without surgery treatment; 16% (8) required operative debridement, an amputation, or the use of a flap or skin graft; 8% (4) underwent distal amputation; 0 required proximal amputation	$N = 50$ ST daily wound care, dressing changes, local debridement, and control infection 0% of patients were healed without surgery treatment; 100% (50) required either operative debridement, an amputation, or the use of a flap or skin graft; 48% (24) underwent distal amputation; 34% (17) required proximal amputation
Abidia et al., 2003 [35]	$N = 9$ (100% oxygen) HBOT 2.4 atmospheres absolute (ATA) for 90 min daily, 5 days per week, totaling 30 sessions Complete epithelialization was achieved in 5 out of 8 ulcers; the median decrease of the wound areas was 100%	$N = 9$(control—air) 2.4 atmospheres absolute (ATA) for 90 min daily, 5 days per week, totaling 30 sessions Complete epithelialization was achieved in 1 of the 8 ulcers; the median decrease of the wound areas was 52%
Kalani et al., 2002 [36]	$N = 17$ 40–60 session of HBOT Investigation the long-term effect of HBOT, 76% of (13) patients had healed; 12% (2) were amputated	$N = 21$ Conventional treatment 48% of (10) patients had healed, 33% (7) were amputated
Faglia et al., 1998 [19]	$N = 51$ Comparison therapy plus treatment in a multiplace HBO chamber Two phases: (1) first (antibacterial) phase uses 100% oxygen at 2.5 ATA for 90 minutes daily; (2) second (reparative) phase uses 100% oxygen at 2.2–2.4 ATA for 90 minutes, 5 days a week	$N = 64$ Debridement, topical antimicrobial agents, and occlusive dressing. Empirical antibiotic therapy modified following sensitivity results. Diabetic control with insulin. PTCA or CABG, if needed
Zamboni et al., 1997 [18]	$N = 5$ Comparison therapy plus treatment in a monoplace HBO chamber with 100% oxygen at 2 ATA for 120 minutes, 30 sessions 5 days a week	$N = 5$ Debridement, silver sulfadiazine dressing twice a day for 5 days, and culture-specific antibiotics
Faglia et al., 1996 [20]	$N = 35$ Comparison therapy plus treatment in a multiplace HBO chamber. Two phases: (1) first (antibacterial) phase uses 100% oxygen at 2.5 ATA for 90 minutes daily; (2) second (reparative) phase uses 100% oxygen at 2.2–2.4 ATA for 90 minutes, 5 days a week. Mean (SD) number of sessions = 38 (8)	$N = 33$ Debridement, topical antimicrobial agents, occlusive dressing. Empirical antibiotic therapy modified following sensitivity results. Diabetic control with insulin. PTCA or CABG, if needed
Doctor et al., 1992 [37]	$N = 15$ Conventional management and 4 sessions of hyperbaric oxygen therapy HBO chamber with 100% oxygen at 3 ATA for 45 minutes, 4 sittings over 2 weeks	$N = 15$ Regular surgical treatment, incision and drainage, debridement, local dressing with boric acid and bleaching powdered solution, or glycerine acriflavine Amputation for gangrene or infection above the knee Cephalosporins, aminoglycosides, and metronidazole with changes made following sensitivity patterns Diabetic control with insulin

Table 1: Continued.

First author and year of publication	Study group (no. of patients)	Control group (no. of patients)
Baroni et al., 1987 [17]	$N = 18$ Comparison therapy plus treatment in a multiplace HBO chamber. Two phases: (1) first (antibacterial) phase uses 100% oxygen at 2.8 ATA for 90 minutes daily; (2) second (reparative) phase uses 100% oxygen at 2.5 ATA for 90 minutes. Mean (SD) number of sessions = 34 (21.8)	$N = 10$ Debridement. Diabetic control with insulin

Table 2: The Wagner classification of diabetic foot ulceration [15].

Grade	Clinical description
0	No open ulcer, high risk
1	Superficial ulcer with subcutaneous involvement
2	Deep ulcer with tendon or joint involvement
3	Deep ulcer with bone involvement
4	Wet or dry gangrene (forefoot), without cellulitis
5	Generalized (whole foot) gangrene

Maggots secrete digestive juices on the outside; necrotic tissue becomes digested and liquefied and absorbed in that form. Additionally, an additional mechanical debridement is caused by the specific mandibles or "mouth hooks" of the maggots and their rough body which both scratch the necrotic tissue. Studies are on the way on chemical composition and mechanism of action of maggot excretions and secretions (ES). It is a blend of collagenases, proteolytic enzymes, serine proteases: trypsin-like and chymotrypsin-like enzymes, metalloproteinase and aspartyl proteinase, carboxypeptidases A and B, and leucine aminopeptidase [42, 44]. Maggots ES contain also allantoin, sulfhydryl radicals, calcium, cysteine, glutathione, embryonic growth stimulating substance, growth stimulating factors for fibroblasts, and other agents (Table 3) [42, 43]. The effect of wound healing stimulation is attributed to allantoin and urea. Ammonia, ammonium bicarbonate, and calcium carbonate contained in ES change medium reaction from acidic into alkaline, inhibiting bacterial growth. Also, tissue irritation by moving maggots also speeds the process of wound healing up [44]. Favourable effect on DFU healing is also associated with MT influence on disturbed mechanisms of the inflammatory process. Monocyte activity change via the cyclic AMP-dependent mechanism causes inhibition of secretion of proinflammatory cytokines TNFα and IL12p40 and the macrophage migration inhibitory factor (MIF) and increased secretion of antiinflammatory IL10. The process leaves phagocytosis untouched [75]. Maggots remove necrotic tissue and do not digest bones, tendons, or viable tissues. They offer a precise, accurate, and delicate debridement [44, 75, 76]. It seems that the level of wound debridement is not achievable with surgical methods. Maggots also clean off microorganisms. Based on the bacteriological analysis of the larval alimentary tract, it was observed that consumed bacteria die in further parts of the larval alimentary tract [77]. Maggots efficiently eradicate *Staphylococcus aureus*, including MRSA strains, Streptococci, and *Pseudomonas aeruginosa*. They have no effect on *Escherichia coli*, *Enterococcus*, *Proteus* [44, 47, 78, 79].

MT may be ineffective in case of highly discharging or dried wounds. Insignificant side effects of the therapy include minor bleeding, pain, excessively induced exudates, increased body temperature, flu-like symptoms, allergic reaction, and skin maceration [44, 76, 80, 81]. There are practically no contraindications for MT in DFU therapy. Patient anxiety may constitute a limitation. *Lucilia sericata* maggot cultures are kept on sterile media, with sterile air flow, ensuring aseptic conditions. 1–3 mm long maggots are used for dressings. They are provided in two forms: open, for direct application on wound, or closed in a biobag. The network of the biobag is permeable and permits the migration of maggot ES to the wound. A wound qualified for MT requires no special preparation; edges of the wound are covered with an ointment protecting against digestive enzymes. Applied maggots, approximately 5–10 for one centimetre squared of the wound, may be covered by a nylon net, ensuring they remain in the wound area, and a humid dressing. A dry dressing is applied on top. As the outer dressing gradually becomes soaked, it should be changed [76, 77]. The dressing with maggots is maintained for 3 days, on average. After that period, the maggots should be removed by washing out the wound by saline. The procedure is repeated 1–4 times, if necessary. Closed dressings are more comfortable to use, because maggots cannot get out; however they often cannot be applied on deep DFU with small, irregular area [44, 45, 76, 82].

The beneficial effects of using larvae in wounds were first noticed by Ambrose Paré in 1557. While treating battle wounds in Napoleon's army, Baron Larrey observed that maggots enhanced granulation formation. The first clinical application of maggot therapy was performed by J. F. Zacharias and J. Jones during the American Civil War [48].

In 1931, Baer published results of his attempts to apply MT in therapy of osteitis in children [85]. However, sterility of maggots, their transport survival, and application of appropriate dressings constituted a problem. MT was difficult from the logistic point of view, costly, and burdened with a risk of infection, for example, with tetanus.

Only in the 1980s, when the number of patients with chronic ulceration grew rapidly and standard therapeutic methods often proved inefficient, increased MT application was observed. Methods of sterile and industrial culture of

Table 3: Statement of research on MT and PRPT.

Methods	Cell cultures	Animal trials	Clinical trials
Maggot therapy (MT)	Prete, 1997 [42] Gupta, 2008 [43]		Jarczyk et al., 2008 [44] Sherman, 2003 [45] Armstrong et al., 2005 [46] Bowling et al., 2007 [47] Chan et al., 2007 [48] Game et al., 2012 [49]
Platelet-rich plasma therapy (PRPT)	Ross et al., 1974 [50] Cenni et al., 2005 [51] Kark et al., 2006 [52] Borzini and Mazzucco, 2007 [53]	Knighton et al., 1986 [54] Pietramaggiori et al., 2008 [55] Borzini and Mazzucco, 2007 [53]	Knighton et al., 1982 [56] Krupski et al., 1991 [57] Margolis et al., 2001 [58] McAleer et al., 2006 [59] Driver et al., 2006 [60] Gandhi et al., 2006 [61] Borzini and Mazzucco, 2007 [53] Scimeca et al., 2010 [62] Villela and Santos, 2010 [63] Frykberg et al., 2010 [64] Carter et al., 2011 [65] de Leon et al., 2011 [66] Slesaczeck et al., 2012 [67] Game et al., 2012 [49]

maggots were developed, along with efficient transport and new types of dressings, which led to the application of MT in inpatient and outpatient settings.

Since the beginning of the 21st century MT, called also the maggot debridement therapy (MDT), biosurgical debridement (BD) with maggots "biosurgeons," or simply larval therapy, has been in the renaissance [76, 84].

MT uses maggots fed on necrotic tissue only. They are *Lucilia sericata*, *Lucilia cuprina*, *Phormia regina,* and *Musca domestica*. The most commonly used species in MT is *Lucilia sericata. Lucilla sericata* maggots are highly voracious and mobile; they have a herd instinct and become even more active if they sense competition. In a natural environment, those flies lay their eggs in carrion. Maggots hatch on the next day. They raven for 5–7 days, grow, and pupate into mature flies in 10–14 days [76, 86].

DFU patients are a major indication for MT, and some comparative studies have been published. Jarczyk et al. [44] used MT in 4 DFU patients at risk of amputation, with non-healing ulcerations (2–9 months). Complete healing of ulcers was achieved in 3 of those 4 patients. Sherman [45] compared the efficacy of conventional treatment (frequent changes of dressings, local antiseptics and antibiotics, hydrogel and hydrocolloid dressings, and surgical wound debridement) with larval therapy in patients with diabetic feet. After 5 weeks of therapy, wounds subjected to conventional treatment remained covered with necrotic tissue (33% of the surface of the wound), while in the case of larval therapy all the ulcerations were cleaned after 4 weeks. Another controlled cohort study demonstrated that MT was more effective and efficient in debriding DFU than the conventional therapy. MT was also associated with better wound granulation and epithelialisation [45]. Armstrong et al. [46] mentioned the benefits connected with larval therapy in the case of patients with peripheral vascular lesions and diabetic foot ulcerations. The previously mentioned authors investigated 60 patients, demonstrating more rapid wound healing, a threefold lower percentage of limb amputations (10% *versus* 33%), and shorter antibiotic therapy in patients subjected to biosurgical therapy, in comparison to the control group. Many other studies proved that MT may reduce time of therapy, reduce short-term morbidity, and reduce the risk of major amputation by as much as 50% in patients with DFU [44, 87–89].

Despite lack of unanimous evidence, clinical experience suggests that MT is effective and safe. Even more, MT used in wound bed preparation can be effective both clinically and financially, if used appropriately [90, 91].

Although indications for wound debridement with maggots are all chronic wounds and some acute ones, that logistically difficult therapy is best suited to DM patients in whom surgical excision of necrotic tissue, application of enzymatic preparations, and dressings speeding autolysis of necrotic tissue up is impossible, or if those methods are not sufficiently effective. In those conditions, MT seems to be a therapy of choice.

4. Autologous Platelet-Rich Plasma Therapy (PRPT)

Making effective wound healing possible is the basic stage of the DFU curing process. Use of autologous platelet-rich plasma (PRP) in the form of local application of a gel obtained by centrifugation of full blood and addition of an activator, clotting agent, is designed for the creation of local conditions favourable to healing processes. PRP is defined as plasma fraction of autologous blood with a platelet count concentrated above the baseline [92]. It is a repository of growth factors, cytokines, adhesion molecules and clotting agents, and leukocytes.

Platelets contain numerous natural growth factors released from their α granulations and stimulating healing processes (Table 3). In 1974, Ross et al. [50] in the *in vitro* study noted thrombin-activated platelets as a source of growth factors that could initiate the body's natural healing. Added to platelet-poor plasma, they increased activity of smooth muscle cells and fibroblasts.

PRP is obtained by repeated centrifugation of autologous full blood [93]. The resulting concentrate, combined with activating bovine thrombin, forms a gel that seals the wound. The gel is placed on wound bed and protected by a cover dressing. The dressing may stay in place for up to 7 days.

Various production systems are suggested, with various abilities of aggregation of platelets and leukocytes. For that reason, there are various preparations available, slightly different from each other [83, 94–96]. Sufficient cellular response to platelet concentrations first began when a 4-5-fold increase over baseline platelets' number was achieve, at least one million platelets per microlitre [97]. Platelets release over 30 factors responsible for healing processes, including three isomers of the platelet-derived growth factor (PDGF $\alpha\alpha$, $\beta\beta$, $\alpha\beta$), vascular endothelial growth factor (VEGF), transforming growth factor-β1 (TGF-β1), transforming growth factor-β2 (TGF-β2), epidermal growth factor (EGF), insulin-like growth factor-1 (IGF-1), and others. PRP contains also proteins responsible for cellular adhesion, fibrin, fibronectin, vitronectin, and also osteocalcin and osteonectin [83, 98, 99]. PRP contains additionally leukocytes that increase its antibacterial properties and synthesize interleukins as part of a nonspecific immune response [95, 96]. PDGF is a growth factor found also in macrophages and endothelial cells. TGF is also found in macrophages, and EGF is present in macrophages, monocytes, and keratinocytes. VEGF is found mostly in endothelium, and IGF-1 is predominantly produced in the liver. Besides activity of leukocytes, the antibacterial effect of PRP is a result of activity of PGDF, by activation of macrophages, and VEGF, by stimulation of macrophages and monocytes. Re-epithelialisation and fibroblast proliferation are mostly the effect of PDGF, TGF, EGF, and IGF-1, stimulating the deposition of extracellular matrix (ECM). PDGF and IGF-1 are responsible for stimulation of other growth factors and cytokines, and angiogenic effect is shown mostly by VEGF, EGF, and PDGF [100, 101]. All these functions have been demonstrated through specifically designed *in vitro* experiments [51–53].

Platelets attach to the connective tissue, and growth factors are released via degranulation of α granulation in just 10 minutes after initiation of blood clotting processes. The majority of them is released during the first hour and is bound to membranous receptors in surrounding cells, activating intracellular signalling pathways.

Following a rapid release of growth factors, platelets contained in PRP synthesise and secrete their additional quantities for subsequent 7 days. After that time, the healing function is taken over by macrophages. Experimental and clinical studies demonstrated the most profound accelerating effect on wound healing in the 3rd week after PRP application [55, 102].

In 1982, Knighton et al. [56] in their experimental *in vivo* animal study demonstrated that a thrombin-activated autologous platelet concentrate stimulated neoangiogenesis, collagen synthesis, epithelial cells proliferation, fibroblast proliferation, and fibrin decomposition products stimulated leukocyte activity. Result of those experiments was a clinical study published four years later on the effective use of local PRP application and PRP-contained growth factors on therapy of chronic wounds, including DFU [54]. The percutaneous delivery of platelet-rich plasma (PRP) Gandhi et al. [61] used in the diabetic BB Wistar femur fracture model. PRP delivery at the fracture site normalized the early (cellular proliferation and chondrogenesis) parameters while improving the late (mechanical strength) parameters of diabetic fracture healing.

A complete clinical evaluation of PRP is still pending. Preliminary results of basic studies and preclinical and clinical trials have not been confirmed in large controlled studies. Available analyses in case reports [62, 67], case series [59, 64], randomised controlled studies [57, 60], cohort studies [58], and metaanalyses [65, 66] are based on small samples. However, they indicate that PRPT applied for nonhealing DFU is a more effective method compared to local conventional therapy.

In the multicentre study by Villela and Santos, and de Leon et al. [63, 66] on a group of 200 patients with 285 wounds, PRPT could restart the healing process in the majority of cases. Rapid treatment response was observed in 275 of 285 wounds, and the size of the reply was high with reported statistically significant outcomes. Carter et al. [65] completed a metaanalysis on the use of PRPT on wound healing patients with DFU, which led to the conclusion that autologous PRP gel promises as an effective treatment for severe DFU. Dougherty [103] statistically analysed efficacy versus cost and quality of life of a patient with DFU treated with PRP compared to conventional or other alternative therapeutic methods. Cost analysis completed in the group of over 200 thousand patients involved quality of life, recurrence, necessary amputation, and mortality. The study demonstrated the highest cost effectiveness with the consideration of quality of life for patients treated with PRP. According to the analysts, PRPT is potentially the most attractive alternative for DFU, that may reduce the cost burden and health effects of nonhealing DFU.

DFU was demonstrated to be associated with reduced activity of many growth factors—hence the concept of their exogenous supply. DM patients demonstrate deficiency of biological stimulators. It is a result of their metabolic and ischaemic problems, and that deficiency inhibits reparative processes and facilitates or intensifies development of infection.

Other studies demonstrated synergistic cooperation of growth factors contained in PRP, and their optimal proportions influence processes of normal healing [104–107].

Therefore, PRP seem to be an effective—if not the most effective—and safe preparation used for therapy of DFU. PRP is an autologous product, therefore constitutes no risk of viral hepatitis or HIV infection. Observed abnormal reactions to clotting activators are very rare [106].

Table 4: Wound healing process and the alternative methods.

Elements of wound healing	Methods		
	HBOT	MT	PRPT
Inflammation	Bactericidal and bacteriostatic effects on both aerobic and anaerobic bacteria through the action of the super oxide enzyme*	Antibacterial potential effect of alkaline pH of maggot secretion [77, 78] Wound bacteria are killed as they pass through the maggot's digestive tract* Presence of a potent bactericide present in maggot secretions* Cytokine regulation and enhanced phagocytosis [75]	Suppresses cytokine release and limits the amount of inflammation, interacting with macrophages to improve tissue healing Enhances phagocytosis and chemotaxis [54]* Antimicrobial host defence enriched with growth factors and other active substances [83]*
Granulated tissue formation—epithelialization	Increases epidermal cells and fibroblast proliferation and differentiation [29]	The healing of wounds is an interactive process (regulators as growth factors, cytokines and chemokines) [42] Synthesized and released locally proteins or polypeptides [42, 43] Increases fibroblast proliferation through maggots excretions and secretions [75]	Influences on chemotaxis, mitogenesis, and differentiation Promotes healing by stimulating fibroblast and keratinocyte proliferation Promotes granulation tissue formation [55, 61]* and epithelialisation
Matrix formations	Increases fibroblast proliferation and collagen production	Stimulates extracellular matrix and remodeling processes [45]	Stimulates the deposition of extracellular matrix and collagen [56]*
Angiogenesis	The oxygen gradient promotes the formation of new vessels required for wound healing [28, 35, 68]	Growth factors, cytokines, and chemokines provide significant vasodilation and increased capillary permeability to the wound site, allowing the infusion of recruited polymorphonuclear leucocytes (PMNs) and macrophages [48, 84]	Promotes new capillary growth [55, 56]*

*Animal models.

PRPT is a rich source of locally active (bioregulating) growth factors and cytokines that improve conditions of wound healing. Relatively simple and cheap production of PRP argues for continued interest in that adjunct method. It seems that specific cellular therapy constitutes an additional and valuable option in therapy of DFU resistant to the conventional therapy.

5. Conclusions

Prevention of DFU is the main point to reduce the associated high morbidity and mortality rates among patients with DM. DFU management involves a multidisciplinary approach from prevention (health educators) to treatment (diabetologist, surgeon, vascular surgeon, orthopaedist, radiologist, shoemaker, and kinesiotherapist).

Proper, effective treatment of DFU prolongs life and improves its quality. The gold standard for DFU includes debridement of the wound, infection cure, revascularization when needed, and other new therapies [49].

Among the adjunct DFU treatment methods are HBOT, MT, and PRPT. The new research on advanced therapy in DFU included stem cells, growth factors, skin substitutes, and gene therapy as well. The preliminary results are often promising, but randomised controlled trials are needed. When considering the adjunct treatment (HBOT, MT, PRPT, and others), it is clear that more patients suffered from refractory wounds, which lead to more frequent hospitalizations from sepsis, gangrene, amputation, and death. The combination therapy should improve the rate of healing and prolong the time of complications (Tables 4 and 5).

Table 5: Clinical relevance in DFU according to the alternative methods.

Clinical relevance	Methods		
	HBOT	MT	PRPT
Reduced area in diabetic foot	Yes**	No	Yes**
Antiedema effect	Yes**	No	No
Decreased risk amputation	Yes**	Yes**	Yes
Shortening time of therapy	Yes**	Yes**	Yes**

**Clinical studies.

The conclusion of the International Working Group of the Diabetic Foot (IWGDF) systematic review is that with the exception of HBOT and possibly negative pressure wound therapy, there is little published evidence to justify the use of other therapies [49]. Conclusions were as follows.

(1) DFU treatment should be strictly applied according to the gold standard accepted by the International Working Group on the Diabetic Foot [108, 109].

(2) The gold standard for DFU treatment includes debridement of the wound, management of any infection, revascularization procedures when indicated, and off-loading of the ulcer [108].

(3) HBO can be applied as an adjunctive therapy for patients with severe soft tissue foot infections and osteomyelitis who have not responded to conventional treatment, though the available data are insufficient [32, 71, 72, 109, 110].

(4) Recent reports suggest that MT is effective in the elimination of drug-resistant pathogens [78, 84].

(5) PRPT is reserved as a second-line therapy similar to HBO, especially in the treatment of refractory wounds [63, 102].

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Research Article

Anti-Diabetic Activities of Jiaotaiwan in db/db Mice by Augmentation of AMPK Protein Activity and Upregulation of GLUT4 Expression

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Jiaotaiwan (JTW), which is composed of *Coptis chinensis* (CC) and cinnamon (CIN), is one of the most well-known traditional Chinese medicines. In this study, we investigated the antidiabetic effects and mechanism of JTW in db/db mice. Results showed that JTW significantly decreased the level of fasting blood glucose and improved glucose and insulin tolerance better than CC or CIN alone. JTW also effectively protected the pancreatic islet shape, augmented the activation of AMP-activated protein kinase (AMPK) in the liver, and increased the expression of glucose transporter 4 (GLUT4) protein in skeletal muscle and white fat. AMPK and GLUT4 contributed to glucose metabolism regulation and had an essential function in the development of diabetes mellitus (DM). Therefore, the mechanisms of JTW may be related to suppressing gluconeogenesis by activating AMPK in the liver and affecting glucose uptake in surrounding tissues through the upregulation of GLUT4 protein expression. These findings provided a new insight into the antidiabetic clinical applications of JTW and demonstrated the potential of JTW as a new drug candidate for DM treatment.

1. Introduction

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by deregulation of glucose and lipid metabolism [1]. With the development of the social economy, DM, especially type 2 diabetes mellitus (T2DM), has become a serious public health problem [2]. Numerous drugs, such as rosiglitazone (ROS) and metformin (MET), have been used in the treatment of DM. However, treatment with synthetic drugs has been reported to lead to various side effects [3]. Therefore, searching for better agents from herbs or natural products that can be used to treat diabetes is necessary [4].

Traditional Chinese medicines (TCMs), which have been used by the Chinese to treat illnesses for thousands of years, are combination drugs comprising several different active compounds. TCMs are better at controlling complex disease systems such as diabetes and are less prone to causing drug resistance development [5]. Jiaotaiwan (JTW) is one of the most well-known TCMs from *Han's Book on Medicine* compiled by Mao Han. JTW is composed of *Coptis chinensis* (CC) and cinnamon (CIN). In China, JTW is mainly used to treat insomnia. The major active constituents of CC and CIN reduce blood glucose levels [6–8]. Berberine chloride, which is isolated from CC, possesses anti-diabetic activity [9, 10]. Cinnamaldehyde is a major active constituent isolated from CIN. Studies show that cinnamaldehyde can reduce the fasting blood glucose level in rats treated with streptozotocin [11]. The therapeutic effect of JTW is also reportedly better than that of CC or CIN alone for treating type 2 diabetes mellitus (DM) in rats [12]. However, the mechanism of the glucose-decreasing effect of JTW is equivocal.

Hepatic glucose production and glucose uptake in surrounding tissues are the key in glucose homeostasis. AMP-activated protein kinase (AMPK) has a critical function in this process because it can suppress gluconeogenesis in the liver and promote glucose uptake in peripheral tissues [13]. The glucose uptake in surrounding tissues is mediated by glucose transporter4 (GLUT4) [14]. Research shows that as a result of the up-regulation of GLUT4 protein expression in adipose cell and skeletal muscle in a state of insulin resistance, glucose uptake in the adipose cell and skeletal muscle is promoted, and glucose tolerance and insulin resistance are improved [15, 16].

In this study, we investigated the anti-diabetic effects of JTW in typical T2DM model db/db mice. We detected the AMPK protein levels in the liver and the GLUT4 protein levels in skeletal muscle and white fat to validate the beneficial effects of JTW as an anti-diabetic agent and to clarify the mechanisms of its action.

2. Materials and Methods

2.1. Animals. The animal protocols used in this study were approved by the Shanghai University of Traditional Chinese Medicine for Animal Studies (Approval number 10032). Female db/m and db/db mice (C57BL BKS cg-M+/+ lepr−/−) purchased from the SLAC Laboratory (Shanghai, China) were housed at 22 ± 2°C and 55% ± 5% relative humidity, with a dark cycle of 12 h (19:00 to 07:00) and a light cycle of 12 h (07:00 to 19:00). Eight-week-old db/db mice were used in the experiment. The db/db mice were randomly divided into five groups, namely, model control group (Model), JTW-treated group (JTW), CC-treated group (CC), CIN-treated group (CIN), and ROS-treated group (ROS). The db/m mice not included in the five groups were designated as the normal control group (Normal). Each group comprised eight mice. JTW (8.4 g/kg), CC (7.6 g/kg), CIN (0.76 g/kg), ROS (5 mg/kg), or water was intragastrically administered to the mice for four weeks. The model control (Model) and normal control (Normal) groups were treated with water.

2.2. Preparation of JTW, CC, and CIN. Composition proportions of JTW prescription: the composition of CC : CIN was equal to 10 : 1. The drugs were purchased from the Yanghetang Decoction Pieces Limited Company (Shanghai, China) and extracted by the Analyses and Testing Laboratory in Shanghai University of Chinese Medicine. The extraction steps of JTW were performed as follows: CIN was soaked with 6 volumes of water for 2 h. The volatiles (A), drug liquid (B), and drug residue (C) were obtained with a simultaneous distillation and extraction (SED) equipment for 5 h. D (CC, B, and C) was soaked with 5 volumes of water for 0.5 h and then subsequently boiled for 1 h and extracted twice to obtain the extraction solution (E). Drug concentrations (F) were obtained from E using low heat. A and F were mixed to form JTW with a concentration of 0.84 g/mL.

The extraction steps of CC concentrations were indicated as follows: CC was soaked with 5 volumes of water for 0.5 h and then subsequently boiled for 1 h and extracted twice to obtain the extraction solution. Subsequently, the CC concentrations were obtained from the extraction solution using low heat.

The extraction steps of CIN concentrations were indicated as follows: CIN was soaked with 6 volumes of water for 2 h. The volatiles (A), drug liquid (B), and drug residue (C) were obtained with an SED equipment for 5 h. A and B were mixed to form CIN concentrations. The concentrations of CC and CIN were 0.76 and 0.076 g/mL, respectively.

2.3. Intraperitoneal Glucose Tolerance Test and Intraperitoneal Insulin Tolerance Test. After two and three weeks of treatment, db/db mice were fasted overnight (12 h). Glucose levels were determined from the tail vein (0 min) before the injection of glucose (1 g/kg body weight) or insulin (1 u/kg body weight). Additional blood samples were collected at regular intervals (15, 30, 60, and 120 min) for glucose measurement.

2.4. Histology. All mice were sacrificed after four weeks of treatment, and their pancreas were immediately dissected. All of the mice pancreases were fixed in 10% neutral formalin, desiccated, and then embedded in paraffin. The pancreases were sectioned (3 μm thick), and the sections were transferred to gelatin-coated slides. Then, the slides were stained with hematoxylin and eosin (HE) and examined under light microscope.

2.5. Immunohistochemistry. The sections were deparaffinized with xylene, rehydrated in graded ethanol (100% to 95%), and rinsed with water and 0.1 M phosphate-buffered saline (PBS, pH 7.4). Following 10 min treatment with 3% H_2O_2 at room temperature, the sections were washed with 0.1 M PBS. The sections were incubated with an anti-insulin monoclonal antibody (Boster, China; diluted 1 : 200) at 37°C for 1 h. After they were washed with PBS, the sections were allowed to react with goat anti-rabbit IgG with horse radish peroxidase (HRP) (MiaoTong, China, diluted 1 : 500) at room temperature for 20 min and then washed with PBS. HRP activity was developed by DAB. Sections were then counterstained with hematoxylin and examined under a light microscope.

2.6. Transmission Electron Microscopy. The mice pancreases (1 mm^3) were fixed in 2% glutaraldehyde in 0.1 M D-PBS for 2 h at 4°C. Following three washes with 0.1 M PBS was postfixed in 1% osmium tetroxide for 2 h and dehydration in ascending concentrations of ethanol and acetone (30% to 50% ethanol, 70% ethanol-uranyl acetate, 80% ethanol, 100% ethanol-acetone, and 100% acetone). Subsequently the pancreases were embedded in Epon 618. Thin 80 nm sections were prepared on a LEICA ULTRACUT R ultramicrotome and stained with lead citrate. The sections were examined with a transmission electron microscope.

2.7. Western Blot Analysis. Equal amounts of protein from each sample, 120 μg from hepatic tissue and skeletal muscle, and 240 μg from white fat were separated on SDS-PAGE gels and then transferred to PVDF membranes. Blots were blocked with 5% nonfat milk in Tris-buffered saline with 0.1%

Tween-20 (TBST, pH 8.0 25 mM Tris, 137 mM NaCl, 2.7 mM KCl, and 0.1% Tween-20) at room temperature for 1 h, followed by overnight incubation with primary antibodies at 4°C. The blots were hybridized with secondary antibody-conjugated HRP in 5% nonfat milk dissolved in TBST at room temperature for 2 h after they were washed with TBST three times. Protein expression was visualized using the ECL Western Blotting Detection System after three washes with TBST.

2.8. Statistical Analysis. The results were expressed as mean ± standard error of mean ($x \pm$ SE). Data analyses were performed using SPSS15.0 software. t-test and one-way ANOVA were adopted for general data analysis. LSD method was applied for comparisons between groups. Data were considered statistically significant when $P < 0.05$.

3. Results

3.1. HPLC Profiles of JTW. To investigate the stability of the JTW water decoction, we repeated the same extraction step times to obtain JTW and then determined the berberine hydrochloride content in JTW by HPLC analysis. The berberine concentration in the JTW samples was 25.61 mg/mL based on the formula, which indicated that the berberine hydrochloride content of JTW was stable (Figure 1).

3.2. Effects of JTW on Body Weight, Food Intake, and Water Intake in db/db Mice. No significant weight gain was observed in the JTW and CC groups after four weeks of treatment. By contrast, the mice began to gain weight after two weeks of treatment in the CIN group and after one week of treatment in the ROS group (Figure 2(a)). All of the drugs used reduced the level of water intake in db/db mice, but only JTW and CC decreased the level of food intake (Figures 2(b) and 2(c)).

3.3. JTW Ameliorates Glucose Tolerance and Insulin Tolerance in db/db Mice. To understand the effects of JTW on blood glucose of db/db mice, fasting blood sugar levels were examined and shown in Figure 3(a). Blood glucose levels in JTW-treated db/db mice were significantly lowered. However, the levels were not significantly changed in the CC- and CIN-treated groups. T2DM commonly coexists with impaired glucose tolerance and insulin tolerance [17]. To verify whether JTW improved glucose tolerance and insulin tolerance *in vivo*, we measured glucose tolerance, insulin tolerance in JTW-treated db/db mice. Figure 3(b) showed that glucose tolerance in JTW mice improved at 0, 15, 30, 60, and 120 min, and CC and CIN improved glucose tolerance at 15, 30, 60, 120 min, 60 and 120 min, respectively. Upon comparison of the CC- and CIN-treated groups with the JTW-treated group, we observed that glucose tolerance in JTW mice was better than that in the CC group at 30 min and that in the CIN group at all time periods, indicating that JTW could ameliorate glucose tolerance and exhibit better performance than CC and CIN. Figure 3(c) showed that the insulin tolerance in JTW mice improved at 0, 15, 30, 60, and 120 min compared with that in the model group. Figure 3(d) also indicated that the downward shift of blood glucose levels in the JTW group mice after treatment with insulin was more noticeable at 15 and 30 min than in the CIN group.

3.4. JTW Ameliorates Islets Morphology and Function. To understand the effects of JTW on islet morphology and function in db/db mice, HE staining, transmission electron microscopy (TEM), and immunohistochemistry analyses were performed. HE stain results showed that the islets in the JTW group had a relatively regular shape and reduced infiltration of exocrine glands. A larger number of islet cells and occasional vacuolar degeneration than the model group were also observed (Figure 4(a)).

We also observed β-cell morphology by TEM. Damage to the pancreatic islet β-cell structure and function results from insulin resistance [18]. In normal group, TEM generally showed numerous β cells in the islet center, and these cells secreted many global granules (β-SGs) (Figure 4(b) (black arrow)). Few α cells also existed in the islet periphery, and these cells secreted α secretory granules (α-SGs). The space between the membrane and core of β-SGs was large, clear, and bright. The electron density of α-SGs was higher than that of β-SGs, and the space between the membrane and core was small.

The results showed numerous α-SGs in the islet center in the model group (Figure 4(b)(white arrow)), which indicated that the β cells were disabled and occupied by α cells. However, some residual β-SGs were observed in the β cells of the JTW group (Figure 4(b) (black arrow)), which indicated that JTW may exert a protective effect on the beta cells. Insulin immunohistochemistry was used to determine the pancreatic insulin content, and the I-solution Image Analysis System was used to test the deeply stained insulin-positive cells in the pancreatic islets. The results showed that the pancreatic insulin content of the JTW group increased compared with the model group but not compared with the ROS group (Figures 4(c) and 4(d)). This finding indicated that the main mechanism of the glucose-decreasing effect of JTW may differ from that of ROS.

3.5. JTW Induced AMPK in the Liver and Increased the Expression of GLUT4 in Skeletal Muscle and White Fat in db/db Mice. AMPK activation and GLUT4 expression are crucial for the treatment of diabetes [19, 20]. To evaluate whether JTW has a positive effect on AMPK and GLUT4 protein, hepatic tissue, skeletal muscle, and abdominal fat were dislodged, and then phosphorylation of AMPK in the liver and expression of GLUT4 in muscle or fat were determined by Western blot analysis with anti-pAMPK-Thr172 antibody and anti-GLUT4 antibody. As expected, the results showed that JTW induced phosphorylation of AMPK in hepatic tissues, and its performance was more effective than that of single herbs (Figure 5(a)). This finding indicates that JTW can effectively contribute in the inhibition of gluconeogenesis and is regulated by AMPK. The glucose uptake in the adipose cell and skeletal muscle is mediated by GLUT4. The experimental findings proved that the expression of GLUT4 significantly

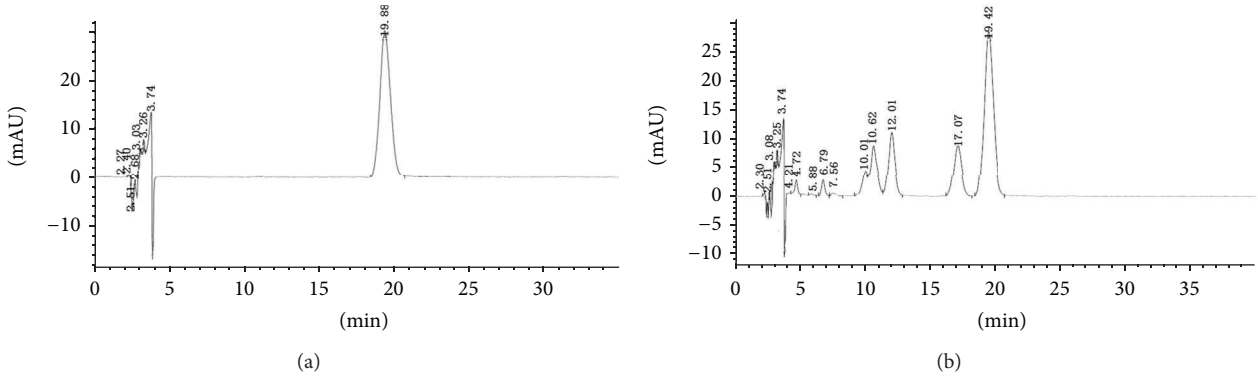

Figure 1: HPLC profiles of JTW and reference material of berberine hydrochloride. The chromatographic conditions were indicated as follows: chromatographic column, C18 (4.6 × 250 mm^2, 5 μm); flow rate, 1 mL/min; mobile phase, 0.05 M methyl cyanide and potassium dihydrogen phosphate (25 : 75); column temperature, 30°C; examination wave length, 346 nm; and sample volume, 20 μL. (a) The peak area of the reference material of berberine hydrochloride was 1481.31 and the retention time was 19.39 min. (b) The peak area of JTW was 1402.66 and the retention time was 19.41 min. Given the formula ($A_1/C_1 = A_2/C_2$), the berberine concentration in the three JTW samples was 25.61 mg/mL (A_1 = 1481.31, C_1 = 0.02, and A_2 = 1402.66).

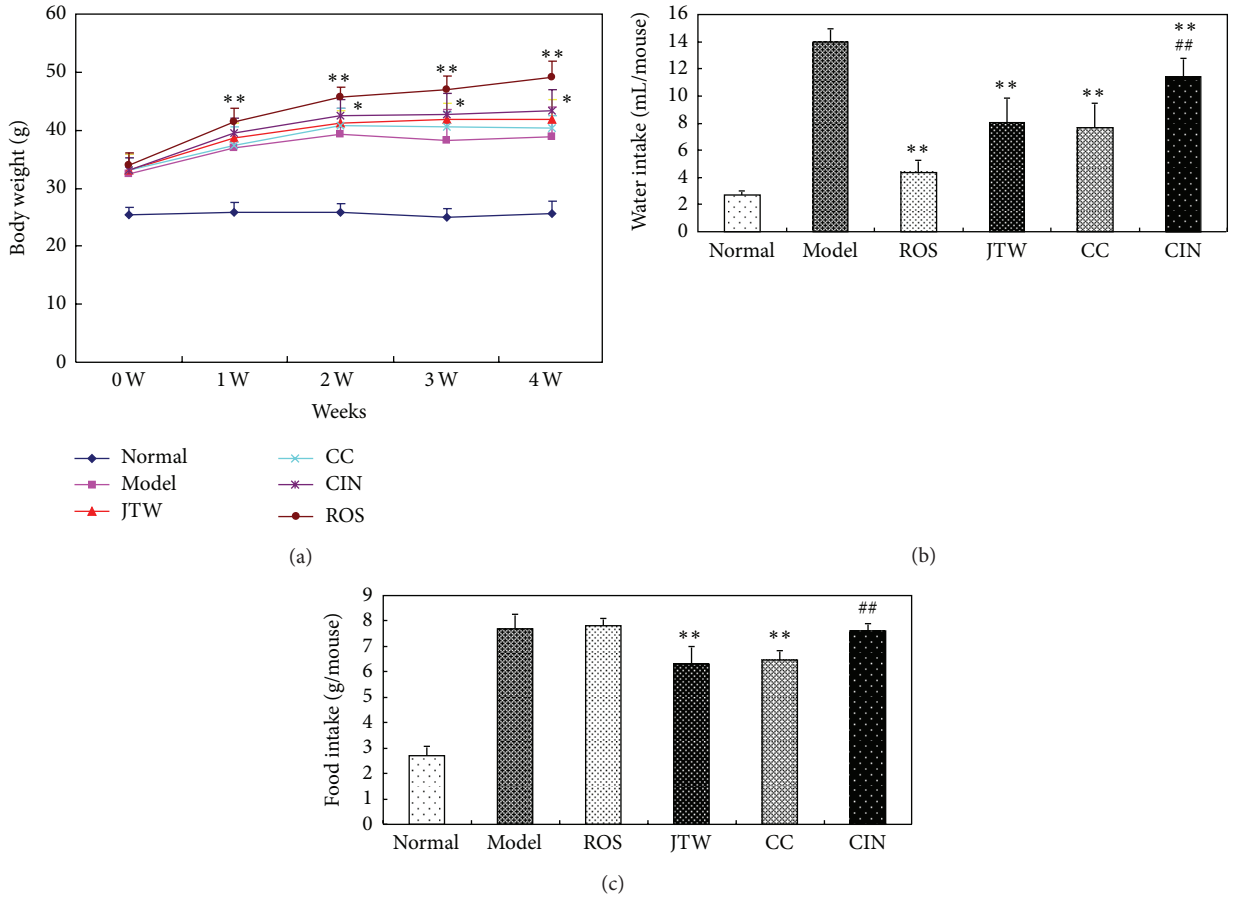

Figure 2: Effects of JTW on body weight, water intake, and food intake in db/db mice. (a) Body weight: body weight was measured every week after treatment (n = 8). (b) Water intake: mice were administered with JTW each day for two weeks (8.4 g kg^{-1} day^{-1}), CC (7.6 g kg^{-1} day^{-1}), CIN (0.76 g kg^{-1} day^{-1}), or ROS (5 mg kg^{-1} day^{-1}) in a vehicle using oral gavage. The water intake amount was recorded every 24 h throughout the treatment (n = 8). (c) Food intake: after treatment with gastric infusion for two weeks, water intake was recorded every 24 h throughout the treatment (n = 8). The data were shown as mean ± SE. $^{*}P < 0.05$ compared with the model control group; $^{**}P < 0.01$ compared with the model control group; $^{\#\#}P < 0.01$ compared with the JTW-treated group.

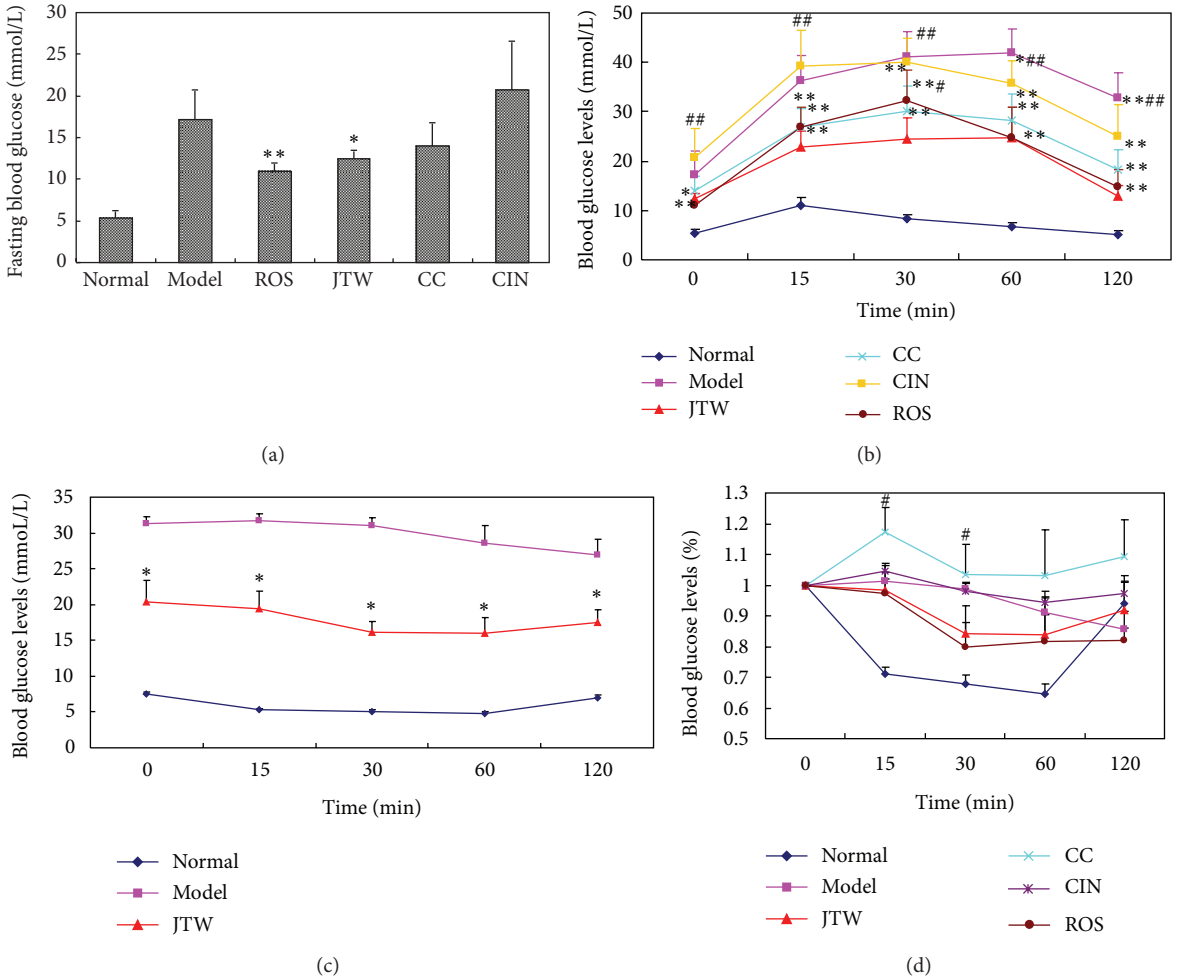

FIGURE 3: Effects of JTW on fasting blood glucose, glucose tolerance, insulin tolerance, and fasting serum insulin level in db/db mice. (a) Fasting blood glucose levels after two-week treatment ($n = 8$). (b) Intraperitoneal glucose tolerance test (IPGTT) after two-week treatment. The mice were fasted for 12 h before measuring blood glucose levels at 0 min. A total of 1 g/kg body weight of glucose was intraperitoneally injected, and glucose levels were tested at regular intervals of 15, 30, 60, and 90 min ($n = 8$). (c), (d) Intraperitoneal insulin tolerance test (IPITT) was performed after three-week treatment. Glucose levels were tested in the same way after intraperitoneally injecting 1 μ/kg body weight of insulin ($n = 8$). Data are presented as mean ± SE. $^{*}P < 0.05$, $^{**}P < 0.01$ versus the model control group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus JTW-treated group.

increased in both skeletal muscle and abdominal fat of db/db mice compared with those of the model control group (Figures 5(b) and 5(c)), revealing that JTW enhanced glucose uptake in surrounding tissues by upregulating the protein expression of GLUT4.

4. Discussion

JTW is one of the most well-known TCMs formulae; it is composed of CC and CIN. In this study, we investigated the hypoglycemic action of JTW, and the results confirmed that JTW possessed anti-diabetic activities *in vivo*. To determine whether the activity of JTW was formed by the overprint of CC and CIN, db/db mice were administered equal volumes of CC and CIN and designated as the control group. The results showed that the administration of single herb did not lower the fasting blood glucose levels in db/db mice after two weeks of treatment. The findings of this study differed from those of other researchers who found that CC and CIN had anti-diabetic properties [6–8]. This result may be due to the disparity in experimental subjects, dosages, and methods for testing. The dosage of JTW was predetermined in another experiment. Two JTW doses were designed for the treatment of mice. One dose was 2.1 g/kg (JTW1) and the other was 8.4 g/kg (JTW2). Results showed that JTW (8.4 g/kg) significantly decreased the level of fasting blood glucose (see Supplementary Material a available online at http://dx.doi.org/10.1155/2013/180721) and improved glucose tolerance (see Supplementary Material b), although both doses of JTW reduced the level of water intake (see Supplementary Material c), food intake (see Supplementary Material d), and urine volume (see Supplementary Material e) in db/db mice. Therefore, we used 8.4 g/kg in subsequent experiments. We decided on the dosage of CC and CIN based on the fact

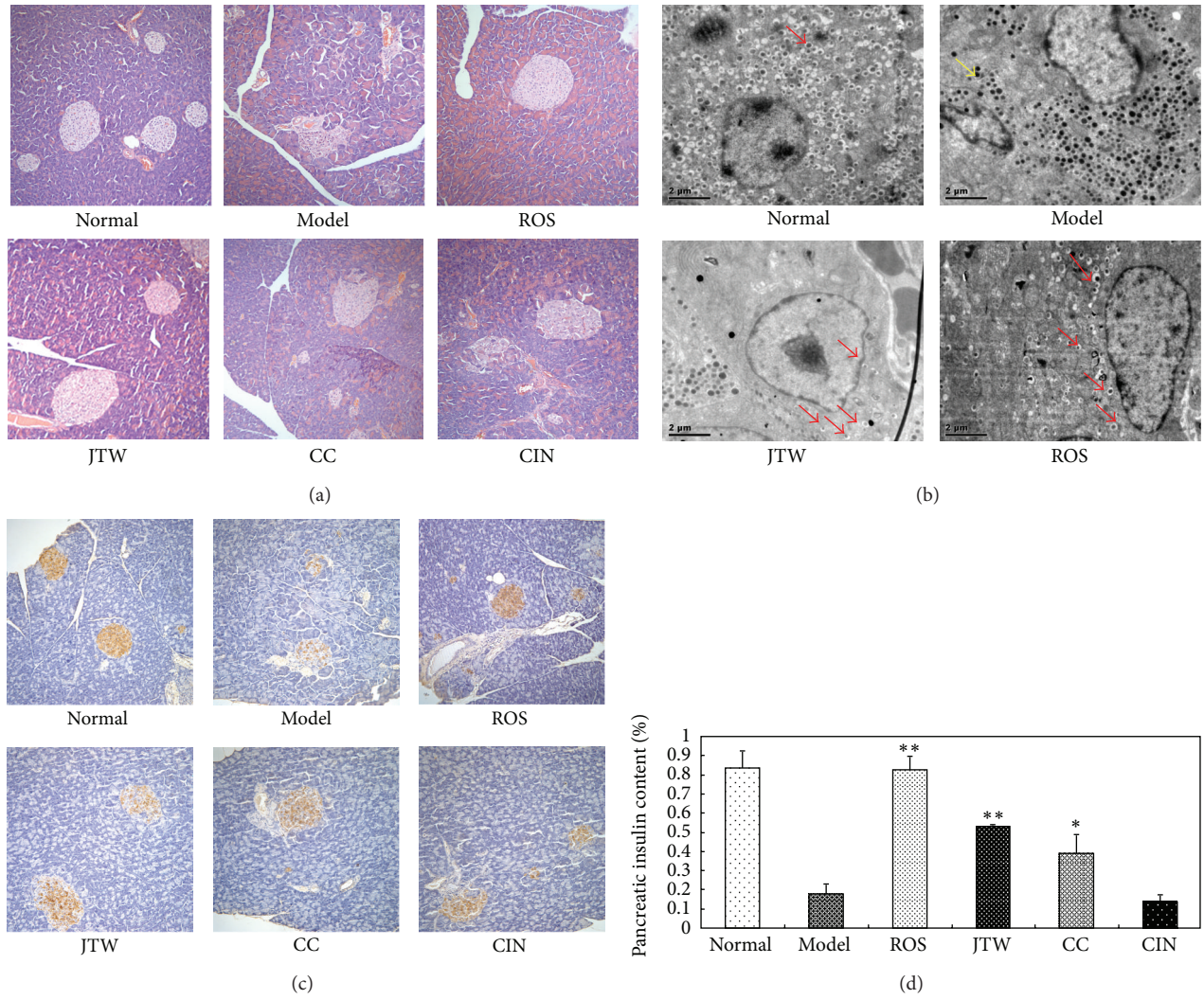

FIGURE 4: JTW ameliorates islet morphology in pancreas and β-cell function. (a) HE stain of pancreas sections, 200x (n = 8). (b) Images of pancreatic islet β cells under TEM, 6000x. The samples were prepared as described in Section 2, and pictures of the cells in the islet center were taken to observe β cell morphology by TEM; (black arrow)β-SGs, (white arrow)α-SGs (n = 8). (c) Immunohistochemical stain of insulin in pancreas sections, 200x (n = 8). (d) Pancreatic insulin content. After insulin immunohistochemical staining, the deeply stained insulin-positive cells in pancreatic islets were tested with an I-solution Image Analysis System (n = 8). Data are presented as the mean ± SE. $^{*}P < 0.05$, $^{**}P < 0.01$ versus the model control group.

that 8.4 g/kg JTW was composed of 7.6 g/kg CC and 0.76 g/kg CIN. In our study, we found that water intake, food intake, and fasting blood glucose levels significantly decreased in the JTW-treated group. CC and CIN did not improve the common symptoms of diabetes in db/db mice, although water and food intake decreased in the CC group. Conversely, water intake, food intake, and body weight increased in the CIN group. This result revealed that the compatibility of CC and CIN had scientific significance.

T2DM typically coexists with impaired glucose tolerance and insulin tolerance. Thus, we measured glucose tolerance and insulin tolerance in JTW-treated db/db mice. The result showed that glucose tolerance in JTW mice significantly improved at 0, 15, 30, 60, and 120 min and were better than those of CC and CIN groups, confirming that JTW could ameliorate glucose tolerance and have more powerful action than CC and CIN. This result agreed with the insulin tolerance results. Our findings suggest that the compatibility of CC and CIN has scientific significance, which may be associated with the bioavailability enhancement of the main component as a result of interaction with each other, indicating that this topic is worthy of further study.

Protection of β cells is significant in T2DM treatment [21, 22]. To understand the effects of JTW on the pancreas shape in db/db mice, HE staining, TEM, and immunohistochemistry test were performed to observe the histomorphology of the pancreas. The results showed that pancreatic islet shape and β-cell function in the JTW group improved to a certain extent, but the effect of this improvement was not really as good as that of ROS, especially in the result of pancreatic

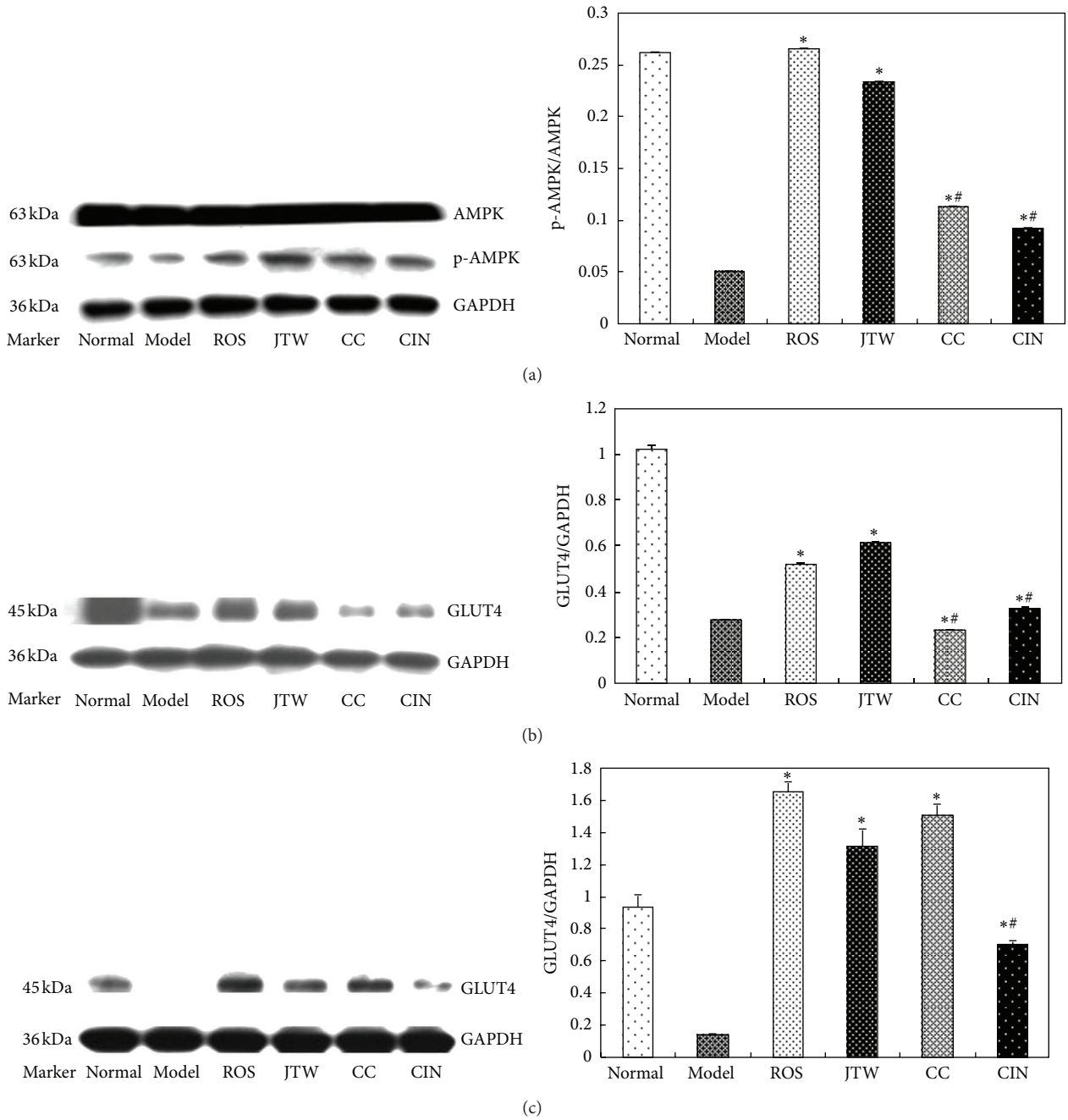

Figure 5: JTW induced AMPK in the liver and increased the expression of GLUT4 protein in skeletal muscle and white fat in db/db mice. (a) Expression of AMPK protein and p-AMPK protein in the liver ($n = 3$). (b) Expression of GLUT4 protein in abdominal fat ($n = 3$). (c) Expression of GLUT4 protein in skeletal muscle ($n = 3$). Data are presented as mean ± SE for twelve-week-old mice per group. $^{*}P < 0.05$ versus model control group. $^{\#}P < 0.05$ versus JTW-treated group.

insulin content. Therefore, we hypothesized that JTW has other hypoglycemic mechanisms. ROS belongs to a class of drugs known as peroxisome proliferator-activated receptor (PPAR) agonists. PPARγ has major functions in regulating glucose homeostasis and lipogenesis [23, 24]. Studies have proved that PPARγ agonists are potent insulin-sensitizing agents for treating T2DM but can induce body weight gain in patients [25, 26]. The conclusions were in agreement with the ROS results in this study. Significantly, our results showed that JTW did not increase body weight upon blood sugar reduction in db/db mice, revealing that JTW blocked the side effect of weight gain. Therefore, we posited that the hypoglycemic mechanism of JTW may not be directly connected to PPAR, and the hypoglycemic mechanism of JTW from other signaling pathways should be studied.

Hepatic glucose production and glucose uptake in surrounding tissues are highly important in body glucose homeostasis. We assumed that JTW is effective in inhibiting

hepatic gluconeogenesis and promoting glucose uptake of peripheral tissue. Increased hepatic glucose production is a major cause of hyperglycemia in T2DM. Gluconeogenesis and glycogenolysis are two methods of hepatic glucose production [27], but gluconeogenesis is more important. AMPK is an $\alpha\beta\gamma$ heterotrimer that has a key function in regulating glucose homeostasis and lipogenesis, comprising an α-catalytic subunit with $\beta\gamma$-regulatory subunits. AMPK is important in regulating gluconeogenesis [13]. AMPK phosphorylation can directly phosphorylate CREB-regulated transcription coactivator 2 on Ser171, which would be antagonistic to the induction of gluconeogenic genes [28]. On the other hand, glucose uptake in surrounding tissues is mediated by GLUT4 [14]. GLUT4 is the major glucose transporter of muscle and adipose tissues and facilitates glucose delivery to intracellular from extracellular, thus augmenting glucose uptake. GLUT4 mRNA and protein content can decrease in peripheral tissues, which may be one of the reasons for insulin resistance [29]. To evaluate the effect of JTW on AMPK protein in the hepatic tissue and GLUT4 protein in the peripheral tissue, Western blot analysis was used in our study. As expected, the data showed that JTW significantly induced AMPK phosphorylation in hepatic tissues and increased the expression of GLUT4 protein in both skeletal muscle and abdominal fat of db/db mice compared with the control group. The results showed that JTW increased the expression of GLUT4 that may reflect functional GLUT4 located in cellular surface, but this result requires further study. Anyway, these results indicated that JTW effectively inhibited AMPK-regulated gluconeogenesis and enhanced glucose uptake in surrounding tissues by upregulating the expression of GLUT4. The effects of JTW on downstream protein and AMPK gene require further studies.

5. Conclusion

We prove in this study that JTW reduces the blood glucose levels, food intake, and water intake and ameliorates glucose tolerance and insulin tolerance in db/db mice. As a result of its safety and low cost, especially its fewer side effects, JTW has high potential in regulating glucose metabolism. Our data prove that JTW has multiple targets in the hypoglycemic mechanism, such as suppressing gluconeogenesis through AMPK activation in the liver and affecting glucose uptake of surrounding tissues by upregulating the protein expression of GLUT4. These findings suggest that JTW may be used as a potential candidate for T2DM therapy.

Conflict of Interests

All the authors have no conflicts of interests in their submitted paper.

Acknowledgments

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Research Article

Citrus junos Tanaka Peel Extract Exerts Antidiabetic Effects via AMPK and PPAR-γ both *In Vitro* and *In Vivo* in Mice Fed a High-Fat Diet

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The antidiabetic effect of the *Citrus junos* Tanaka (also known as yuja or yuzu) was examined. Ethanol extract of yuja peel (YPEE) significantly stimulated 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (2-NBDG) uptake in C2C12 myotubes. However, ethanol extract of yuja pulp (YpEE) and water extract of yuja peel (YPWE) or pulp (YpWE) did not stimulate glucose uptake. In addition, peroxisome proliferator-activated receptor gamma (PPAR-γ) and AMP-activated protein kinase (AMPK) activities were increased by YPEE in a dose-dependent manner. Pretreatment of AMPK inhibitor decreased the glucose uptake stimulated by YPEE in C2C12 myotubes. We confirmed the anti-diabetic effect of YPEE in mice fed a high fat-diet (HFD). Compared with control mice on a normal diet (ND), these mice showed increased body weight, liver fat, insulin resistance, triacylglycerol (TG), and total cholesterol content. Addition of 5% YPEE significantly reduced the weight gain and rise in liver fat content, serum triacylglycerol (TG), total cholesterol, and insulin resistance found in mice fed a high-fat diet (HFD). Moreover, YPEE reduced the secretion of HFD-induced adipocytokines such as leptin and resistin. YPEE also resulted in increased phosphorylation of AMPK in muscle tissues. These results suggest that ethanol extract of yuja peel exerts anti-diabetic effects via AMPK and PPAR-γ in both cell culture and mouse models.

1. Introduction

Metabolic syndrome is a serious public health problem, characterized by several metabolic risks such as diabetes mellitus, obesity, and hypertension. Diabetes mellitus is characterized by elevated blood glucose levels and is associated with the development of secondary diseases. Available therapeutic agents, including sulphonylureas and thiazolidinediones, have been widely used for treating diabetes; however, most of these drugs are associated with side effects [1]. Therefore, recently, natural products with antidiabetic activity have been highlighted for the treatment of diabetes and are believed to have minimal side effects. These natural products appear to be able to control metabolic syndrome and diabetes by reducing disease-related biomarkers [2, 3].

AMP-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor gamma (PPAR-γ) are well-known biomarkers for antidiabetic drugs that contribute to insulin-sensitizing activities [4–6]. AMPK is a metabolic protein that triggers catabolic pathways, including glycolysis, for generating adenosine 5′-triphosphate (ATP). In contrast, anabolic pathways, including fatty acid and cholesterol synthesis, are blocked by AMPK, inhibiting ATP-consumption [4, 5]. Currently, AMPK is believed to act as a marker in the treatment of diabetes using natural ingredients or compounds [4, 5]. Another target for antidiabetic action is PPAR-γ, which regulates glucose metabolism via gene expression [6]. Currently, several insulin-sensitizing drugs, including thiazolidinediones, are used to treat diabetes by targeting PPAR-γ [7]. As in case of AMPK, a number of

ingredients or compounds derived from plants have been developed to treat diabetes by targeting PPAR-γ [8, 9].

Development of insulin resistance is also affected by several cytokines, including leptin and resistin [10, 11]. Leptin is believed to play a role in insulin resistance, since its levels are abnormally high in the plasma of diabetic patients [10]. Resistin is an inflammatory cytokine that plays a role in the regulation of glucose metabolism [11] and several studies have suggested that resistin leads to insulin resistance *in vivo* [11, 12].

Yuja (*Citrus junos* Tanaka), also known as yuzu in Japanese, is a yellow-colored citrus fruit that has traditionally been used to improve blood circulation and treat the common cold in Korea, Japan, and China. Recently, yuja has been found to be effective in preventing certain diseases, including those associated with oxidative stress and inflammation [13, 14]. However, its antidiabetic effects have not yet been elucidated.

In this study, we examined the effect of yuja extracts on glucose uptake in C2C12 myotubes via targeting the AMPK and PPAR-γ signaling pathways. Furthermore, we demonstrated that yuja extracts improve insulin resistance and reduce adipocytokine production in the mice fed a high-fat diet.

2. Materials and Methods

2.1. Materials. C2C12 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were purchased from WelGene (Daegu, Republic of Korea). The compound 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-d-glucose (2-NBDG) was purchased from Invitrogen (Carlsbad, CA, USA). Phosphospecific antibodies against AMP-activated protein kinase (AMPK) and beta-actin were purchased from Cell Signaling Technology (Beverly, MA, USA). SuperFect Transfection Reagent was purchased from Qiagen (Valencia, CA). DNA expression plasmids for PPAR-γ and RXR-α and luciferase reporter vectors containing PPAR-response elements (PPRE) were a kind gift from Dr. Kim Jae Bum (Seoul National University, Republic of Korea). Yuja was provided from Goheung County Office (Republic of Korea), where a voucher specimen was deposited.

2.2. Extraction and Lyophilization. Yuja pulp and peel powders (6 kg, resp.) were extracted with 10-fold volume of 70% ethanol or 100% water by shaking for 24 h at 25°C, and precipitates were removed by centrifugation at 8,000 × g for 30 min (Beckman, USA). Supernatants were lyophilized using a freeze dryer (Il Shin, Republic of Korea).

2.3. HPLC Analysis of Flavonoids. A YMC ODS-AM (250 mm × 4.6 mm ID; particle size, 5 μm) reversed-phase column (Kyoto, Japan) was used for HPLC analysis. The mobile phase consisted of solvent A, 0.1% acetic acid in water, and solvent B, 0.1% acetic acid in acetonitrile. The following gradient was used: initial 0 min A/B (88 : 12, v/v), 18 min A/B (78 : 22), 28 min A/B (72 : 28), 35 min A/B (62 : 38), 48 min A/B (52 : 48), 54 min A/B (32 : 68), 58 min A/B (0 : 100), 60 min A/B (0 : 100), and 62 min A/B (88 : 12). The column was equilibrated for 15 min prior to each analysis. The mobile phase flow rate was 1.0 mL/min, the column temperature was 35°C, the injection volume was 20 μL, and the UV detector was operated at 285 nm.

2.4. Quantitative Analysis of Phenolic Compounds. Analysis of phenolic compounds was performed using Folin-Ciocalteu reagent. Dry samples (10 mg) were mixed with 1 mL of 70% ethanol, followed by addition of 0.1 mL Folin-Ciocalteu reagent. After 3 min, 10% (w/v) Na_2CO_3 was added to each reaction mixture. Reactions were performed in the dark for 60 min, and absorbance at 725 nm was recorded using a UV spectrophotometer (JASCO, Japan). Total phenolic compound contents were quantified according to a calibration standard curve of caffeic acid (2–10 mg/L).

2.5. Muscle Differentiation and Glucose Uptake Assay. C2C12 cells were maintained in DMEM containing 10% fetal bovine serum in a CO_2 incubator. Differentiation was induced by incubation with normal medium containing 1% horse serum for 6 days. Fully differentiated cells were incubated overnight in serum-free medium containing low glucose and were added to 2-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (2-NBDG) in the presence or absence of samples for 24 h. The 2-NBDG uptake assay was performed with a fluorometer at excitation and emission wavelengths of 485 and 535 nm, respectively.

2.6. Western Blot Analysis. Cells were washed with ice-cold PBS and lysed with lysis buffer (50 mM Tris HCl, 1% Triton X-100, 0.5% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM sodium orthovanadate, 1 mM NaF, and 0.2% protease inhibitor cocktail; pH 7.2). Protein expression was detected by western blot analysis.

2.7. PPAR-γ Transcriptional Activity Assay. HEK293 cells were cotransfected with 1 μg of total DNA-expression plasmids for PPAR-γ or retinoid X receptor alpha (RXR-α) and luciferase reporter vectors containing PPAR-response elements (PPRE) and β-galactosidase. After transfection, the cells were treated with the indicated stimuli for 24 h. PPAR-γ and RXR-α activities were assessed using the Luciferase Assay System (Promega, Madison, WI, USA). The results were normalized to β-galactosidase activity.

2.8. Animal Experiments. Three-week-old male C57BL/6J mice were obtained from Nara Biotech (Seoul, Republic of Korea). Mice were housed in a climate-controlled environment (24 ± 1°C at 50% relative humidity) with 12-h light/12-h dark cycles. The mice were freely fed a 10% fat normal diet (ND, D12450B, Research Diets, New Brunswick, NJ, USA), a 60% kcal high-fat diet (HFD, D12492, Research Diets, New Brunswick, NJ, USA), a 60% kcal high-fat diet plus 1% YPEE, or a 60% kcal high-fat diet plus 5% YPEE. The mice had free access to autoclaved tap water. The diet composition is

Table 1: Composition of the diets.

Ingredients	ND (g)	HFD (g)	YPEEL (g)	YPEEH (g)
YPEE	0	0	25	125
Casein, lactic	473.9	646.1	646.1	646.1
L-Cystine	7.1	9.7	9.7	9.7
Corn starch	746.4	0.0	0.0	0.0
Maltodextrin 10	82.9	403.8	403.8	403.8
Sucrose	829.3	222.3	222.3	222.3
Cellulose, BW200	118.5	161.5	161.5	161.5
Soybean oil	59.2	80.8	80.8	80.8
Lard	47.4	791.5	791.5	791.5
ETC	135.2	184.3	184.3	184.3
Total	2500	2500	2525	2625

ND: normal diet; HFD: high-fat diet; YPEEL: high-fat diet plus 1% ethanol extract of yuja peel/kg diet; YPEEH: high-fat diet plus 5% ethanol extract of yuja peel/kg diet. ETC: et cetera.

provided in Table 1. The weight and total food intake of the mice were measured every week. After 9 weeks, the mice were starved for 12 h and blood was drawn from the orbital vein, followed by serum separation. The mice were then sacrificed, and their livers were removed. All animal experiments were performed and approved by the Institutional Animal Care and Use Committee of the Korea Food Research Institute (approval number: KFRI-M-11011, validity date: 18 May 2011).

2.9. Insulin Resistance (HOMA-IR). The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated using the methods described in the paper of Matthews et al., 1985 [15].

2.10. Histology Studies. Liver tissue was fixed in 4% buffered formalin and cut into 4 μm thick sections. Sections were stained with hematoxylin and eosin (H&E) and examined by microscopy.

2.11. Quantification of Serum Cholesterol, Triglyceride (TG), Leptin, and Resistin. Cholesterol and triacylglycerol were quantified by enzymatic methods using commercial kits (Asan Pharm, Seoul, Republic of Korea). Leptin and resistin levels were measured using enzyme-linked immunosorbent assay (ELISA) kits (R&D systems, Minneapolis, MN, USA), as per the manufacturer's instructions.

2.12. Statistical Analyses. Statistical analyses were conducted using SPSS 9.0 (SPSS Inc., Chicago, IL, USA). All results are presented as mean ± standard deviation (SD) values. Statistical differences between means were evaluated by one-way analysis of variance (ANOVA) followed by the Bonferroni test. The accepted level of significance was $P < 0.05$ for all analyses in cell and animal experiments.

3. Results

3.1. Effect of Ethanol Extract of Yuja Peel (YPEE) on 2-NBDG Uptake in C2C12 Myotubes. To determine whether yuja extract can stimulate glucose uptake, we first examined its effect on 2-NBDG uptake in C2C12 myotubes. As shown in Figure 1(a), treatment with ethanol extract of yuja peel (YPEE) significantly increased 2-NBDG uptake in a dose-dependent manner. However, ethanol extract of yuja pulp (YpEE) or water extract of yuja peel (YPWE) or pulp (YpWE) did not stimulate glucose uptake. These results suggest that ethanol extract of yuja peel (YPEE) is effective for facilitating glucose uptake. Next we used HPLC to evaluate the flavonoid content of YPEE. As shown in Figure 1(b) and Table 2, the total phenolic content of YPEE was 47.8 ± 0.5 mg/100 g. Among flavonoids contained in YPEE, rutin (2.7 mg/100 g), quercetin (1.7 mg/100 g), tangeretin (0.7 mg/100 g), naringin (11.6 mg/100 g), and hesperidin (36. 3 mg/100 g) appeared to be the major compounds.

3.2. Activation of AMPK and PPAR-γ by YPEE. Because AMPK and PPAR-γ signaling pathways are well-known targets of antidiabetic drugs, we examined whether YPEE-stimulated glucose uptake is accompanied by the activation of AMPK, and PPAR-γ. Treatment with YPEE dramatically increased the phosphorylation of AMPK in C2C12 myotubes, and pretreatment with compound C (C.C), a pharmacological inhibitor of AMPK completely decreased the phosphorylations of AMPK stimulated by YPEE (Figure 2(a)). Moreover, pretreatment of compound C inhibited the glucose uptake stimulated by YPEE (Figure 2(b)). 5-amino-1-β-Dffff-ribofuranosyl-imidazole-4-carboxamide (AICAR) was used as a positive control of AMPK activation. This result demonstrates that AMPK activation is necessary for the YPEE-stimulated glucose uptake in C2C2 myotubes.

On the other hand, we also examined the transcriptional activity of PPAR-γ upon treatment with YPEE. As shown in Figure 2(c), YPEE stimulated the transcriptional activity of PPAR-γ in a dose-dependent manner. Combined, these results suggest that YPEE-stimulated glucose uptake is accompanied by the activation of AMPK and PPAR-γ.

3.3. Effect of YPEE Supplementation on Body Weight, Liver Fat Contents, Serum TG, and Total Cholesterol Levels. To further assess the *in vitro* antidiabetic effects of YPEE, we employed a HFD-induced obese mouse model. The initial body weights of four groups were not statistically different; however, final body weights were significantly lower in the YPEEH group (Table 3). The total food-intake values showed no significant changes (Figure 3(b)) in all the groups. As shown in Figure 3(a), the body weight gain in the HFD group was higher than that in the normal diet (ND) group. Under identical conditions, higher intake of YPEE (5%) induced greater weight loss. Levels of cholesterol and TG in serum were significantly higher in the HFD group than in the ND group, yet this was alleviated significantly by YPEE supplementation (Table 3). Serum glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) levels were not significantly changed in the YPEE group compared with the ND group (data not shown). In addition, liver tissue fat was significantly decreased in the HFD plus YPEE group compared with the HFD group (Figure 3(c)).

Table 2: Total phenolic compounds and major compounds content, expressed as mg/100 g of YPEE.

	Total phenolic compounds	Rutin	Naringin	Hesperidin	Quercetin	Tangeretin
YPEE	47.8 ± 0.5	2.7 ± 0.0	11.6 ± 1.7	36.3 ± 4.3	1.7 ± 0.1	0.7 ± 0.0

All experiments were performed with 3 replicates. Data are expressed as mean ± SD.

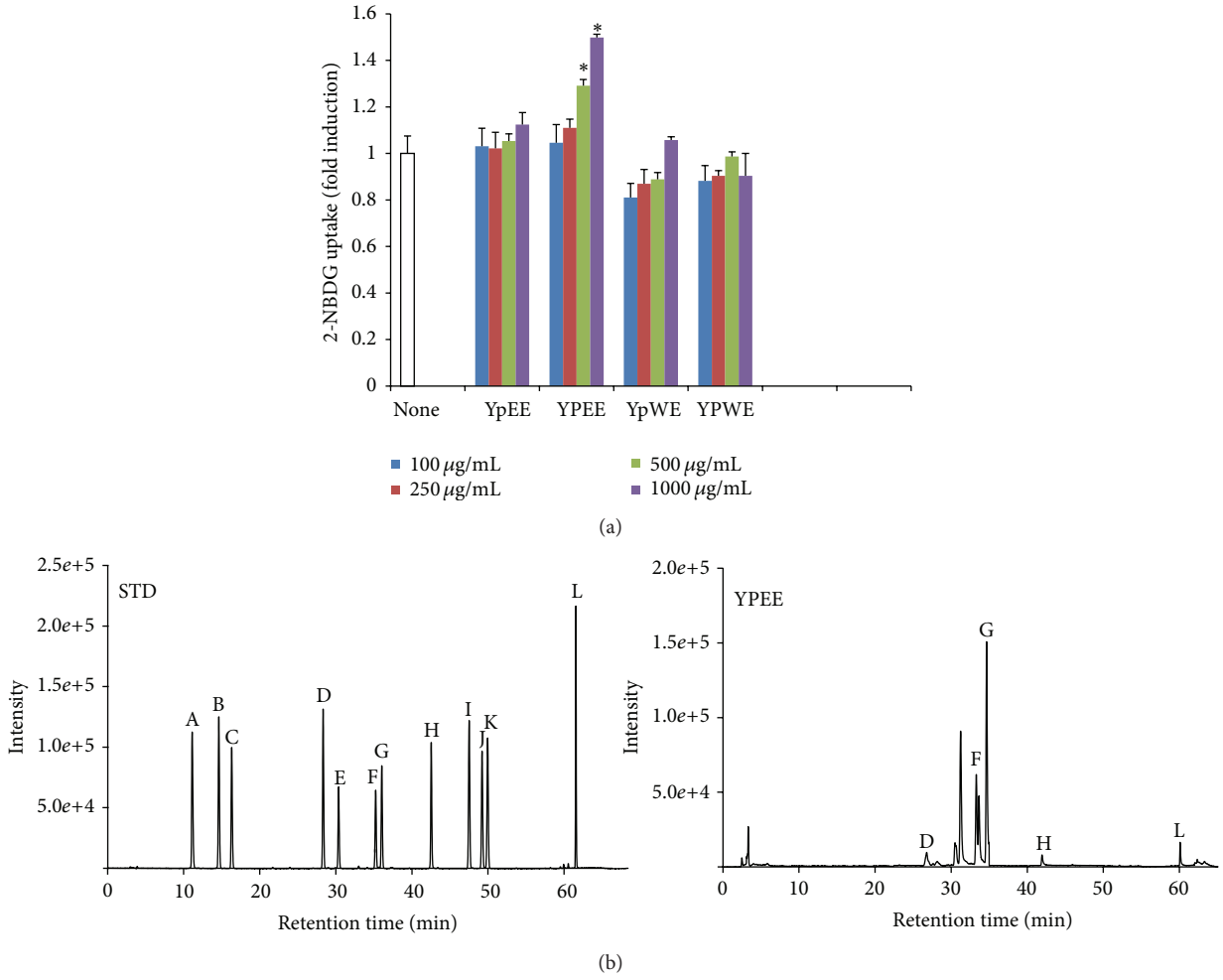

Figure 1: Effect of ethanol extract of yuja pulp (YpEE), ethanol extract of yuja peel (YPEE), water extract of yuja pulp (YpWE), and water extract of yuja peel (YPWE) on glucose uptake in C2C12 myotubes. Cells were treated with the indicated stimulus in a dose-dependent manner, and then, the 2-NBDG uptake assay was performed, as described in Section 2 (a). Data are expressed as mean ± SD. $^{*}P < 0.05$ versus none. HPLC was performed as described in Section 2. Peaks: A, chlorogenic acid; B, caffeic acid; C, epicatechin; D, rutin; E, luteolin-7-glucoside; F, naringin; G, hesperidin; H, quercetin; I, naringenin; J, kemperol; K, apegenin-7-glucoside; L, Tangeretin. STD, standard; YPEE, ethanol extract of yuja peel (b).

3.4. Effect of YPEE on HOMA-IR and Secretion of Adipocytokines. The YPEE group showed lower serum glucose and insulin levels than the HFD group (Table 3). On the basis of these results, HOMA-IR was analyzed to evaluate insulin resistance level in this study. As shown in Table 3, insulin resistance (HOMA-IR) was significantly increased in the HFD group compared with the ND group. Administration of 5% YPEE resulted in a decrease in insulin resistance compared with the HFD group.

Because it is well known that obesity-induced adipocytokine release is important event in insulin resistance, we next examined the effect of YPEE on adipocytokine production increased by HFD. As shown in Table 3, levels of adipocytokines, including leptin and resistin, were increased in the HFD group relative to the ND group. Addition of 5% YPEE to the HFD resulted in significantly decreased levels of leptin. To determine whether AMPK was phosphorylated in the muscle tissue of the mice fed HFD plus 5% YPEE, we performed a Western blot analysis using randomly collected muscle tissue. HFD plus 5% YPEE resulted in increased phosphorylation of AMPK compared with HFD alone (Figure 4). These results show that the antidiabetic effect of YPEE appears to be mediated by AMPK in these mouse models, in addition to the cell culture system.

Table 3: Inhibitory effect of YPEE on high-fat diet-induced cholesterol, TG, resistin, leptin, glucose, and insulin resistance (HOMA-IR).

	ND	HFD	YPEEL	YPEEH
Initial body weight (g)	20.2 ± 1.1	19.9 ± 0.8	20.1 ± 0.9	18.9 ± 1.2
Final body weight (g)	27.8 ± 1.6	40.4 ± 2.0[a]	38.4 ± 3.8	32.4 ± 2.4[b]
HOMA-IR	1.95 ± 0.5	26.2 ± 5.4[a]	24.2 ± 6.4[b]	7.91 ± 3.3[b]
Glucose (mg/dL)	115 ± 3	187 ± 9[a]	195 ± 8[b]	174 ± 11[b]
Serum				
Resistin (ng/mL)	17.5 ± 1.9	29.9 ± 2.1[a]	29.8 ± 3.3	20.6 ± 1.8[b]
Leptin (ng/mL)	1.6 ± 0.2	14.0 ± 1.6[a]	10.1 ± 1.5[b]	3.7 ± 0.9[b]
Total cholesterol (mg/dL)	79.1 ± 3.0	92.0 ± 3.1[a]	77.6 ± 1.7[b]	74.2 ± 1.2[b]
Triglyceride (mg/dL)	68.4 ± 9.8	78.5 ± 6.4[a]	75.6 ± 4.8	67.5 ± 8.4[b]
Insulin (ng/mL)	0.23 ± 0.05	1.85 ± 0.33[a]	1.64 ± 0.39[b]	0.58 ± 0.21[b]

ND: normal diet; HFD: high-fat diet; YPEEL: high-fat diet plus 1% ethanol extract of yuja peel/kg diet; YPEEH, high-fat diet plus 5% ethanol extract of yuja peel/kg diet. Data are expressed as mean ± SD ($n = 8$). [a]$P < 0.05$ versus ND; [b]$P < 0.05$ versus HFD.

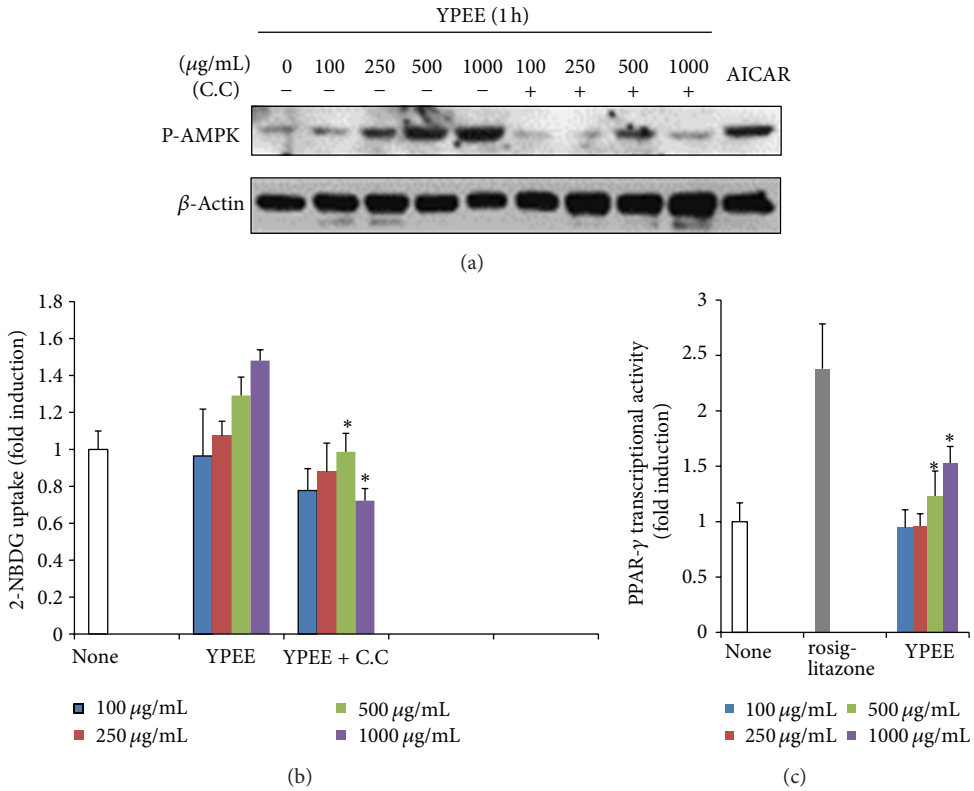

Figure 2: Effect of YPEE on AMPK and PPAR-γ. Cells were pretreated with 10 μM C.C for 30 min and consecutively exposed to YPEE for 1 h in a dose-dependent manner. Western blot analysis was performed with phosphospecific AMPK and normal beta-actin antibodies (a). Cells were pretreated with 10 μM C.C for 30 min and consecutively exposed to YPEE, and then, the 2-NBDG uptake assay was performed, as described in Section 2 (b). Data are expressed as mean ± SD.$^*P < 0.05$ versus YPEE. PPAR-γ expression vector and PPRE-luc vectors were cotransfected in HEK293 cells and exposed to YPEE or 25 μM rosiglitazone. PPAR-γ transcriptional activity was measured with the luciferase assay system (c). Data are expressed as mean ± SD.$^*P < 0.05$ versus none.

4. Discussion

The aim of this study was to evaluate the antidiabetic effect of yuja in a cell culture system and in HFD-fed mice. We found that YPEE treatment produced antidiabetic effects in both C2C12 myotubes and mouse models. In contrast, YpEE, YPWE, and YpWE did not have antidiabetic effects. In addition, AMPK and PPAR-γ were significantly activated by YPEE in our studies, suggesting that YPEE has an antidiabetic effect via activation of the AMPK and PPAR-γ signaling pathways.

Natural plants generally contain high polyphenol content, which has shown positive effects for the prevention of metabolic disorders such as diabetes [16, 17]. Thus, the antidiabetic effect of yuja peels is likely to be related to its phenolic composition, which comprises several major compounds, including rutin, quercetin, tangeretin, naringin, and hesperidin, as shown in Table 2 and Figure 1(b). Rutin

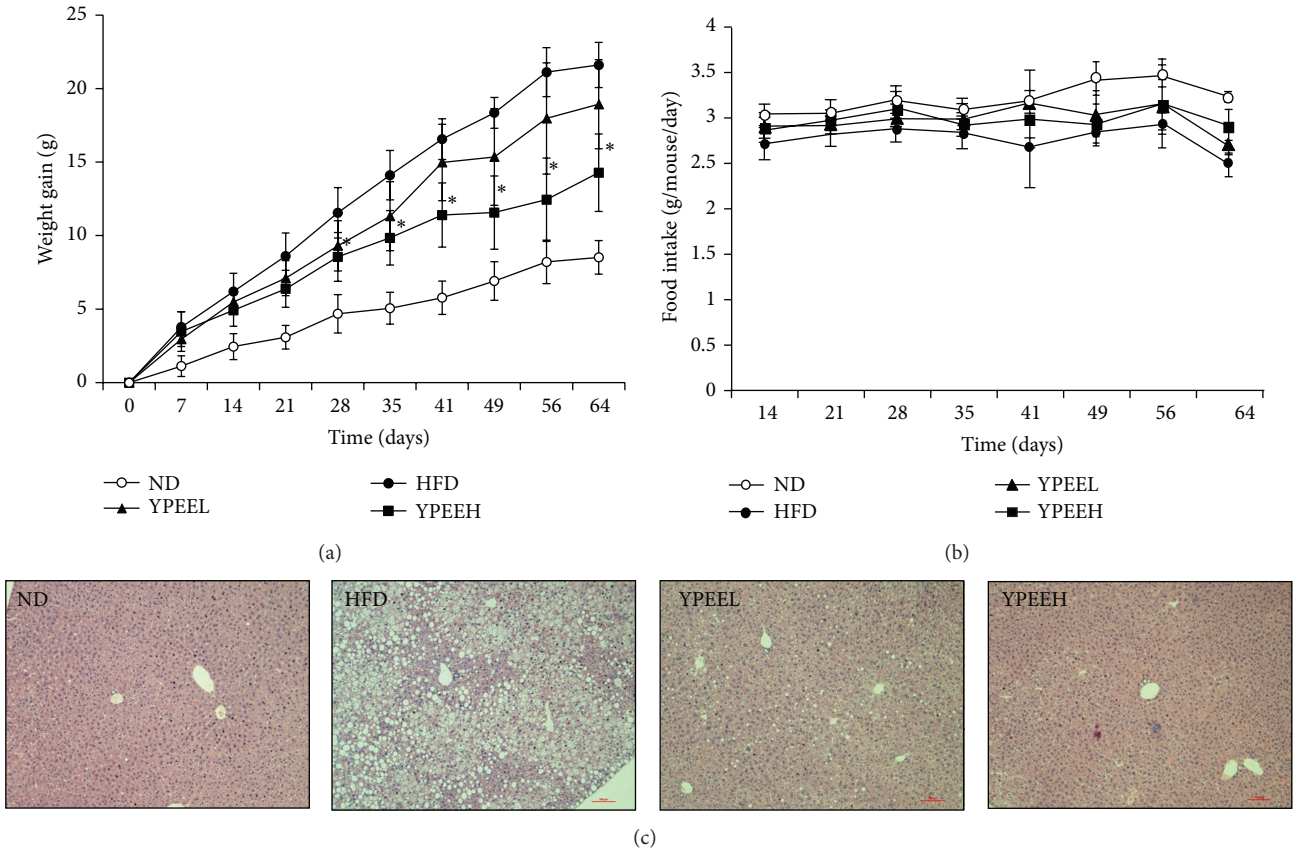

Figure 3: Effect of YPEE on body weight gain, food intake, and liver fat accumulation. Mice were fed a normal diet (ND), high-fat diet (HFD), high-fat diet + 1% YPEE (YPEEL), or high-fat diet + 5% YPEE (YPEEH). Body weight gain (a), food intake (b), and liver fat accumulation (c) were measured, as described in Section 2. Data are expressed as mean ± SD.* $P < 0.05$ versus HFD.

has been reported to exert beneficial health effects in a variety of disease models, such as inflammation, cancer, oxidative stress, and cardiovascular diseases [18–21]. Meanwhile, quercetin, naringin, and hesperidin have also been reported to exhibit beneficial health effects [22–24]. Quercetin has been suggested to prevent the development of various diseases, such as viral infection, cancer, inflammation, and metabolic syndrome [22, 25]. Hesperidin and tangeretin, which are found in citrus fruits such as yuja, also exhibit beneficial health effects against certain diseases, such as hypercholesterolemia, obesity, and diabetes [24–26]. Our previous study also has shown the ability of tangeretin to reduce the levels of circulating lipid mediators, including TG and total cholesterol, in obese mice [26]. Taken together, these results suggest that the phenolic compounds of YPEE can improve insulin resistance and thereby contribute to the amelioration of dyslipidemia, a metabolic complication of diabetes.

To the best of our knowledge, the antidiabetic effect of YPEE arises from the presence of certain flavonoids. However, we did not identify the precise phenolic compounds involved in the antidiabetic effects exerted by YPEE. Further investigations are needed to illustrate the relationship between the major compounds and the antidiabetic effect stimulated by YPEE.

Moreover, it has been reported that yuja peel contains abundant fiber, which can reduce blood glucose and improve insulin resistance [27, 28]. Yuja also contains carotenoids, which have been proposed to improve insulin resistance by stimulating insulin-signaling pathways [29]. In the present study, we speculated that like flavonoids, the minor compounds, such as fiber, carotenoids, and ascorbic acid, present in YPEE contribute to the antidiabetic effect of yuja, but we did not elucidate the underlying mechanism. AMPK and PPAR-γ were significantly activated by YPEE in myotubes. AMPK plays a central role in the metabolic process [4, 5]. Thus, many investigators have focused on the role of AMPK in the development of antidiabetic compounds. In addition, several natural ingredients have been shown to exert antidiabetic effects, accompanied by AMPK activation [4, 5]. The extract of *Malva verticillata* (MV) seeds improves diabetes by increasing glucose uptake via AMPK, dependent signaling pathways [30]. Another study demonstrated that chitosan, a natural biomaterial, exerts a glucose-lowering effect by activating AMPK and Akt in streptozotocin-induced diabetic rats [31]. The authors suggested that the mechanism of the antidiabetic effect is similar to that of metformin, an antidiabetic drug that activates AMPK. This is in consensus with our results concerning the antidiabetic activity of YPEE that activated the AMPK signaling pathway (Figure 2(a)).

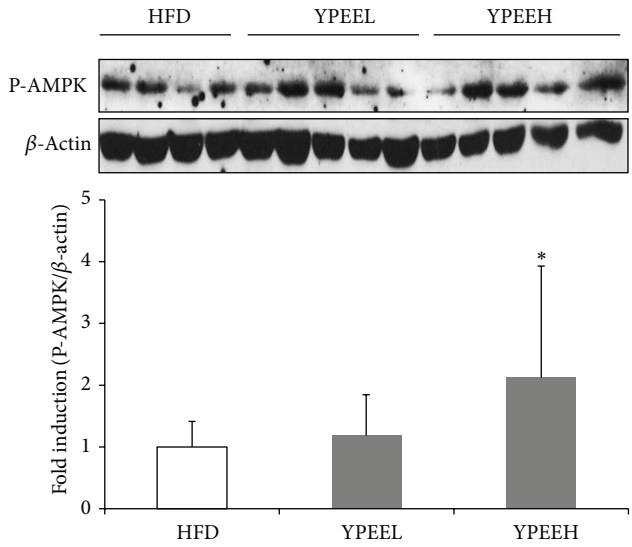

Figure 4: Phosphorylation of AMPK by YPEE in mouse muscle tissue. After finishing animal experiments, the muscle tissues randomly collected and were homogenized with lysis buffer. AMPK phosphorylation was measured using western blot analysis, and bands were analyzed by densitometry. Data are expressed as mean ± SD. $^{*}P < 0.05$ versus HFD.

YPEE also increased the transcriptional activity of PPAR-γ (Figure 2(c)), another well-known antidiabetic target. Numerous studies have demonstrated that targeting PPAR-γ is one of the best strategies for developing antidiabetic drugs [6, 32]. In this study, YPEE exerted an antidiabetic effect by activating PPAR-γ. However, the precise upstream and downstream regulators of AMPK and PPAR-γ stimulated by YPEE remain unclear. Further studies should be performed to clearly determine how YPEE regulates AMPK and PPAR-γ.

A 5% YPEE reduced fat accumulation and adipocytokine production and improved insulin resistance (Figure 3 and Table 3). A number of studies have suggested that adipocyte-derived cytokines are important factors in the etiology of diabetes. In diabetic patients, the levels of several cytokines, including resistin and leptin, are significantly increased [33, 34]. As shown in Table 3, the YPEEH appears to partially and significantly counteract the negative metabolic effects of the HFD corresponding with previous studies. Moreover, YPEE also increased the phosphorylation of AMPK in mouse muscle tissue, similar to cell culture systems. Thus, we speculate that YPEE might alter inflammatory factors or inflammation-induced insulin resistance, and that AMPK might play an important role in this action. Further studies are needed to determine the correlation between adipocytokine secretion and AMPK activity upon treatment with YPEE.

5. Conclusion

This study is the first to demonstrate the antidiabetic activity of YPEE by targeting AMPK and PPAR-γ signaling in myotubes. Moreover, YPEE also improved the abnormal levels of blood glucose, lipid, and adipocytokines in the HFD-fed mice. In addition, the major phenolic compounds in YPEE were found to include rutin, quercetin, naringin, tangeretin, and hesperidin compounds with known health promoting effects or antidiabetic effects. Therefore, YPEE may be useful for preventing diabetes and related diseases.

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Research Article

The rs1142345 in TPMT Affects the Therapeutic Effect of Traditional Hypoglycemic Herbs in Prediabetes

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Therapeutic interventions in prediabetes are important in the primary prevention of type 2 diabetes (T2D) and its chronic complications. However, little is known about the pharmacogenetic effect of traditional herbs on prediabetes treatment. A total of 194 impaired glucose tolerance (IGT) subjects were treated with traditional hypoglycemic herbs (Tianqi Jiangtang) for 12 months in this study. DNA samples were genotyped for 184 mutations in 34 genes involved in drug metabolism or transportation. Multinomial logistic regression analysis indicated that rs1142345 (A > G) in the thiopurine S-methyltransferase (TPMT) gene was significantly associated with the hypoglycemic effect of the drug ($P = 0.001$, FDR $P = 0.043$). The "G" allele frequencies of rs1142345 in the healthy (subjects reverted from IGT to normal glucose tolerance), maintenance (subjects still had IGT), and deterioration (subjects progressed from IGT to T2D) groups were 0.094, 0.214, and 0.542, respectively. Binary logistic regression analysis indicated that rs1142345 was also significantly associated with the hypoglycemic effect of the drug between the healthy and maintenance groups ($P = 0.027$, OR = 4.828) and between the healthy and deterioration groups ($P = 0.001$, OR = 7.811). Therefore, rs1142345 was associated with the clinical effect of traditional hypoglycemic herbs. Results also suggested that TPMT was probably involved in the pharmacological mechanisms of T2D.

1. Introduction

Diabetes is a metabolic disorder characterized by chronic hyperglycemia and abnormalities in carbohydrate, fat, and protein metabolism. The World Health Organization (WHO) reported that diabetes is the fifth leading cause of death in the world and is a serious public health problem worldwide [1]. The prevalence of diabetes in the USA, European Union, China, and India is 8.3%, 6.5%, 9.7%, and 8.6%, respectively, and these numbers increase annually [2–5]. Approximately 366 million people are estimated to develop diabetes-related diseases by 2030 [6]. Type 2 diabetes (T2D) is the most common form of diabetes, accounting for over 90% of all cases.

Prediabetes is a condition in which the glycemic variables are lower than the diabetes threshold but higher than normal (the level of fasting plasma glucose (FPG) is lower than 6.1 mmol/L and the glucose level after a two-hour oral

glucose tolerance test (2 h OGTT) is lower than 7.8 mmol/L). Prediabetes is of three types: impaired fasting glucose (IFG), impaired glucose tolerance (IGT), and combined IFG/IGT [7]. WHO (1999) defined IGT as a prediabetic state in which the level of FPG is lower than 7.0 mmol/L and the glucose level after a 2 h OGTT is between 7.8 mmol/L and 11.1 mmol/L. IFG is defined as a condition in which the level of FPG is between 6.1 mmol/L and 6.9 mmol/L and the 2 h OGTT glucose level is lower than 7.8 mmol/L. Previous study reported that 5% to 10% of prediabetics become diabetes patients every year; thus, prediabetes is a high-risk state for diabetes [8]. Hypoglycemic therapy significantly reduces the risk of developing diabetes [8, 9].

Individual variations in drug response are common in clinical treatments, which result in the low control rate of many diseases [10]. Many pharmacogenetic studies have been conducted to identify the potential mechanism of drug response differences, but only few focused on traditional herbs. Arrays have been developed mainly for pharmacogenetic studies [11–13]. Pharmacogenetic arrays include most of the markers related to drug absorption, distribution, metabolism, and excretion (ADME). Pharmacogenetic arrays help describe the relationship between the ADME markers and the drug under study. Consequently, this type of array has become popular in pharmacogenetic studies.

A traditional hypoglycemic herb known as Tianqi Jiangtang is widely utilized for diabetes treatment in China. The herb is processed and produced in capsules by Heilongjiang Baoquan Pharmaceutical Company, Limited (Hegang, China). This drug is licensed by the State Food and Drug Administration of China as a novel category III drug for lowering blood glucose levels in patients with diabetes [14, 15]. Previous studies have indicated that Tianqi Jiangtang controls diabetes by reducing hyperglycemia and modifying lipid metabolism [16, 17]. Tianqi Jiangtang consists of 10 Chinese herbal medicines, namely, Radix Astragali, Radix Trichosanthis, Fructus Ligustri Lucidi, Caulis Dendrobii, Radix Ginseng, Cortex Lycii Radicis bone, Rhizoma Coptidis, Asiatic Cornelian cherry fruit, Ecliptae Herba, and Chinese gall. Many of these herbal medicines are correlated with diabetes-related parameters. For example, Rhizoma Coptidis and astragalin in Radix Astragali reduce glucose, similar to Diformin [18, 19]. Berberine in Rhizoma Coptidis improves some glycemic parameters [20–22]. Ginsenoside Re in Radix Ginseng has significant antihyperglycemic effects [23–25]. The iridosides of Cornus officinalis in Asiatic Cornelian cherry fruit prevent diabetic vascular complications [26, 27]. Only one major effective component of Tianqi Jiangtang has been identified, namely, berberine hydrochloride ($C_{20}H_{18}ClNO_4$), which has been successfully employed in antidiabetes treatments [28–31].

This study aims to identify a possible prophylactic action against diabetes as well as the genetic factors related to the individual differences in drug response to Tianqi Jiangtang. A total of 194 prediabetes patients from 12 Chinese hospitals were treated with traditional hypoglycemic herbs for 3 to 12 months. The condition of the subjects during the trial was determined, and a correlation analysis was conducted to identify the genetic markers related to the hypoglycemic effect of traditional hypoglycemic herbs.

2. Materials and Methods

2.1. Subjects. This study was approved by the Institutional Review Board of Beijing Guang'anmen Hospital, Chinese Academy of Medical Sciences, and by Central South University. All subjects were IGT patients from 12 hospitals in China. The hospitals are Beijing Guang'anmen Hospital, Chinese Academy of Medical Sciences; Zhejiang Chinese Medical University Affiliated Hospital; Kwong Hing/Hangzhou Municipal TCM Hospital; The First Affiliated Hospital of Guangzhou University of Traditional Chinese Medicine; Chinese Medicine Hospital of Foshan City, Guangdong Province; Qinghai Hospital of Traditional Chinese Medicine, Qinghai Province; Shenzhen Traditional Chinese Hospital; Affiliated Hospital of Changchun University of Traditional Chinese Medicine; Chinese Medicine Hospital of Xuzhou; Chinese Medicine Hospital of Shantou; Guangzhou Huangpu Chinese Medicine Hospital; and Guangzhou Tianhe Chinese Medicine Hospital. All subjects were overweight or obese. The subjects signed an informed consent form before participating in this study. The inclusion and exclusion criteria are provided below.

Inclusion criteria are (1) having an FPG level lower than 7.0 mmol/L and a glucose level between 7.8 mmol/L and 11.1 mmol/L after a two-hour OGTT; (2) being Qi- and yin deficient according to traditional Chinese medicine and suffering from excess body heat (manifested by weakness, fatigue, dry mouth, bitter taste in mouth, red tongue with teeth prints, thin and white hair, and taut, rapid, or thready pulse); (3) having never used an anti-diabetic drug; (4) having body mass index (BMI) of 24 kg/m^2 to 30 kg/m^2; (5) aging 25 to 70; (6) not participating in drug trials in the past three months.

Exclusion criteria are (1) having suffered from acute cardiocerebrovascular events or myocardial infarction in the past six months; (2) having proliferative retinopathy treated by laser; (3) having stress or secondary hyperglycemia; (4) being unwilling to cooperate; (5) having mental illness; (6) being pregnant or lactating, or planning pregnancy or lactation; (7) being allergic to Tianqi Jiangtang; (8) having systolic blood pressure higher than or equal to 160 mmHg and diastolic blood pressure higher than or equal to 100 mmHg (i.e., secondary hypertension); (9) having cholesterol higher than or equal to 6.22 mmol/L or low-density lipoprotein higher than or equal to 4.14 mmol/L; (10) taking other hypoglycemic drugs.

2.2. Herbs and Preparation Method. Tianqi Jiangtang capsules are produced by Heilongjiang Baoquan Pharmaceutical Company, Limited (Hegang, China; Z20063799). The exact amount of each of the herbs in the drug is listed in Table 1. The preparation of the drug was described in detail by Zhang et al. [16]. The batch number of the drug is 080103. The berberine hydrochloride content of each capsule was measured by high-performance liquid chromatography to

Table 1: Exact amount of each component required to produce 1000 capsules of Tianqi Jiangtang.

Herb name	Amount	Herb name	Amount
Radix Astragali	400 g	Radix Trichosanthis	400 g
Fructus Ligustri Lucidi	334 g	Caulis Dendrobii	200 g
Radix Ginseng	134 g	Cortex Lycii Radicis bone	267 g
Rhizoma Coptidis (steamed)	200 g	Asiatic Cornelian cherry fruit	200 g
Ecliptae Herba	334 g	Chinese gall	200 g

Table 2: Genes in the Veracode ADME core panel.

Phase I enzymes		Phase II enzymes		Transporters		Other
CYP1A1	CYP2C9	DPYD	SULT1A1	ABCB1	SLC22A6	VKORC1
CYP1A2	CYP2D6	GSTM1	TPMT	ABCC2	SLCO1B1	
CYP2A6	CYP2E1	GSTP1	UGT1A1	ABCG2	SLCO1B3	
CYP2B6	CYP3A4	GSTT1	UGT2B15	SLC15A2	SLCO1B1	
CYP2C19	CYP3A5	NAT1	UGT2B17	SLC22A1		
CYP2C8		NAT2	UGT2B7	SLC22A2		

control drug quality. Exactly 0.5 g of the sample was added to a conical flask containing 50 mL of acidified (1% HCl) methanol. The mixture was heated ultrasonically (250 W, 40 kHz) for 30 min and then naturally cooled and filtered. Ten milliliters of the filtered mixture was placed in a neutral alumina column (100 mesh to 200 mesh, 5 g, inner diameter of 0.9 cm). Ethanol (30 mL) was utilized to elute the mixture. The eluent was collected and transferred to a 50 mL measuring flask. Ten microliters of the eluent was injected into the liquid chromatograph (Beckman Instruments, Inc.) to determine berberine hydrochloride content. The average berberine hydrochloride content was 7.8 (±0.13) mg per capsule.

2.3. Study Design. This study is a double-blind, multicenter, randomized clinical trial (registration number: ISRCTN90063632). The 194 volunteers participated in a one-month placebo treatment before the actual treatment. Afterward, the subjects were treated with Tianqi Jiangtang at a dosage of 5 capsules (1.6 g, based on the instruction of the drug) per time, three times daily for 3 months to 12 months. Smoking and drinking alcohol were prohibited during the treatment. The subjects were subjected to blood glucose tests every three months and were divided into healthy, maintenance, and deterioration groups according to the test results. The WHO criteria were employed to diagnose and classify the samples [32]. The healthy group included subjects whose FPG and 2 h OGTT levels returned to normal (the FPG level was less than 6.1 mmol/L and the glucose level after the 2 h OGTT was less than 7.8 mmol/L). This group then underwent a consolidation period of 3 months, taking the medicine at a dosage of 3 capsules per time, three times daily. Treatment was stopped after this period, but the group was monitored for six months. The maintenance group included subjects who still had IGT (the FPG level was less than 7.0 mmol/L and the glucose level after the 2 h OGTT was between 7.8 mmol/L and 11.1 mmol/L) after the trial. This group received treatment until the end of the study. Subjects who developed T2D (the FPG level was more than or equal to 7.0 mmol/L or the glucose level after the 2 h OGTT was more than or equal to 11.1 mmol/L) during the trial were assigned to the deterioration group. The treatment for this group was substituted with Western medicine. The research process is illustrated in Figure 1.

2.4. Genotyping. A commercial isolation kit (Qiagen, Inc.) was utilized to extract genomic DNA from peripheral blood leukocytes. DNA concentration and purity were assessed with Nanodrop 2000 (Thermo Fisher Scientific, Inc.). An Illumina VeraCode ADME core panel (Illumina, Inc.) was utilized according to the manufacturer's protocol to genotype the subjects. With this core panel, the genotypes of the 32 samples were recorded. The DNA of each sample was divided into three portions and the target regions into three optimized assay pools by utilizing three different biotinylated primer mixes. Paramagnetic particles were employed to isolate the products. The allele-specific extension and ligation of the products were conducted by universal polymerase chain reaction amplification. The products were then hybridized with VeraCode beads and scanned by a BeadXpress Reader (Illumina, Inc.). The data were managed and analyzed through Verascan. The VeraCode ADME core panel is described in detail at http://www.illumina.com/documents/products/datasheets/datasheet_veracode_adme_core_panel.pdf. The VeraCode ADME core panel includes 184 markers (173 single-nucleotide polymorphisms (SNPs), 10 copy number variations, and 1 three-base deletion) in 34 genes, covering over 95% of the PharmaADME Core List. All genes were functionally significant in drug ADME (Table 2). SNPs with a minor allele frequency (MAF) lower than 5%, a Hardy-Weinberg equilibrium (HWE) test P value lower than 0.001, and those that were not genotyped were excluded before the statistical analyses. Out of the initial full set of 173

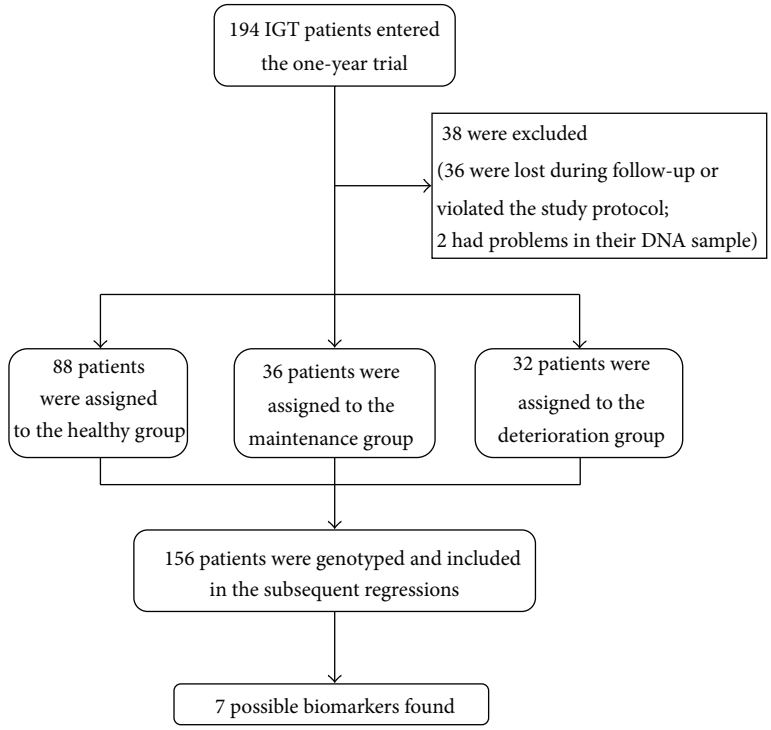

Figure 1: Research process.

SNPs, 3 were not genotyped, 127 had MAF lower than 0.05, and none had an HWE test P value lower than 0.001. The 43 SNPs that remained were employed for subsequent analysis.

2.5. Statistical Analyses. Stepwise regression was performed (add $P = 0.05$, remove $P = 0.1$) before the correlation analysis to screen for possible covariants. The analysis parameters were age, sex, height, weight, body mass index, systolic and diastolic blood pressure, heart rate, and waistline and hip measurements. Multinomial and binary logistic regressions were performed with SPSS 17 (Chicago, IL) to identify the association between the genetic markers and the hypoglycemic effects of Tianqi Jiangtang. The Bonferroni correction was utilized for the multiple testing corrections to adjust the raw P values. Plink 1.07 (http://pngu.mgh.harvard.edu/~purcell/plink/) was utilized to calculate the MAFs and HWE test P values of the SNPs.

3. Results

The basic characteristics of the subjects including sex, age, height, weight, BMI, and FPG and 2 h OGTT levels are summarized in Table 3. Among the initial 194 prediabetes patients, the 156 patients who finished the entire treatment were selected for subsequent analysis. The average FPG and 2 h OGTT glucose levels were 6.15 ± 0.54 mmol/L and 9.22 ± 1.03 mmol/L, respectively. Among the selected 156 patients, 88 were cured (healthy group), 36 still had IGT (maintenance group), and 32 developed T2D (deterioration group). The efficiency of the Tianqi Jiangtang capsules was 56%. The healthy group included 46 males and 42 females aged 29 to 67. The average FPG and 2 h OGTT glucose levels of this group were 5.64 ± 0.59 mmol/L and 6.86 ± 1.12 mmol/L, respectively. The corresponding average decrease was 0.40 ± 0.81 mmol/L for average FPG and 2.13 ± 1.41 mmol/L for 2 h OGTT glucose. The maintenance group consisted of 17 males and 19 females aged 32 to 69. The average FPG and 2 h OGTT glucose levels were 6.06 ± 0.58 mmol/L and 9.01 ± 1.56 mmol/L, respectively. The deterioration group included 15 males and 17 females aged 32 to 69. The average FPG and 2 h OGTT glucose levels were 7.34 ± 1.08 mmol/L and 11.00 ± 1.76 mmol/L, respectively. The corresponding average increase was 1.03 ± 1.17 mmol/L for FPG and 1.41 ± 1.86 mmol/L for 2 h OGTT glucose.

Age ($P = 0.032$) was screened as the covariate of subsequent multinomial logistic regressions. Seven SNPs were found to be significantly associated with the hypoglycemic effect of Tianqi Jiangtang after the multinomial logistic regressions. These SNPs were rs1142345 ($P = 0.001$) in the TPMT (thiopurine S-methyltransferase) gene, rs2306168 ($P = 0.003$) in the ABCG2 (adenosine triphosphate ATP-binding cassette, subfamily G, member 2) gene, rs2231142 ($P = 0.021$) in the SLCO2B1 (solute carrier organic anion transporter family, member 2B1) gene, rs717620 ($P = 0.023$) in the ABCC2 (ATP-binding cassette, sub-family C, member 2) gene, rs1799931 ($P = 0.024$) in the NAT2 (N-acetyltransferase 2) gene, rs4244285 ($P = 0.027$) in the CYP2C19 (cytochrome P450, family 2, sub-family C, polypeptide 19) gene, and rs4124874 ($P = 0.044$) in the UGT1A1 (uridine diphosphate glucuronosyltransferase 1 family, polypeptide A1) gene. Only rs1142345 in TPMT passed the multiple testing correction (adjust $P = 0.043$). The

Table 3: Basic characteristics of the subjects in the study.

Traits	Total sample	Healthy group	Maintenance group	Deterioration group	*P* value
Number (male/female)	156 (78/78)	88 (46/42)	36 (17/19)	32 (15/17)	—
Age (years)	51.96 ± 9.78	50.59 ± 9.49	52.83 ± 9.22	54.7 ± 10.74	0.10
Height (cm)	163.20 ± 8.21	162.74 ± 8.49	163.25 ± 7.63	164.41 ± 8.18	0.62
Weight (kg)	66.65 ± 9.79	66.60 ± 10.42	66.21 ± 6.60	67.31 ± 11.16	0.90
BMI (kg/m^2)	24.99 ± 3.12	25.06 ± 2.99	24.90 ± 2.76	24.91 ± 3.84	0.96
FPG (mmol/L)	6.15 ± 0.54	5.64 ± 0.59	6.06 ± 0.58	7.34 ± 1.08	0.02
2 h OGTT (mmol/L)	9.22 ± 1.03	6.86 ± 1.12	9.01 ± 1.56	11.00 ± 1.76	0.01

The values are presented as means ± standard deviation.
The *P* values in all three groups were calculated by ANOVA.

Table 4: Significant SNPs for multinomial logistic regression.

SNP	Gene	Allele	Chr	Position	MAF*	*P*	FDR *p*
rs1142345	TPMT	A/G	6	18130918	0.229	0.001	0.043
rs2306168	SLCO2B1	T/C	11	74907582	0.305	0.003	0.129
rs2231142	ABCG2	A/C	4	89052323	0.260	0.021	0.903
rs717620	ABCC2	C/T	10	101542578	0.230	0.023	0.989
rs1799931	NAT2	A/G	8	18258370	0.153	0.024	1
rs4244285	CYP2C19	A/G	10	96541616	0.344	0.027	1
rs4124874	UGT1A1	G/T	2	234665659	0.337	0.044	1

*Minor allele frequency calculated from the subjects.

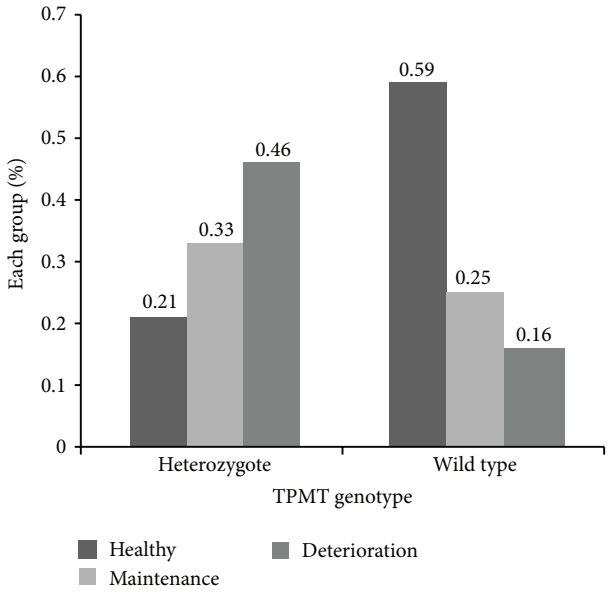

Figure 2: Percentage of each group in rs1142345 heterozygote (AG) and wild-type (AA) patients.

analysis results and the basic characteristics of the seven SNPs are summarized in Table 4. The "G" allele frequencies of rs1142345 in the healthy, maintenance, and deterioration groups were 0.094, 0.214, and 0.542, respectively. These frequency values increased progressively. Figure 2 shows the percentage of each group in the heterozygote (AG) carriers (TPMT rs1142345 mutated) and homozygote (AA) carriers (wild-type allele). No mutated homozygotes (GG) of TPMT rs1142345 were found in the subjects. The effective ratio of subjects with the wild-type allele of TPMT rs1142345 was 2.8 times higher than that of subjects with TPMT heterozygotes. Binary logistic regression was also employed. Between the healthy and maintenance groups and between the healthy and deterioration groups, rs1142345 was significantly associated with the hypoglycemic effect of the drug ($P = 0.027$, OR = 4.828 and $P = 0.001$, OR = 7.811, respectively).

4. Discussion

To our knowledge, this study is the first to utilize the ADME gene chip in the pharmacogenetic study of traditional hypoglycemic herbs. The seven SNPs were associated with the hypoglycemic effect of Tianqi Jiangtang. The genes containing these SNPs are all pharmacokinetics related, including phase I enzyme CYP2C19; phase II enzymes TPMT, NAT2, and UGT1A1; transporter ABCC2, SLCO2B1, and ABCG2. Only rs1142345 in TPMT passed the multiple testing corrections.

TPMT catalyzes the S-methylation of some drugs, such as azathioprine, mercaptopurine, and thioguanine. Research on TPMT genetic polymorphisms is one of the most advanced pharmacogenetic studies [33]. The TPMT activity exhibits genetic polymorphism: approximately 90% of individuals inherit high activity, 10% exhibit intermediate activity because of heterozygosis, and approximately 0.3% have low or no detectable enzyme activity because they inherited two nonfunctional TPMT alleles [34]. The SNP rs1142345, also called TPMT*3B, alters Ala154Thr and reduces the TPMT activity. In this study, TPMT*3B was associated with the

hypoglycemic effect of Tianqi Jiangtang. The effective ratio of subjects with homozygotes (AA) of the wild-type allele of TPMT rs1142345 was 2.8 times higher than that of subjects with TPMT heterozygotes (AG). This association is due to the possible involvement of TPMT with one of the active components of Tianqi Jiangtang. Reduced TPMT activity increases the concentration of the active components and improves their pharmacological effect. However, the relationship between TPMT and berberine hydrochloride (the major effective active component of Tianqi Jiangtang) remains unclear. The hypoglycemic effect on subgroup patients with a TPMT mutated allele and berberine hydrochloride from Tianqi Jiangtang should be investigated further.

Although the other six polymorphisms did not pass the multiple testing corrections, they still provide information for further study. The polymorphism rs717620 (−24C>T) in ABCC2 was found to be associated with the hypoglycemic effect of Tianqi Jiangtang. ABCC2 encodes the excretive transporter MRP2, mediating the reversed concentration gradient excretion of some exogenous and endogenous compounds. The polymorphism rs717620 enhances transport activity and substrate excretion [35, 36]. Subjects with a mutated allele (T) of rs717620 tended to progress from IGT to diabetes mellitus (the "T" allele frequency of the healthy group was 0.35 and that of the deterioration group was 0.52). The enhanced excretion of the active components of mediated reversed concentration gradient excretion decreased cell concentration and worsened the pharmacological reaction. The polymorphism rs2231142 (421C>A) in the excretive transporter ABCG2 was associated with the suitable hypoglycemic effect of mediated reversed concentration gradient excretion because rs2231142 possibly weakened the transport function and increased the plasma concentration of its substrate [37]. One polymorphism in the uptake transporter gene associated with the hypoglycemic effect of Tianqi Jiangtang was rs2306168 (1457C>T) in SLCO2B1 (encoded OATP2B1). This polymorphism reduced transport activity [38, 39] and improved the pharmacological reaction. OCT1 is an organic cation transporter, whereas OATP2B1 is an anion transporter. This difference suggests that Tianqi Jiangtang has more than one kind of active compound. The polymorphisms rs4244285 (682C>A) in CYP2C19, rs1799931 (857G>A) in NAT2, and rs4124874 (−3279T>G) in UGT1A1 were also associated with the hypoglycemic effect of Tianqi Jiangtang. The subjects with the mutated alleles of these three polymorphisms exhibited reduced catalytic activity [40–42] and appropriate pharmacological reactions. Although these six polymorphisms did not pass the multiple testing corrections, their results may provide clues for the screening of possible active components.

The rate of prediabetes developing into diabetes was 20.5% in this study, a value that is higher than that reported in a previous study (5% to 10%) [8]. The higher rate could have been caused by the difference in the design of the studies. The subjects in this present study were selected from the Chinese Han population, whereas the previous study focused on Caucasians. The ethnic differences between the Chinese and Caucasian populations may have caused the rate to vary. The small sample size of the present study may have also caused the variation. Moreover, the traditional hypoglycemic herbs administered in this study might have been unsuitable for the IGT patients because of their genetic background. For these patients, taking traditional hypoglycemic herbs increased the risk of diabetes, which could have caused the rate to increase.

5. Conclusion

The SNP rs1142345 was found to be associated with the clinical consequence of Tianqi Jiangtang. TPMT is possibly involved in the pharmacological mechanisms of T2D.

Conflict of Interests

None of the authors has any conflict of interests regarding this study.

Authors' Contribution

Xi Li and Feng-Mei Lian equally contributed to this work.

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Research Article

Supplementation of *Lactobacillus plantarum* K68 and Fruit-Vegetable Ferment along with High Fat-Fructose Diet Attenuates Metabolic Syndrome in Rats with Insulin Resistance

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Lactobacillus plantarum K68 (isolated from *fu-tsai*) and fruit-vegetable ferment (FVF) have been tested for antidiabetic, anti-inflammatory, and antioxidant properties in a rat model of insulin resistance, induced by chronic high fat-fructose diet. Fifty rats were equally assigned into control (CON), high fat-fructose diet (HFFD), HFFD plus K68, HFFD plus FVF, and HFFD plus both K68 and FVF (MIX) groups. Respective groups were orally administered with K68 (1×10^9 CFU/0.5 mL) or FVF (180 mg/kg) or MIX for 8 weeks. We found that HFFD-induced increased bodyweights were prevented, and progressively increased fasting blood glucose and insulin levels were reversed ($P < 0.01$) by K68 and FVF treatments. Elevated glycated hemoglobin (HbA1c) and HOMA-IR values were controlled in supplemented groups. Furthermore, dyslipidemia, characterized by elevated total cholesterol (TC), triglyceride (TG), and low-density lipoproteins (LDLs) with HFFD, was significantly ($P < 0.01$) attenuated with MIX. Elevated pro-inflammatory cytokines, interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α (TNF-α), were controlled ($P < 0.01$) by K68, FVF, and MIX treatments. Moreover, decreased superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) activities were substantially ($P < 0.01$) restored by all treatments. Experimental evidences demonstrate that K68 and FVF may be effective alternative medicine to prevent HFFD-induced hyperglycemia, hyperinsulinemia, and hyperlipidemia, possibly associated with anti-inflammatory and antioxidant efficacies.

1. Introduction

The major recent challenge in medical or food science in developed/developing counties is to combat against the diet related disorders, especially diseases connected to insulin resistance (IR) syndrome. IR represents a cluster of metabolic disorders, including obesity, glucose intolerance, and predisposes to type 2 diabetes [1, 2]. Occurrence of IR is mainly due to the urbanization and consequent changes in lifestyle, particularly, shifting the regular diet to "Western-style diet" which contains high fat [1, 3, 4]. Chronic high fat intake has been proved as a key responsible factor for metabolic syndrome [5]. In addition, high fructose consumption progresses to dietary model of type 2 diabetes that is associated with obesity, IR, hyperglycemia, and dyslipidemia [2]. Intake of either fat or fructose diet has been shown to trigger the free radicals or reactive oxygen species (ROS) production, thus ruining the antioxidant and inflammatory systems [5–7].

Many traditional ethnicities in East Asia and around the world believe that functional foods (fermented) are rich in nutrients and used as alternative medicine. Preserved foods have been used as condiments to enhance the overall

flavor of the meal. Fermented food products contain several useful bacteria, including lactic acid bacteria (LAB), and easier to digest than unfermented foods [8]. Various strains of LAB are used in the manufacture of fermented foods, including milk, bread, vegetables, and other edible plant materials [9, 10]. Certain strains of LAB, particularly strains from the genera *Lactobacillus*, showed several health promoting effects, including antidiabetic property [11, 12]. Among several *Lactobacillus* species, *L. plantarum* is versatile species that possesses several therapeutic applications. *L. plantarum* 299v is reported as cardioprotective agent by decreasing the plasma insulin, leptin, and IL-6 concentrations in smokers [13]. However, only few studies demonstrated the applications of *L. plantarum* in diabetic rat model. In view of the global threat from obesity and diabetes, it is highly essential to treat/prevent the diet-induced metabolic syndrome by enhancing the intake of proper nutrients, which has been considered as alternative medicine.

Our previous findings demonstrated the diversity of LAB from various kinds of Taiwanese fermented foods, such as stinky tofu, *suan-tsai,* and *fu-tsai* [14, 15]. *L. plantarum* K68 (K68), a probiotic strain isolated from *fu-tsai*, has been shown to reduce the production of pro-inflammatory cytokines (TNF-α, IL-β, and IL-6) in dextran sulfate sodium (DSS)-induced ulcerative colitis BALB/c mice [16]. Frohlich et al. [17] reported the protective effect of fermented food substance against the development of cancer.

Since indigenous fermented fruits and vegetables tremendously contribute health promoting effects, it is necessary to validate the scientific facts of their ingredients. Therefore, we made an attempt to evaluate the medicinal values of *L. plantarum* K68 and fruit-vegetable ferment (FVF) against high fat-fructose diet (HFFD)-induced metabolic syndrome in rats. In this study, we fed rats with HFFD to induce insulin resistance and simultaneously supplemented K68 and FVF to examine their therapeutic effects on the progression of obesity, hyperglycemia, hyperinsulinemia, and hyperlipidemia. To emphasize the significance of traditional food as a complementary and alternative medicine, we further investigated the anti-inflammatory and antioxidant properties of these substances in IR rats.

2. Materials and Methods

2.1. Preparation of L. plantarum K68 from Fermented fu-tsai. In this study, *L. plantarum* K68 was prepared as described in the previous studies [15, 16]. Briefly, K68 was prepared from the locally available fermented food, *fu-tsai*, and preserved in our LAB bank. For the experiments, K68 was inoculated in MRS broth and cultured at 30°C for 21 hrs. The isolated bacteria was harvested using centrifugation for 10 min at 1500 g, then washed twice with sterile PBS, and then resuspended to a final concentration of 1×10^9 CFU/mL. The entire procedure was carried out under hygienic conditions at the Institute of Biochemistry and Molecular Biology, National Yang-Ming University. In our previous study, we used different doses of *K68* in cell culture and animal studies and demonstrated that oral administration of K68 is effective in reverting the DSS-induced ulcerative colitis in mice through the anti-inflammatory and immunomodulatory activities [16]. Based on these evidences, the dose 1×10^9 CFU/0.5 mL of K68 was selected in the present study to evaluate the antidiabetic, antioxidant, and anti-inflammatory properties.

2.2. Preparation of Fruit and Vegetable Ferment (FVF). Fruit and vegetable ferment (FVF) was obtained from the Fu Gui Bioscience Co. Ltd., Chayi City, Taiwan. FVF used in this study is commercially available food in Taiwan, which contained several fruits and vegetables, mainly available in Taiwan area. All fruits and vegetables were cut into small pieces and then preserved for one year at 25°C. The fruits in FVF include jujube, mini melon, apple, strawberry, grapefruit, orange, lemon, pears, pineapple, papaya, longan, litchi, mango, hami melon, watermelon, tomato, plum, mulberry, avocado, kiwi, coconut, guava, sugar cane, fig, banana, and passion fruit. The vegetables (herbs) are such as cucumber, pumpkin, *Benincasa hispida*, mushroom, eggplant, cabbage, spinach, seaweed, Chinese cabbage, water spinach, celery, *Crataegus pinnatifida, Artemisia capillaries, Houttuynia cordata, Polygonatum odoratum* (Mill.) Druce, *Anemarrhena asphodeloides*, *Eleutherococcus senticosus*, *Taxillus parasitica, Lophatherum gracile,* and *Ligustrum lucidum* Fructus. The detailed nutrition values and bioactive compounds existed in FVF were determined at Food Industry Research and Development Institute, Hsinchu, Taiwan, and the values were represented in Table 2. The final gummy-like substance was dissolved in water and dose equivalent to 180 mg/kg bodyweight was administered orally to rats. According to the calculations of US Food and Drug Administration (FDA), Center for Drug Evaluation and Research (CDER), July 2005 [18], the dose of FVF was calculated for average human bodyweight. The following equation was employed to convert the animal dose to human.

Human Equivalent Dose (mg/kg) = Animal dose (mg/kg) × Animal Km/Human Km (0.162).

3. Animal Care and Maintenance (*In Vivo*)

Fifty healthy male Sprague-Dawley rats weighing 155 ± 5 g were obtained from the National Laboratory of Animal Breeding and Research Center, Taipei, Taiwan. All the rats were housed in clean polypropylene cages and maintained in animal house with controlled temperature (23 ± 2°C) and alternating 12 h dark and 12 h light cycle. After one week acclimatization to laboratory conditions, all rats in experimental groups had free access to high fat-fructose diet, while control animals were fed a standard diet (AIN-93M, PMI Nutritional International LLC, Saint Louis, MO, USA) and water *ad libitum*. The entire study design and all experimental protocols used in this study followed the guidelines of Care and Use of Laboratory Animals, and the study was approved by the Animal Care and Ethics Committee of Shih-Chien University.

3.1. Preparation of High Fat-Fructose Diet (HFFD, High Calorie Diet). The HFFD used in this study was modified from

Table 1: Composition of standard and experimental diets.

AIN-93M	Standard diet (w/w)	High fat-fructose diet (w/w)
Corn starch	46%	46%
Dextrin	15.5%	15.5%
Casein vitamin free	14%	14%
Sucrose	10%	28%
Fructose	—	20%
Powdered cellulose	5%	5%
Soybean oil	4%	4% + 18% (lard)
AIN 93M mineral mix	3.5%	3.5%
AIN 93 vitamin mix	1%	1%
Choline bitartrate	0.25%	0.25%
L-Cystine	0.18%	0.18%
t-Butylhydroquinone	0.0008%	0.0008%
Energy (Kcal/30 g/day)	114	141

Table 2: Quantified bioactive compounds in fruit-vegetable ferment.

Name of the component	Content/100 g
Crude fat	0.48 g
Crude protein	2.12 g
Total carbohydrate	148.97 g
Dietary fiber	2.70 g
Sodium (Na)	87.44 mg
Potassium (K)	490.04 mg
Calcium (Ca)	48.43 mg
Magnesium (Mg)	39.4 mg
Ferrous (Fe)	1.35 mg
Phosphorous (P)	61.08 mg
Vitamine B-1	0.03 mg
Vitamin B-2	0.16 mg
Vitamin B-6	0.32 mg
Vitamin E	0.10 mg
Pantothenic acid	0.29 mg
Folic acid	34.14 mg
Choline	14.55 mg
Inositol	100.77 mg
Biotin	9.15 μg

All the values are expressed with respective units per 100 g of FVF.

the standard diet AIN-93M formula. Briefly, the standard diet contained 10% sucrose and 4% fat which were raised to 28% and 22%, respectively, by adding the additional 18% sucrose and 18% animal oil (lard) to the sucrose and fat portions. In addition to that, 20% fructose was additionally added to the standard diet in order to get high energy diet that is, 141 Kcal/30 g, where the standard diet comprised 114 Kcal/30 g only. The detailed components in high fat-fructose (modified) diet were shown in Table 1. Food and water intakes were recorded daily. Food conversion efficiency (FCE) ratio was calculated accordingly (FCE = food intake (g)/weight gain (kg) $\times 10^2$). Change in bodyweight on every other day was also recorded for all groups throughout the study.

3.2. Grouping and Treatment. All the rats were assigned into five groups, ten in each, and treated as follows.

Group I. Control (CON). Ten rats in this group consumed the standard laboratory diet and served as healthy control. For equivalent handling to other experimental groups, 0.9% saline was orally administered to all rats instead of supplements.

Group II. High Fat-Fructose Diet (HFFD). Rats in this group ($n = 10$) consumed high fat-fructose diet for 8-week period and received saline solution similar to control.

Group III. High Fat-Fructose Diet Plus K68 (HFFD + K68). In addition to the high fat-fructose diet, rats in this group ($n = 10$) were supplemented with K68 for a period of 8 weeks at the dose of 1×10^9 CFU /0.5 mL per day. K68 was provided orally via an orogastric tube.

Group IV. High Fat-Fructose Diet Plus FVF (HFFD + FVF). All rats in this group were fed on a high-fat-fructose diet and treated with FVF product for 8 weeks. Based on the *in vitro* studies, 180 mg/kg bodyweight was provided orally by an orogastric tube.

Group V. High Fat-Fructose Diet Plus MIX (HFFD + MIX). Rats in this group were fed a high-fat-fructose diet along with the mixture of both K68 plus FVF substances (MIX). The dose and rout of administration was similar to groups III and IV.

4. Biochemical Evaluations

4.1. Measurement of Fasting Blood Glucose, Serum Insulin, and HbA1c. On every week from week 0 to week 8, fasting blood samples were collected from the tail vein. Fasting blood glucose levels were determined immediately by using the glucose analyzer (LifeScan, Milpitas, CA, USA). Simultaneously, about 200 μL blood sample was transferred into labeled centrifuge tubes and then centrifuged at 3500 rpm for 10 min to obtain serum for insulin assay. According to the protocol provided by Mercodia Insulin ELISA kit (Uppsala, Sweden), serum insulin levels were assayed. The endpoint was read spectrophotometrically on an ELISA plate reader at 450 nm (BioTek, PowerWave XS2, Vermont, USA). Both blood glucose and insulin levels were measured on every week.

Furthermore, the homeostasis model assessment of basal insulin resistance (HOMA-IR) was calculated at week 6 and week 8 by using fasting serum insulin and blood glucose levels according the following equation:

$$\text{HOMA}-\text{IR index} = \frac{\text{insulin}\,(\mu\text{U/mL}) \times \text{glucose}\,(\text{mmol/L})}{22.5}. \quad (1)$$

After 8-week treatment, a fraction of whole blood sample was used to detect the glycated hemoglobin (HbA1c) as described by the Randox HbA1c Assay Kit (Randox, Antrim, United Kingdom).

4.2. Oral Glucose Tolerance Test (OGTT). Oral glucose tolerance test was performed after 6-week treatment in this study. All the rats were fasted for 12 h prior to test, and OGTT was conducted in the morning between 7.00 and 9.00 AM under fasting condition. One gram of glucose solution (50%, w/v) was orally administered to all rats before performing the test. Blood samples were collected from the tail vein by tail milking at 0 min (fasting sample) and 30, 60, 120, and 180 min time points after oral glucose administration (1 g/kg bodyweight). Glucose tolerance was determined by measuring the blood glucose and serum insulin concentrations for every 30 min interval. The glucose analyzer was used to determine the blood glucose levels (Lifescan, Milpitas, USA), and serum insulin levels were quantified on an ELISA analyzer as described in the previous section.

4.3. Assessment of Serum Lipid Profiles and Adipokines. Lipid profiles, including total cholesterol (TC), triglycerides (TG), low-density lipoprotein (LDL), and high-density lipoprotein (HDL) levels, were estimated in the serum of freshly collected blood sample. Prior (12 h) to the blood collection, the feed was removed from each cage, and samples were collected under fasting condition. TC, TG, and HDL levels were detected by commercial kits provided by the Randox (Randox Laboratories Limited, BT29 4QY, UK). LDL levels were calculated accordingly by using the Friedewald equation [19]. All the values were expressed as mg/dL.

Important adipokines, adiponectin, and leptin, which are associated with bodyweight changes, were determined in the serum. As per the Assaypro Rat Adiponectin ELISA kit's protocol, serum adiponectin levels were estimated (St. Charles, MO, USA). Circulating leptin levels were determined according to the protocol provided by RayBio rat leptin ELISA kit (Norcross, GA, USA). Absorbance of the sample was read immediately after adding the stop solution at 450 nm in a spectrophotometer (BioTek, PowerWave XS2, VT, USA). Adiponectin and leptin values were represented as ng/mL and pg/mL, respectively.

4.4. Determination of Circulating Pro-Inflammatory Cytokines. Circulating pro-inflammatory cytokines, including interleukin-1α (IL-1α), IL-6, and tumor necrosis factor-α (TNF-α), concentrations were evaluated by commercially available kits obtained from eBioscience (San Diego, CA, USA). The final absorbance for all cytokines (IL-1α, IL-6, and TNF-α) were monitored at 450 nm on an ELISA plate reader (BioTek, PowerWave XS2, Vermont, USA). The concentrations of all inflammatory markers were expressed as pg/mL of serum.

4.5. Assessment of Antioxidant Enzyme Activities. Three major antioxidant enzymes in serum including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) activities were assayed by using the Cayman assay kits (Anna Arbor, MI, USA). Serum SOD activity was determined on a ELISA plate reader. The final absorbance was read at 450 nm (Tecan Genios, A-5082, Austria), and activity was expressed as units/mL. CAT activity was measured by adding the H_2O_2 to the sample as per the protocol described in Cayman Catalase Assay Kit, and the activity was expressed nanomoles/mL/min. By using the NADPH, serum GPx activity was assayed. The reduction in the absorbance was read at 340 nm for every minute to get at least 5 time points by using a plate reader (Tecan Genios, A-5082, Austria). GPx activity was presented as units/mg protein. Protein concentrations in the samples were determined by Bio-Rad protein assay.

5. Assessment of FVF Antioxidant Capacities (*In Vitro*)

5.1. DPPH Scavenging Activity. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity was determined by the method of Liyana-Pathirana and Shahidi [20]. The absorbance of the mixture was measured spectrophotometrically at 517 nm. The ability of the FVF extract to scavenge DPPH radical was calculated by the equation

$$\text{DPPH radical scavenging activity} = \left[\text{Abs control} - \frac{\text{Abs sample}}{\text{Abs control}}\right] \times 100. \quad (2)$$

5.2. Superoxide Radical ($O_2^{\bullet -}$) Scavenging Activity. Superoxide anion scavenging activity was measured by method described by Robak and Gryglewski [21]. $O_2^{\bullet -}$ were generated in PMS-NADP system by oxidation of NADH and assayed by the reduction of nitro blue tetrazolium (NBT). Decreased absorbance of the reaction mixture indicates the increased superoxide anion scavenging activity. The percentage inhibition of superoxide anion generation was calculated using the following formula: superoxide anion scavenging activity (%) $= [(A_{cont} - A_{test})/A_{cont}] \times 100$, where A_{cont} was the absorbance of control reaction, and A_{test} was the absorbance of the extract or standards.

5.3. Angiotensin-Converting Enzyme (ACE) Activity. ACE activity was measured by using the substrate hippuryl-L-histidyl-L-leucine (HHL). ACE cleaves the substrate to expose to a free N-terminus, which could fluorogenically be labeled with o-phthaldialdehyde (OPA). Fluorescent was read at 355 nm excitation and 535 nm emission on a fluorescent spectrophotometer.

5.4. Total Phenol. The phenolic content present in FVF was determined spectrophotometrically by the Folin Ciocalteu modified method as described by Wolfe et al. [22]. Sample absorbance was measured spectrophotometrically at 765 nm (BioTek, PowerWave XS2, VT, USA). The measurements were conducted in triplicate. The obtained results were expressed as mg/g tannic acid equivalent from the calibration curve.

The vitamin E levels were evaluated by HPLC (Hitachi L2400), and selenium levels were estimated by atomic absorbance spectrophotometer (Perkin Elmer 5100). Both assays were performed at Food Industry Research and Development Institute, Hsinchu, Taiwan.

5.5. Statistical Analyses. The obtained data were analyzed by MS Office Excel and SPSS software. All the data were expressed by means ± SEM for ten replicates. The significance among the groups was achieved by two-way ANOVA along with Tukey's multiple-range post hoc test. The significance level was set as $P < 0.05$.

6. Results

6.1. Bioactive Ingredients in FVF. All active ingredients existing and responsible for the pharmacological effects of fermented fruit-vegetable (FVF) are estimated. In addition to proteins and carbohydrates, FVF is rich with several minerals, such as sodium (Na, 87.44 mg), potassium (K, 493 mg), calcium (Ca, 48.3 mg), magnesium (Mg, 39.4 mg), ferrous (Fe, 1.35 mg), and phosphorous (61 mg/100 g). Furthermore, FVF also possesses vitamin B-complex (B-1 (thiamin), B-2 (riboflavin), B-6, B-7 (biotin), and B-9 (folic acid)) and other antioxidants, including vitamin E and selenium (Se). The detailed nutrition values with bioactive compounds were listed in Table 2.

6.2. Confirmation of Insulin Resistance (IR). Circulating insulin and glucose concentrations were regularly monitored for all groups under fasting condition. We found higher serum insulin levels (1.5 ng/dL) after 4 weeks along with elevated blood glucose concentrations in HFFD-fed rats (Figures 2(a) and 2(b)). The progressively increase in serum insulin (hyperinsulinemia) and blood glucose (hyperglycemia) levels with HFFD considered as insulin resistance in rats.

6.3. Impact of Chronic High Fat-Fructose Diet on Metabolic Parameters. Collected data indicates that progressively increased whole bodyweight from week 0 to week 8 with high fat-fructose diet clearly reveals the negative impact of chronic HFFD intake in rats (Figure 1). Table 4 shows that weight gain in HFFD group was 341.6 ± 5.73 g, where it was only 288.7 ± 1.6 g in control group that was fed a standard diet. The weight management effects of K68, FVF, and MIX supplements have been noticed from week 3 with a significant ($P < 0.01$) lower bodyweights compared to the untreated HFFD group. This trend was continued until week 8 (Figure 1).

Individual organ weights, including liver, kidney, and epididymal fat weights, were recorded at the end of the study. We found that liver weight was partially, and epididymal fat weight was significantly ($P < 0.05$), increased with HFFD compared to standard diet. However, these changes were controlled in all supplemented groups. Particularly, epididymal fat weight was significantly reduced. Although the average daily food intake was not significantly altered among the groups, FCE ratio was marginally decreased in HFFD and FVF as well as MIX supplemented groups compared to control group (Table 4).

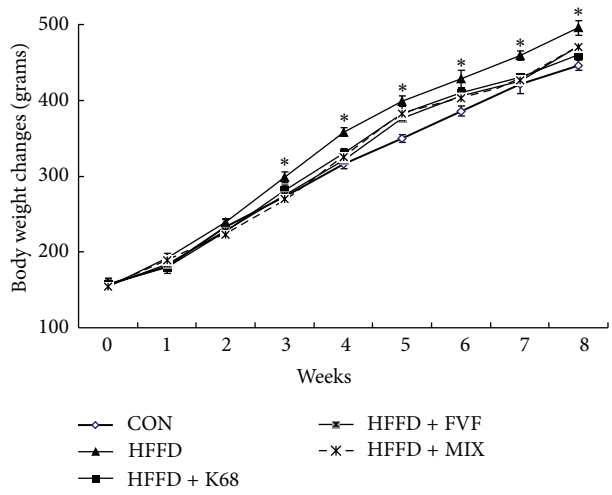
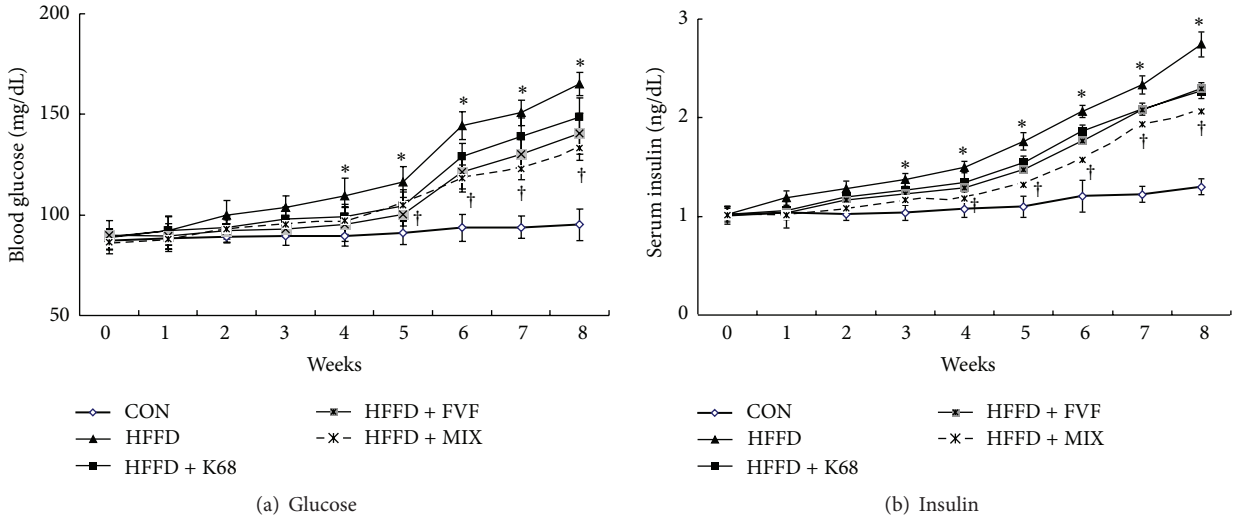


Figure 1: Changes in whole bodyweight over a period of 8 weeks with high fat-fructose diet, and K68, FVF, and MIX supplements in rats ($N = 10$). *: significant compared to CON group.

6.4. Antihyperglycemic and Antihyperinsulinemic Effects of K68 and FVF. In order to determine the antidiabetic properties K68 and FVF, fasting blood glucose, insulin, and HbA1c concentrations were measured. Data clearly showed that intake of diet with high fat-fructose affects the circulating blood glucose and insulin concentrations. Elevated blood glucose and insulin levels at week 6 were 54% and 61.4%, respectively, and that was progressed to 75.5% and 110.7% at week 8 compared to the control diet group. Calculated HOMA-IR values were also significantly ($P < 0.01$) increased in HFFD group (3.28 ± 0.08) than CON group (1.32 ± 0.04) at week 6. Interestingly, supplementation of K68 and FVF to HFFD groups showed a trend in decreasing the blood glucose, insulin, and HOMA-IR values at week 6. This decrease trend was statistically significant with combination of K68 plus FVF (MIX) supplement compared to HFFD (Table 5).

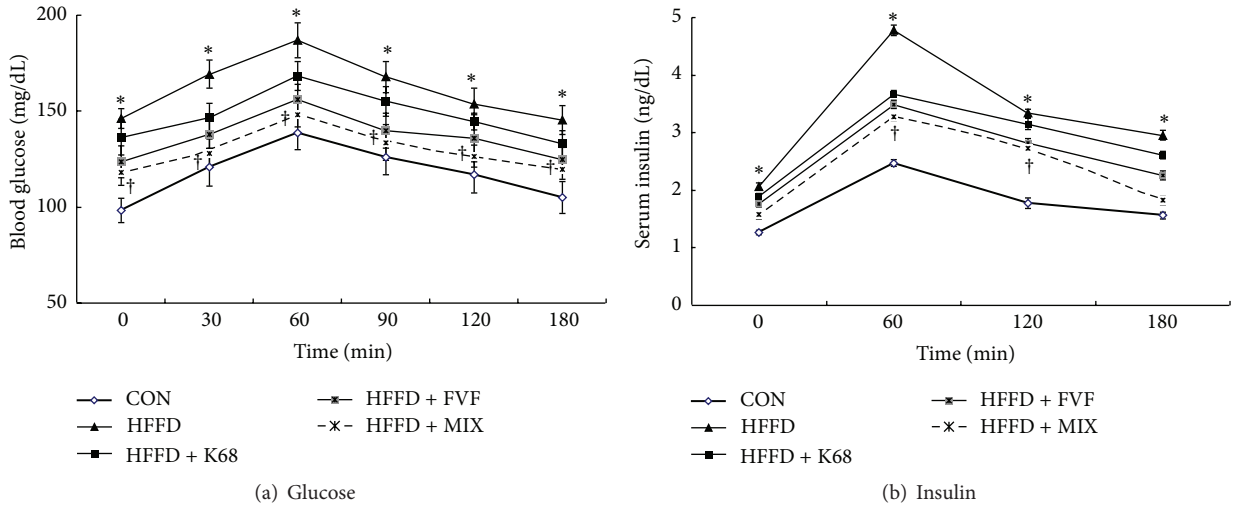
Statistical analyses showed that at week 8 blood glucose, insulin, HbA1c, and HOMA-IR values were recorded significantly ($P < 0.01$) lower with all supplements (K68, FVF, and MIX) (Table 5). Although supplements were unable to reduce those diabetes indicators completely, however, the treatment was effective to avoid the further progression of diabetic condition. This was further convinced by weekly monitored fasting blood glucose and insulin concentrations, which showed supplements setback of the diet-induced elevated glucose and insulin levels from week 4 till the end of the study (Figures 2(a) and 2(b)).

Furthermore, blood glucose and insulin levels under an oral glucose challenge (OGTT) were significantly ($P < 0.01$) higher at all time points in HFFD group and unable to reach baseline until 180 min. However, K68, FVF, and MIX



(a) Glucose (b) Insulin

Figure 2: Changes in blood glucose (a) and insulin (b) levels over a period of 8 weeks with K68, FVF, and MIX supplements along with HFFD in rats ($N = 10$). *: significant compared to CON; †: significant compared to HFFD group.



(a) Glucose (b) Insulin

Figure 3: Effect of K68, FVF, and MIX supplementation on oral glucose tolerance test (OGTT) in rats fed a HFFD for 6 weeks ($N = 10$). *: significant compared to CON; †: significant compared to HFFD group.

supplementation to HFFD rats maintained the lower glucose and insulin levels against an oral glucose challenge (Figures 3(a) and 2(b)).

6.5. Effect of K68 and FVF on Dyslipidemia and Adipokines with HFFD. Estimated lipoprotein levels, including total TC, TG, LDL, and HDL, were represented in Table 6. As we expected, Tukey's post-hoc analyses showed that circulating TC, TG, and LDL levels were significantly ($P < 0.01$) elevated with HFFD compared to standard diet. The drastic increase in TG levels was prominent (321%), followed by TC (41.5%) and LDL (40%) in HFFD group. In accordance with hypoglycemic property, both K68 and FVF were collectively attenuated hyperlipidemia. All treated groups exhibit decreased ($P < 0.01$) TG levels. Elevated TC and LDL were attenuated by synergetic effect K68 and FVF. Nonetheless, HDL levels were not significantly altered with any of supplement.

The analyzed two adipokines, adiponectin, and leptin levels responded inversely to each other, where adiponectin levels were significantly ($P < 0.01$) decreased and leptin levels were increased with HFFD. Interestingly, decreased adiponectin concentrations were partially restored and increased leptin levels were prominently ($P < 0.01$) inhibited with K68, FVF, and MIX supplements (Table 6).

6.6. Influence of K68 and FVF Supplementation on Pro-Inflammatory Cytokines. HFFD-induced harmful effects on circulating inflammatory cytokines were clearly evidenced by elevated ($P < 0.01$) serum pro-inflammatory cytokines, including IL-1β, IL-6, and TNF-α concentrations. Among

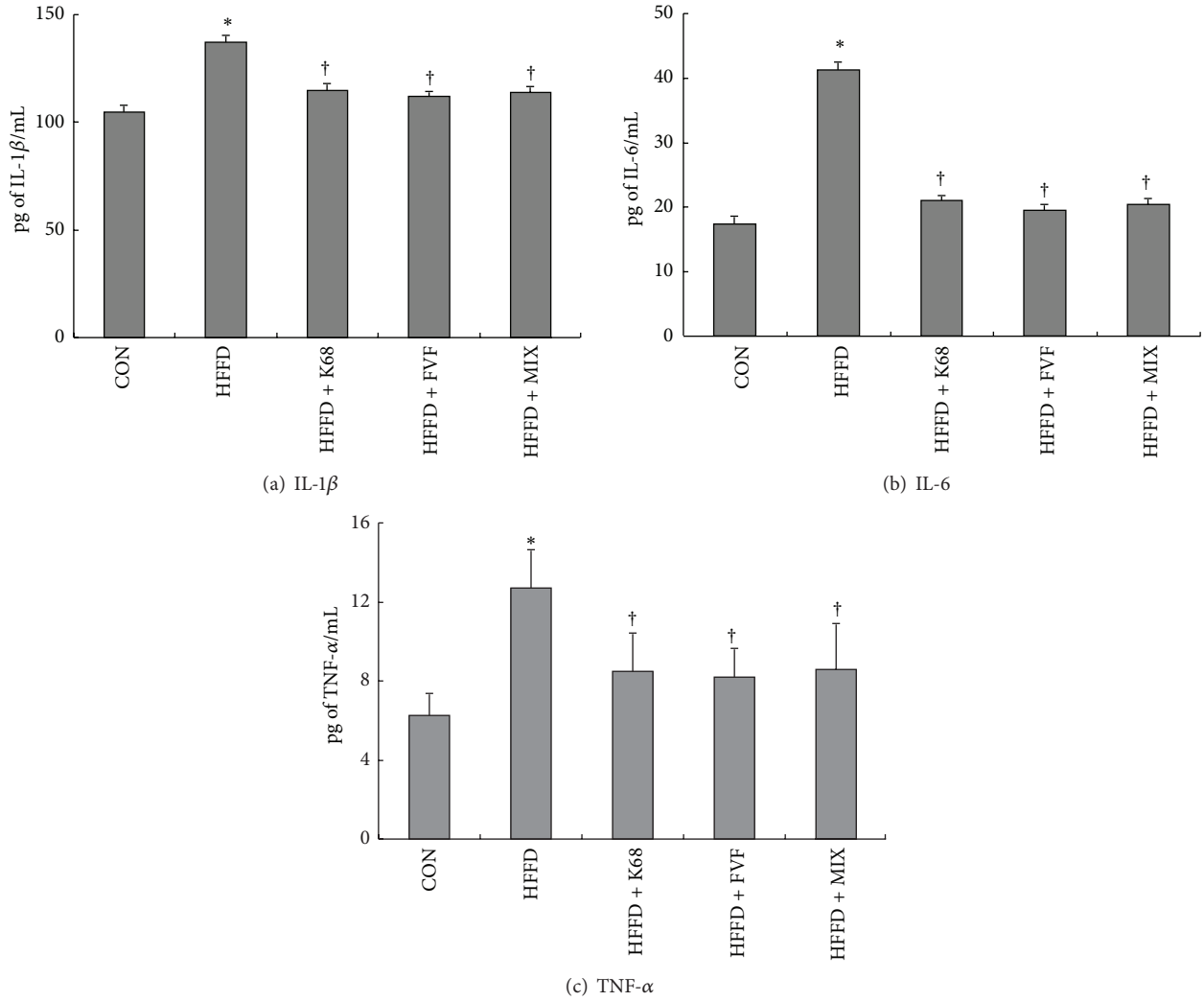


Figure 4: Influence of K68, FVF, and MIX supplementation for 8 weeks on inflammatory cytokines (IL-1β, IL-6, and TNF-α) in rats fed a HFFD ($N = 10$). *: significant compared to CON; †: significant compared to HFFD group.

three pro-inflammatory mediators, the levels of IL-6 and TNF-α were predominantly increased with HFFD as they reached 137% and 105% elevations, respectively, more than the control diet rats. Another key finding of this study is that elevated inflammatory cytokines were inhibited by K68, FVF, and MIX supplements. The mean values of IL-1β, IL-6, and TNF-α in supplemented groups were almost similar to the control group. In particular, decreased IL-6 concentration with K68, FVF and MIX treatments was ~50% compared to untreated HFFD group (Figures 4(a), 4(b), and 4(c)).

6.7. Beneficial Effect of K68 and FVF on Antioxidant Enzyme Activities. To demonstrate whether K68 and FVF and/or combination of both supplements could improve the antioxidant status against hyperglycemic, SOD, CAT, and GPx activities were measured in serum samples. Similar to many other studies, we found a significant ($P < 0.01$) drop in SOD, CAT, and GPx activities in HFFD group. However, decreased antioxidant enzyme activities were significantly ($P < 0.05$) restored with additional supplementation of K68, FVF, and MIX. This data indicates that chronic oral administration of K68, FVF, and MIX are able to cope the oxidative stress caused by HFFD (Figures 5(a), 5(b), and 5(c)).

The antioxidant property of FVF was confirmed by free radical scavenging activity, which was achieved by *in vitro* studies. *In vitro* data convincingly demonstrated that FVF is able to scavenge the free radicals, including DPPH and $O_2^{\bullet -}$ anion. DPPH scavenging activity was found to be 81% and $O_2^{\bullet -}$ scavenging activity was indicated as 0.81 IU/200 mg of FVF. Furthermore, FVF possesses ACE inhibitory activity (40%). The total phenol present in the FVF was recorded as 92 mg/200 mg (Table 3).

7. Discussion

In this study, we fed a high fat-fructose diet (HFFD) to rats to induce a rat model of insulin resistance. Chronic HFFD apparently showed the basic phenotypes of metabolic

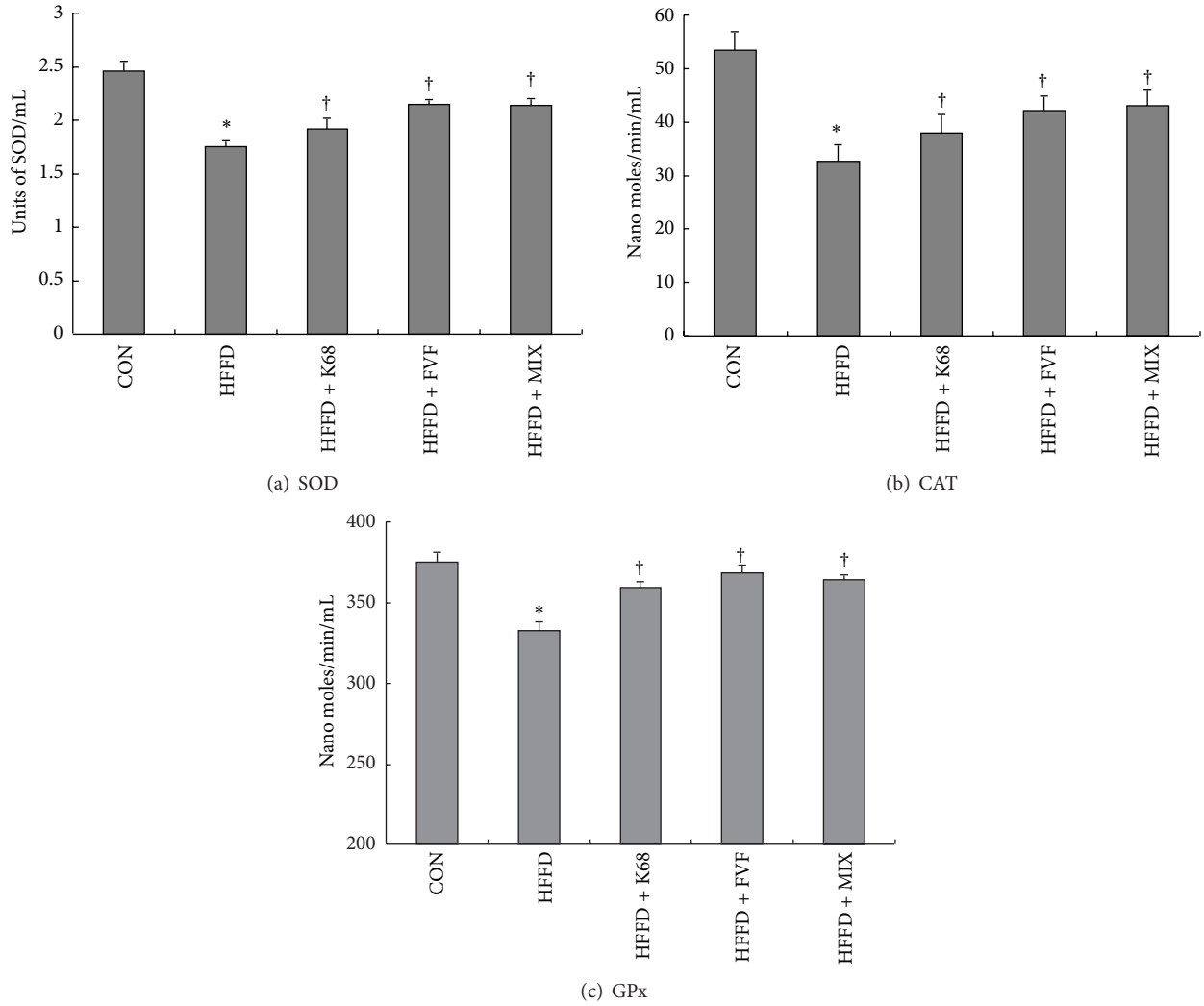


Figure 5: Beneficial effects of K68, FVF, and MIX supplementation for 8 weeks on antioxidant enzyme activities (SOD, CAT, and GPx) in rats fed a HFFD ($N = 10$). *: significant compared to CON; †: significant compared to HFFD group.

Table 3: Existence of antioxidant compounds and free radical scavenging properties (*in vitro*) of FVF.

Antioxidant compounds/capacity	Units
Vitamin E	0.10 mg/100 mg
Selenium	0.06 ppm/100 mg
DPPH scavenging ability, 100% (Vit C 1 mg/mL)	81%/200 mg
Superoxide radical scavenging activity	0.81 IU/200 mg
Total phenols	92 mg/200 mg
ACE inhibition	40%/200 mg

syndrome, including obesity, hyperglycemia, hyperinsulinemia, and hyperlipidemia. Evidences from our study clearly demonstrated that supplementation of *L. plantarum* K68 and FVF along with HFFD significantly attenuated the obesity, hyperglycemia, hyperinsulinemia, and hyperlipidemia. For the first time, here we provided experimental evidences for antidiabetic properties of K68 and FVF. Concomitantly, increased serum pro-inflammatory cytokines, IL-1β, IL-6, and TNF-α, were significantly alleviated by K68, FVF, and MIX supplements, which indicate the potent anti-inflammatory effect. Furthermore, ruined antioxidant system as shown by decreased SOD, CAT, and GPx activities was restored to normal levels, and this restoration was pronounced with MIX. *In vitro* studies further confirmed the antioxidant efficacies of FVF as it showed the DPPH and $O_2^{\bullet -}$ radical scavenging activities.

It is well known that obesity may influence overall blood parameters, which are responsible for metabolic syndrome. Chronic high fat and fructose consumption, in terms of increased energy intake, has been shown a greater increase in bodyweights [23, 24]. Enhanced fat deposition with high fat-fructose diet may result in weight gain over a period of time. This phenomenon was evidenced by increased epididymal fat weight in HFFD group, which can be considered as a risk factor for cardiovascular disease (CVD). A human study suggested that adolescent overweight will increase CVD among future young and middle-age adults [25]. In this context,

Table 4: Bodyweight gain over a period of 8 weeks and the final liver, kidney, and epididymal fat weights in HFFD, K68, FVF, and MIX supplemented groups. All the values are expressed in grams for 10 replicates ($N = 10$).

Parameters	CON	HFFD	HFFD + K68	HFFD + FVF	HFFD + MIX
Bodyweight gain (g) (final-initial weight)	288.7 ± 1.6	341.6 ± 5.73*	302.5 ± 2.4*†	316 ± 5.8*†	315.62 ± 4*†
Liver (g)	2.49 ± 0.10	2.79 ± 0.05*	2.33 ± 0.13†	2.49 ± 0.08†	2.61 ± 0.09
Kidney (g)	0.50 ± 0.01	0.54 ± 0.02	0.54 ± 0.02	0.53 ± 0.01	0.55 ± 0.01
Epididymal fat (g)	2.06 ± 0.08	2.48 ± 0.03*	2.35 ± 0.06	2.26 ± 0.06	2.13 ± 0.03†
Average food intake (g/day)	28.4 ± 0.6	29.5 ± 0.2	27.5 ± 0.6	27.3 ± 0.4	27.3 ± 0.6
FCE ratio	0.097 ± 0.002	0.087 ± 0.003*	0.09 ± 0.002	0.087 ± 0.002*	0.087 ± 0.002*

*Significant compared to CON group.
†Significant compared to HFFD group.
Food conversion efficiency (FCE) ratio = food intake (g)/weight gain (kg) × 10^2.

Table 5: Changes in blood glucose, insulin, HbA1c, and HOMA-IR values over a period of HFFD feeding and effect of K68, FVF, and MIX supplementation ($N = 10$).

Parameters	CON	HFFD	HFFD + K68	HFFD + FVF	HFFD + MIX
Week 6					
Glucose (mg/dL)	93.6 ± 3.0	144.4 ± 2.8*	138.4 ± 3.2*	133.8 ± 2.6*†	128.1 ± 2.4*†#
Insulin (ng/mL)	1.27 ± 0.03	2.05 ± 0.04*	1.89 ± 0.03*	1.77 ± 0.02*†	1.58 ± 0.03*†#
HOMA-IR	1.32 ± 0.04	3.28 ± 0.08*	2.92 ± 0.10*†	2.63 ± 0.05*†	2.26 ± 0.07*†#
Week 8					
Glucose (mg/dL)	95.1 ± 2.7	165 ± 2.0*	152.5 ± 3.5*†	140.4 ± 3.6*†	137.1 ± 2.3*†#
Insulin (ng/mL)	1.3 ± 0.03	2.74 ± 0.05*	2.3 ± 0.04*†	2.29 ± 0.04*†	2.13 ± 0.03*†
HbA1c (%)	4.8 ± 0.2	5.8 ± 0.2*	5.4 ± 0.2*	5.2 ± 0.2†	5.1 ± 0.2†
HOMA-IR	1.39 ± 0.05	5.08 ± 0.09*	3.94 ± 0.12*†	3.62 ± 0.15*†	3.27 ± 0.08*†#

*Significant compared to CON group.
†Significant compared to HFFD group.
#Significant compared to K68 and FVF groups.

controlled bodyweight by *L. plantarum* K68, FVF, and MIX supplements implies the reduced risk of CVD. Soypro, a fermented soymilk with lactic acid bacteria, isolated from Kimchi, was showed to inhibit the expression of transcription factors of adipocyte differentiation [23]. Yadav and colleagues [12] showed that dahi (curd), which contained lactobacillus bacteria prevent the bodyweight gain against high fructose diet. The decreased bodyweight with supplements might be associated with the hypolipidemic property and/or altered adiponectin and leptin concentrations in this study.

Intake of chronic high fat-fructose diet confirmed the IR in rats as we found greater serum insulin and blood glucose levels. Insulin plays a unique role in regulating blood glucose levels and fat metabolism. Recent studies showed that rodents fed a high fat and/or fructose diet for 8 weeks increased the levels of insulin, glucose, and HOMA-IR values that were preceded to IR [24, 26]. Higher insulin levels in fructose group may impair β-cells function, since the cells could not cope with the increased insulin demand due to insulin resistance [7]. Impaired insulin homeostasis or glucose tolerance with HFFD is an important predictor of type 2 diabetes, which was reflected by increased risk factors, including HbA1c and HOMA-IR. Decreased insulin sensitivity in type 2 diabetes either by β-cells dysfunction or by obesity may affect the circulating lipids [2]. Furthermore, hyperglycemia triggers the ROS production and inflammatory cytokines that are closely associated with CVD [27]. Supplementation of K68 and FVF along with HFFD reversed the hyperglycemia and hyperinsulinemia along with HbA1c and HOMA-IR mean values. These results confirmed that lactobacillus bacteria and FVF products synergistically play a key role in delaying the onset of type 2 diabetes. We assumed that supplements may improve the β-cell function. A fermented milk product known as dahi, contained *L. acidophilus* and *L. casei*, was shown to delay the progression of high fructose-induced hyperglycemia, hyperinsulinemia, dyslipidemia, and oxidative stress in rats [12]. Another study showed that oral administration of *L. casei* significantly decreased the blood glucose in KK-Ay mice [28]. Similarly, Tabuchi et al. [11] reported improved glucose tolerance in *L. rhamnosus* GG treated streptozotocin-induced diabetic rats. Antidiabetic properties of bacteria and fermented fruit-vegetable products further suggest the cardioprotective properties against metabolic syndrome.

On the other hand, it has been shown that adipose tissue participates in regulation of bodyweight, glucose, and lipid metabolism via number of secreted proteins, including adiponectin and leptin [29]. Adiponectin exerts a potent insulin-sensitizing effect, activates the glucose uptake, protects against insulin resistance, and acts as anti-inflammatory

Table 6: Effect of K68, FVF, and MIX supplementation on altered lipid profile (TC, TG, HDL, and LDL) and adipokines (adiponectin and leptin) in HFFD-induced IR rats ($N = 10$).

Parameters	CON	HFFD	HFFD + K68	HFFD + FVF	HFFD + MIX
TC (mg/dL)	68.9 ± 1.1	97.5 ± 2.2*	93.5 ± 2.6*	92.6 ± 1.7*	86.8 ± 1.9*†#
TG (mg/dL)	87.8 ± 1.9	203.6 ± 2*	181.5 ± 1.6*†	183.5 ± 1.5*†	181.5 ± 1.6*†
LDL (mg/dL)	15.3 ± 1.0	21.3 ± 2.5*	21.4 ± 2.8*	21 ± 1.7*	15.3 ± 1.7†#
HDL (mg/dL)	36.1 ± 0.5	35.5 ± 0.4	35.8 ± 0.3	34.9 ± 0.7	35.2 ± 0.4
Adiponectin (ng/mL)	44.5 ± 0.7	32.1 ± 0.7*	38.4 ± 0.9†	37.8 ± 1.1*†	39.2 ± 0.8†
Leptin (pg/mL)	39.8 ± 1	68.1 ± 1.1*	48 ± 1*†	43.8 ± 0.8*†	49.5 ± 1*†

*Significant compared to CON group.
†Significant compared to HFFD group.
#Significant compared to K68 and FVF groups.

protein [30, 31]. In this study, decreased adiponectin with HFFD confirmed the impaired insulin sensitivity. However, K68, FVF, and MIX treatments restored the adiponectin concentrations. This data indicates that augmented adiponectin levels may play a vital role in improving the insulin sensitivity and decrease obesity-mediated metabolic complications. Elevated leptin concentrations with high fat-fructose diet were attenuated by K68, FVF, and MIX treatments. In a human study, Naruszewicz et al. [13] reported significantly reduced blood leptin concentrations in smokers after *L. plantarum* 299v contained drink intake.

The important CVD risk factor in type 2 diabetes is dyslipidemia, which is characterized by elevated plasma triglycerides and LDL [32, 33]. Elevated serum TC, TG, and LDL levels with high fat-fructose diet in this study reflect the preoccurrence of CVD. Attenuated dyslipidemia through the synergetic effect of supplements suggests that functional food could be useful to prevent the CVD. Previous studies showed that supplementation of diet with functional food product containing fruit juice, fermented oat, and *L. plantarum* significantly lowers the LDL concentrations in patients with moderately higher cholesterol levels [34]. Another study reported that serum TC levels were significantly decreased in rats fed fermented milk with both *L. casei* and *Streptococcus thermophilus* TMC 1543, while TG levels were decreased only with fermented milk [35]. The hypolipidemic activity of functional foods may be due to the specific action on cholesterol metabolizing enzymes in liver, promotion of cholesterol excretion though feces, and inhibition of cholesterol absorption by binding of cholesterol to LAB cells [36–38].

HFFD-induced altered lipid profiles and hyperglycemia are closely associated with elevated pro-inflammatory cytokines, as we recorded increased concentrations of IL-1β, IL-6, and TNF-α. Increased pro-inflammatory mediators can tip the crucial balance between pro- and anti-inflammatory mediators, thus results in inflammation and influences the normal physiological functions. TNF-α, contributing to development of IR, has been shown to elevate in obese rodents [39]. Our findings are in agreement with previous results, which showed the increased pro-inflammatory cytokines with high fat and/or fructose diets in rodents [24, 40]. The major therapeutic applications of K68 and FVF supplements were evidenced by decreasing all pro-inflammatory cytokines. Recently, we reported that production of pro-inflammatory cytokines, IL-1β, IL-6, and TNF-α, was significantly decreased by oral administration of *L. plantarum* K68 in DSS-induced ulcerative colitis mice. Increased mRNA expression of TNF-α in DSS group was also found to lower in K68 treated groups [16]. Chon et al. [41] showed that ultrafiltrates of *L. plantarum* KFCC11689P metabolic products inhibit IL-6 and TNF-α production in lipopolysaccharide (LPS)-induced RAW 264.7 cells. Since elevated IL-1β, IL-6, and TNF-α were referred to as independent predictors of diabetes [42], decreased concentrations reveal the antidiabetic properties of functional food. The combined effect of K68 and FVF as anti-inflammatory substances may play a key role to attenuate the inflammation. However, the detailed mechanism behind this anti-inflammatory activity needs further investigations.

Hyperglycemia-induced negative impact on antioxidant status was revealed by decreased antioxidant enzyme activities. Emerging evidences show that chronic fat or fructose intake triggers the reactive oxygen species (ROS) production, including superoxide radicals ($O_2^{\bullet-}$) that mediate insulin resistance [5, 43]. Typically, $O_2^{\bullet-}$ radicals scavenge into hydrogen peroxide (H_2O_2) by SOD, and then H_2O_2 rapidly converted into water and oxygen by CAT and GPx enzymes in a site-specific manner. Decrease in these enzyme activities results in accumulation of ROS and therefore causes oxidative stress. Rise in $O_2^{\bullet-}$ production and/or glycation of active sites of SOD under hyperglycemic condition may be the possible reason for SOD reduction [6, 44]. Similar to our studies, Francini et al. [7] reported decreased CAT activity with 10% fructose diet for 3 weeks, and Kannappan et al. [6] found reduced GPx activity in rats fed with 60% fructose for 60 days. K68 and FVF supplements were found to restore the SOD, CAT, and GPx activities. Since accumulation of $O_2^{\bullet-}$ plays a key role in the progression oxidative stress, normalizing the $O_2^{\bullet-}$ production may prevent the hyperglycemic mediated oxidative stress [45]. Improved antioxidant enzyme activities with supplements may effectively eradicate the excessive ROS and thereby prevent the oxidative stress. Studies have indicated that *L. plantarum* encodes genes for various oxidative stress-related protein such as catalase, thiol reductase, and GPx, while it is not encoded with SOD genes [46, 47]. On the

other hand, antioxidants, including vitamin E and selenium that are present in FVF, may be responsible for its antioxidant activity.

8. Conclusion

For the first time, our study demonstrated that *L. plantarum* K68 and fermented fruit-vegetables supplements contribute to decrease the hyperglycemia, hyperinsulinemia, and hyperlipidemia in HFFD-fed rats. The therapeutic effects may attribute to increased antioxidant status and decreased pro-inflammatory cytokines. These findings conclude that specific LAB, *L. plantarum* K68, isolated from Taiwanese traditional food *fu-tsai*, and FVF products synergistically exert promising antidiabetic, anti-inflammatory, and antioxidant properties. Our results suggest that inclusion of K68 and FVF to the Western style diet may decrease the risk of diabetes. Due to their effective medicinal properties, both K68 and FVF can be considered as alternative medicine to prevent insulin resistance-associated metabolic disorders.

Conflict of Interests

All authors declare no conflict of interests.

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Research Article

A Small Molecule Swertisin from *Enicostemma littorale* Differentiates NIH3T3 Cells into Islet-Like Clusters and Restores Normoglycemia upon Transplantation in Diabetic Balb/c Mice

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Aim. Stem cell therapy is one of the upcoming therapies for the treatment of diabetes. Discovery of potent differentiating agents is a prerequisite for increasing islet mass. The present study is an attempt to screen the potential of novel small biomolecules for their differentiating property into pancreatic islet cells using NIH3T3, as representative of extra pancreatic stem cells/progenitors. *Methods*. To identify new agents that stimulate islet differentiation, we screened various compounds isolated from *Enicostemma littorale* using NIH3T3 cells and morphological changes were observed. Characterization was performed by semiquantitative RT-PCR, Q-PCR, immunocytochemistry, immunoblotting, and insulin secretion assay for functional response in newly generated islet-like cell clusters (ILCC). Reversal of hyperglycemia was monitored after transplanting ILCC in STZ-induced diabetic mice. *Results*. Among various compounds tested, swertisin, an isolated flavonoid, was the most effective in differentiating NIH3T3 into endocrine cells. Swertisin efficiently changed the morphology of NIH3T3 cells from fibroblastic to round aggregate cell cluster in huge numbers. Dithizone (DTZ) stain primarily confirmed differentiation and gene expression studies signified rapid onset of differentiation signaling cascade in swertisin-induced ILCC. Molecular imaging and immunoblotting further confirmed presence of islet specific proteins. Moreover, glucose induced insulin release (*in vitro*) and decreased fasting blood glucose (FBG) (*in vivo*) in transplanted diabetic BALB/c mice depicted functional maturity of ILCC. Insulin and glucagon expression in excised islet grafts illustrated survival and functional integrity. *Conclusions*. Rapid induction for islet differentiation by swertisin, a novel herbal biomolecule, provides low cost and readily available differentiating agent that can be translated as a therapeutic tool for effective treatment in diabetes.

1. Introduction

Diabetes is a devastating disease, affecting millions of people worldwide. Hyperglycemia is a principal signature of both type 1 diabetes (T1D) and type 2 diabetes (T2D). Reversal of hyperglycemia by exogenous insulin may delay or attenuate but never eliminate the risk for developing secondary complications [1]. Islet transplantation is a modern approach that has become more prevalent in clinics nowadays. It offers internal glucose homeostasis with low surgery risk and reduces complications in diabetic patients. However, islets derived from multiple donors require immunosuppressors. Also inadequate islet supply from cadaveric pancreas has limited the widespread utilization of this approach [2].

Cell-based therapy, principally new islets derived from stem cell differentiation, is a new area of research in diabetes. Recent studies have shown that embryonic stem cells, induced pluripotent stem cells, adult bone marrow mesenchymal stem cells, and many other tissue-specific progenitors have the ability to convert into cell of multiple lineages like blood, liver, lung, skin, cardiac, muscles, and neurons including insulin producing β cells upon appropriate

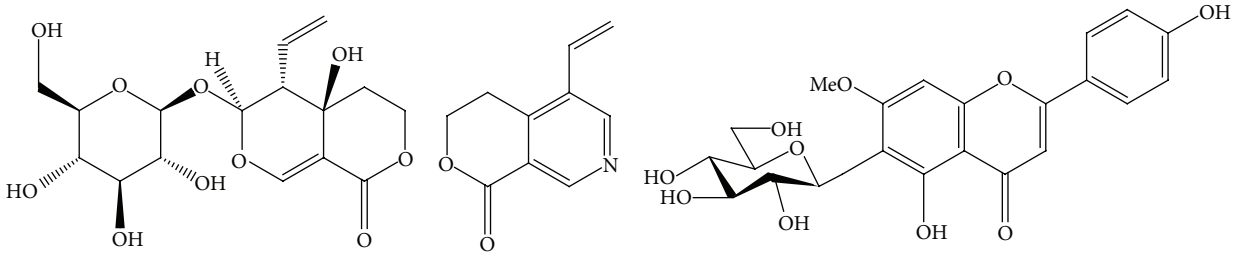

Figure 1: Structure of various *E. littorale* compounds: (a) represents structure of swertiamarin, (b) represents structure of Gentianine, and (c) represents structure of Swertisin molecule.

induction [3, 4]. Although few reports have shown generation of islet mass from various pluripotent and multipotent stem cells, progress has been hampered in increasing differentiated islet yield due to lack of potent and economical islet differentiating agents. Success in exploring various adult tissues to isolate stem cell population has been recorded for autologous transplants. However, not much work has been done for identification of potent differentiating agents that not only accelerate the rate of differentiation but also produce functional cell types in large numbers. If this can be achieved, the utilization of huge islet mass seems to be feasible to withstand the shortage of autologous islet transplant in near future and prevent diabetes and its complications.

There are many growth factors and differentiating agents known to promote differentiation or regeneration of pancreatic β cells [5]. These include nicotinamide, glucagon-like peptide, gastrin, activin A, betacellulin, Reg protein, INGAP, and hepatocyte growth factor (HGF). Practically none of them were translated as therapeutic molecule for islet generation and transplant in clinics, as all of them are associated with high cost and low yield of islet clusters.

Plants are exemplary sources of medicinal values, and an important thing is to properly identify and screen for their miraculous properties. From a practical point of view, low molecular weight compounds are favorable for such cellular interaction studies, because such agents are not immunogenic and may be effective even when administered orally. Numerous plant products have demonstrated antidiabetic activity [6–9]. Reports have appeared in recent times regarding inductive agents that have been shown to stimulate regeneration and replenishment of islet cells from herbal sources. Kojima and Umezawa demonstrated islet differentiation activity of conophylline molecule isolated from *Ervatamia microphylla* with AR42J cells, where they reported conophylline to have activin-A like activity and showed acinar to islet cell transdifferentiation [10]. On similar lines, our group has also reported antidiabetic activity of *Enicostemma littorale* Blume.

E. littorale is a perennial herb, belonging to Gentianaceae family and distributed throughout India. Major chemical constitutes of the plant are swertiamarin and gentianine (Figure 1) [11, 12]. Apigenin, genkwanin, isovitexin, swertisin, saponarin, and gentiocrucine [13] are also reported to be present in minor amounts. Aqueous extract of *E. littorale* demonstrated hypoglycemic potential in alloxan-induced diabetic rats [8, 9, 14]. Hypoglycemic antioxidant with hypolipidemic potential was also reported in newly diagnosed NIDDM patients [7]. The antidiabetic effect of this plant has been reported by other workers too [15, 16]. Apart from these properties, various fractions of *E. littorale* also demonstrated cytoprotective effect in isolated islets and pancreatic regeneration in both T1D and T2D animal models. Based on these observations a preliminary study was conducted by the author's group for islet differentiation property with an active herbal compound isolated from methanolic extract of *E. littorale* [17]. Further more to identify the potent islet differentiating agent, we screened various biomolecules isolated from *E. littorale* and monitored for effective stem cell differentiation with NIH3T3 cells as representative of extra pancreatic stem/progenitor cells. A flavonoid extracted from ethyl acetate fraction was found to be the most potent in increasing islet mass out of various molecules tested. This molecule was characterized by UV, TLC, HPTLC, and mass spectra analysis which was found to be identical to swertisin, reported earlier as one of the constituents of *E. littorale* [13]. Swertisin was further assessed *in vitro* at molecular, immunological, and functional levels for confirming proper differentiation into ILCC. Glucose lowering-effect-of transplanted ILCC was monitored in streptozotocin-(STZ) induced BALB/c mice suggesting functional maturity and integrity of newly generated ILCC.

2. Methods

2.1. Plant Material. Whole dried plant was procured from Bhavnagar district in Gujarat state, India, in the month of August after authentication from taxonomist with voucher specimen number Oza 51, 51 (a) present in the Herbarium, Department of Botany, The M.S University of Baroda, Vadodara, Gujarat, India.

2.2. Isolation and Characterization of Compounds from E. littorale. Methanol extract of *E. littorale* was prepared as discussed in earlier reports [8]. In brief, the extract (50 mL) was dissolved in distilled water (100 mL) and transferred

to a separating funnel. Successive fractionation was carried out with solvents like butanol, chloroform, and ethyl acetate (250 mL each) which lay in descending manner of polarity index. Ethyl acetate fraction yielded a pale white precipitates which were further purified by washing them with acetone whereas other compounds obtained from different fractions were confirmed as swertiamarin and gentianine. This pale yellow compound was further confirmed in terms of purity as described in earlier reported methods using UV spectrum, thin layer chromatography (TLC), high performance thin layer chromatography (HPTLC) [18], and mass fragmentation pattern using ESI-MS/MS under positive and negative ionization mode.

2.3. Characterization by TLC, HPTLC, Ultraviolet Spectrometry, and Mass Fragmentation Pattern. The pale yellow compound was screened with TLC, using1 mg and dissolved in 25 mL of methanol and applied on a precoated plate with Silica gel GF254 (E. Merck). The test solution was loaded and the solvent system of ethyl acetate : methanol (8 : 2) was used for development. Plate was evaporated and visualized at UV 254 nm. For HPTLC spectrum, twenty-five mg of compound was dissolved in small aliquot of methanol and developed in the solvent system mentioned above, scanned densitometrically at 243 nm and recorded using CAMAG TLC SCANNER 3 system. One milligram of compound (swertisin) was dissolved in 1 mL of methanol and scanned for the wavelength ranging between 200 and 800 nm and absorption maxima were compared with previous reports. For mass spectral identification by ESI-MS/MS, 1 mg of this compound was dissolved in methanol and was introduced for direct fragmentation in ionization chamber in positive and negative ionization mode. The base peak was recorded and compared with the molecular weight as reported earlier [18].

2.4. Cell Culture Maintenance and Preparation of Differentiation Medium. NIH3T3 (generous gift from Zydus Research Centre, Ahmedabad, Gujarat, India) was maintained and cultured in high glucose DMEM complete medium with PenStrep. The differentiation medium composed of DMEM Ham's F-12 (1 : 1, 8 mM glucose) without serum, cocktail supplements of insulin 5 mg/L, transferrin 5 mg/L, selenite 5 ug/L (Sigma-Aldrich, USA) and BSA 1.5 g/L with antibiotics penicillin 25 ug/mL and streptomycin 25 ug/mL.

2.5. FACS Analysis. Cells were trypsinized and centrifuged, and one million cells were resuspended in 100 μL of wash buffer (PBS containing 10% serum), washed twice with phosphate buffered saline (PBS) containing 1% bovine serum albumin, and then incubated with primary antibody (for details and dilution see Table 2) at 4°C for 1 h. Cells were again labeled with 100 μL of secondary antibody for counter staining (for details and dilution see Table 2) and incubated for an additional 40 min at 4°C [19]. Data was recorded in three observations.

2.6. Comparative Evaluation of E. littorale Extracts and Compounds for Generation of ILCC from NIH3T3 Cells. ILCC were generated from NIH3T3 cells in a four-step protocol as described in our previous report (Figure 5) [17]. Briefly, 0.1 million cells per ml were allowed to grow till 90% confluency and prior to differentiation, trypsinized with 0.05% trypsin for 60–120 sec to slightly loosen up for migration and efficient cluster formation. Finally, cells were seeded with differentiating medium (described above) and supplemented with various extracts/purified compounds with dose of 15 μg/mL (*in vitro)*, while control cells received differentiation medium alone. Medium was replenished every alternate day till 8 days. On the 8th day, ILCC were collected and observed for morphological changes. Differentiated ILCC were washed three times with ice cold PBS and stained with 10 uL of DTZ stain. Images were recorded with inverted phase contrast microscope, photographed, and evaluated for total islet yield number and size and other morphological features.

2.7. Immunocytochemistry and Confocal Microscopy. Comparative analysis demonstrated swertisin to possess the highest islet neogenic potential depicted by the highest ILCC yield. Hence, swertisin-induced ILCC were further investigated for differentiation markers and islet hormones by immunocytochemistry. For analysis, ILCC were collected and spun at 1200 rpm (Eppendorf 5415R) and kept for adherence on glass coverslip with high glucose DMEM complete medium for 3-4 hours. Upon attachment, clusters were immediately fixed with 3.7% paraformaldehyde solution for 20 min and then washed with 0.1 M PBS. Blocking solution was then added for 30 min to prevent nonspecific binding. Thereafter ILCC were stained with primary antibodies for insulin, C-peptide, glucagon, and PDX-1 at 4°C for 18 h, washed with 0.1 M PBS, and further incubated with secondary antibodies (for details, see Table 1) at 25°C for 1 h. ILCC were photographed with confocal microscope LSM710 (Zeiss, Germany).

2.8. RNA Extraction, Semiquantitative Reverse Transcriptase PCR (RT-PCR), and Quantitative Real-Time PCR (Q-PCR). Total RNA was isolated from differentiated ILCC using TRIzol Reagent (Sigma-Aldrich, USA). Purity of RNA was confirmed by $A_{260/280}$ ratio and checked for integrity. Five μg of total RNA was reverse transcribed into first strand cDNA and subjected to PCR amplification for various genes as mentioned in Table 2. Gradient PCR was performed with a range of annealing temperature from 51 to 60°C. cDNA was amplified using Fermentas 2x master mix (1.5 unit Taq Polymerase, 2 mM dNTP, 10x Tris, glycerol reaction buffer, 25 mM $MgCl_2$) with 20 pM forward and reverse primers (see Table 2). Gapdh served as internal control and negative RT was performed with untranscribed RNA. PCR products were separated on a 10% polyacrylamide gels (Sigma-Aldrich, USA) and visualized and images were captured with Alpha Imager software (UVP Image Analysis Software Systems, USA) for densitometric analysis.

For real-time quantitativePCR of islet specific mRNA transcripts, again 1–1.5 μg reverse-transcribed cDNA template was used from each group harvested in a time-dependent differentiation manner (2–8 days) using Fermentas first strand cDNA Reverse-Transcription kit. SYBR Green

Table 1: List of antibodies used for flow cytometry, immunohistochemistry, and western blotting.

Name of antibody	Company	Target against species	Mono/polyclonal	Molecular weight (kDa)	Source	Dilution for WB	Dilution for ICC	Dilution for flow cytometry
Beta actin	Thermo scientific	Hu, Rt, Mu	Mono	42	Mouse	1 : 5000	NA	NA
p-Smad-2	Cell signalling	Rt, Mu	Poly	60	Rabbit	1 : 1000	NA	NA
Smad-7	R & D systems	Rt, Mu	Mono	50	Mouse	1 : 1000	1 : 100	NA
Nestin	Sigma-Aldrich	Hu, Rt, Mu	Mono	177	Rabbit	1 : 1000	1 : 250	NA
Pdx1	Cell signaling	Hu, Rt, Mu	Mono	42	Rabbit	1 : 1000	1 : 250	NA
Vimentin	Sigma	Hu, Rt, Mu	Mono	53	Mouse	1 : 1000	1/250	NA
CD90-FITC	BD	Hu, Mu	Mono	—	Mouse	—	1 : 100	1 : 10
CD34-FITC	BD	Hu, Mu	Mono	—	Mouse	—	1 : 100	1 : 10
CD45-APC	BD	Hu, Mu	Mono	—	Mouse	—	1 : 100	1 : 10
CD49b-FITC	BD	Hu, Rt, Mu	Mono	—	Mouse	—	1 : 100	1 : 10
CD117-PE	BD	Hu, Rt, Mu	Mono	—	Mouse	—	1 : 100	1 : 10
CD44-PE	BD	Hu, Rt, Mu	Mono	—	Mouse	—	1 : 100	1 : 10
C-peptide	Cell signalling	Hu, Rt, Mu	Poly	4	Rabbit	—	1 : 100	NA
Insulin-Alexa 548	Santa Cruz	Hu, Rt, Mu	Mono	6	Guinea Pig	—	1 : 200	NA
Glucagon	Sigma	Hu, Rt, Mu	Mono	3.5	Mouse	—	1 : 200	NA
Somatostatin	Sigma	Hu, Mu	Poly	12	Rabbit	—	1 : 200	NA

Table 2: List of primer sequences of RT-PCR with annealing temperature and amplicon size.

Name of gene	Gene accession number	Primer sequence forward	Primer sequence reverse	PCR conditions (Tm)	Amplicon size (BP)
α-SMA	NT_039687.7	AGTCGCCATCAGGAACCTCGAG	ATCTTTTCCATGTCGTCCCAGTTG	60	296
Vimentin	NT_039202.7	AGCGGGACAACCTGGCCG	GGGAAGAAAAGGTTGGCAGAGGC	58	744
Nestin	NT_039240.7	GCGGGGCGGTGCGTGACTAC	AGGCAAGGGGGAAGAGAAGGATGT	58	326
PDX-1	NT_039324.7	CTC GCT GGG AAC GCT GGA ACA	GCT TTG GTG GAT TTC ATC CAC GG	55	229
Ngn-3	NT_039500.7	ACTAGGATGGCGCCTCATCCCTTG	GGTCTCTTCACAAGAAGTCTGAGA	57	658
Pax-4	NM_011038.2	TGGCTTCCTGTCCTTCTGTGAGG	TCCAAGACTCCTGTGCGGTAGTAG	62	214
Nkx-6.1	NM_144955.2	ATGGGAAGAGAAAACACACCAGAC	TAATCGTCGTCGTCCTCCTCGTTC	60	280
Insulin	NM_008386.3	GCCCAGGCTTTTGTCAAACA	CTCCCCACACACCAGGTAGAG	55	90
Glucagon	NM_008100.3	ATGAAGACCATTTACTTTGTGGCT	GGTGTTCATCAACCACTGCAC	58	243
Reg-1	NM_009042.1	AAGCTGAAGAAGACCTGCCA	TGTTAGGAGACCCAGTTGCC	60	123
GAPDH	NT_166349.1	CAAGGTCATCCATGACAACTTTG	GTCCACCACCCTGTTGCTGTAG	58	496
List of primer sequences for Q-PCR with annealing temperature and amplicon size					
PDX-1	NT_039324.7	ACTTGAGCGTTCCAATACGG	GCTTTGGTGGATTTCATCCACGG	55	813
INS-2	NM008387	TGTCTCTGGGGAAATGGGATTC	TGCTGCTTGACAAAAGCCTG	59	243

reactions using SYBR Green PCR Master Mix (Fermentas Inc., USA) were assembled along with 250 nM primers according to the manufacturer's instructions and performed with an ABI 7500 real-time PCR machine using standard comparative Ct value detection programme (Applied Biosystems). Relative expression of islet genes encoded mRNA was then determined after normalization to beta actin as endogenous control. Relative quantification value (RQ value) and $\Delta\Delta$Ct values were then calculated using 7500 software v2.0.6 (Applied Biosystems 7500 machine, ABI). All primer sequences used were intron flanking primers, negating the possibility of false amplification from genomic DNA contamination. The details of primer sequences are shown in Table 2.

2.9. Immunoblotting for Islet Differentiation Proteins. ILCC were collected by centrifugation and suspended in lysis buffer (1 mM EDTA, 50 mM Tris-HCl pH 7.5, 70 mM NaCl, 1% Triton, 50 mM NaF) containing 1x proteinase inhibitors (Roche), incubated on ice for 30 min. After centrifugation at 16000 g for 15 min at 4°C, the supernatant was collected

and kept at −80°C for future use. Total protein content was quantified using Bradford assay (Biolrad Bradford Solution, USA). Ten μg of protein was loaded on a 12% polyacrylamide gel and then electrophoretically transferred onto a Hybond-Nitrocellulose membrane (GE Healthcare). The membrane was then incubated for 1 h at room temperature in blocking buffer (TBS-T containing 8% skimmed milk) and further incubated overnight with the primary antibody at 4°C (details in Table 1). Membrane was then washed four times with TBS-T and incubated with HPR-conjugated secondary antibody for 1 h (details in Table 1). Finally, membrane was developed and visualized with Enhanced Chemiluminscence western blotting detection system (Millipore Inc. USA).

2.10. Glucose Induced Insulin/C-Peptide Release Assay. Differentiated ILCC were initially incubated for 30 min in glucose-free Krebs-Ringer bicarbonate buffer (KRB) containing 0.5% bovine serum albumin and then induced with 5.5, 20 mM glucose, and 10 mM L-arginine for additional 3 hours on constant shaking condition at 5% CO_2 and 95% O_2. After brief centrifugation, the supernatant was collected and frozen at −70°C until further analysis. Insulin/C-peptide assay was performed using mouse-insulin ELISA (Mercodia Inc., USA) and mouse C-peptide ELISA (ALPCO Immunoassays, USA).

2.11. Animal Selection and Induction of Diabetes. Male BALB/c mice of 3-4 weeks old weighing 15–20 grams were used for transplantation experiments. All animal experiments were performed in accordance with our institutional Ethical Committee for Animal Experiment and CPCSEA guidelines and regulations. Animals were kept in animal house with 12 hours light and 12 hours dark cycle and allowed to have water and pellet diet *ad libitum*. Diabetes was induced with streptozotocin injection (STZ; 65 mg/kg body weight) intraperitoneally for 5 days with overnight fasting. Diabetic status of animals was confirmed by monitoring FBG using Accu check glucometer (Accu check, Roche, USA) till 30 days for stabilization of hyperglycemia.

2.12. Transplantation of ILCC and Reversal of Hyperglycemia. Transplantation was performed under anesthesia with ketamine and xylazine (as per CPCSEA guidelines). Briefly, the abdomen was incised to expose kidney capsule ($n = 5$) with small incision for implanting ILCC. The abdominal cavity and overlying skin were sutured back and animals were allowed to recover for 2 weeks. Other groups of mice ($n = 5$) were taken as diabetic control. FBG was monitored weekly till four weeks.

2.13. Histological and Immunohistochemistry Assessment of ILCC after Graft Excision. Transplanted ILCC from kidney graft were excised 4 weeks after surgery and were histologically examined. Eight micron thick sections were used, deparaffinized, and rehydrated using varying alcohol grades of 100% for 5 min and 90%, 70%, 50%, and distilled water for 1 min each. Each section was then incubated in blocking solution at room temperature for 1-2 hours. Primary antibodies for insulin, c-peptide, and glucagon (for details, see Table 1) were incubated overnight at 4°C. After washing three times with 0.1 M PBS for 10 min, FITC conjugated secondary antibodies were incubated for 1 h in 0.1 M PBS at room temperature. Nuclei were visualized with 4-9-6-9 diamidino-2-phenylindole (DAPI) and sections were mounted with VECTASHIELD antifade mountant. Images were recorded using confocal microscope (LSM710, Carl Zeiss, Germany).

2.14. Statistical Analysis. The data are given as mean ± SEM. The significance of difference was evaluated by the paired Student's t-test. When more than one group was compared with one control, significance was evaluated according to one-way analysis of variance (ANOVA).

3. Results

3.1. Isolation of Compound from Ethyl Acetate Fraction Characterization: Identified as Swertisin. The compound isolated from ethyl acetate fraction of *E. littorale* was confirmed as a single peak at 344 nm in UV-Vis spectrum analysis (Figure 2) and a single spot at Rf 0.72 of HPTLC plate again at λ_{max} of 344 nm when scanned on CAMAG TLC Scanner-3 system (Figures 2(a) and 2(b)). These results indicated the presence of swertisin compound as reported earlier by Patel and Mishra and Colombo et al. [18, 20]. Mass fragmentation pattern of this compound was also compared with earlier references and found to be identical to swertisin [20]. Our results showed a base peak of m/z 447 [M + H] which corresponded to molecular weight of swertisin in positive ionization mode. Further more, a peak of M + H − 120 was also obtained which indicated a loss of 120 u a characteristic peak of C-glycosyl flavonoids as mentioned byColombo et al. (Figure 3(b)) [20]. The compound was found to be soluble in solvents like DMSO and dioxane, while sparingly soluble in methanol and insoluble in water. All the above results shown by us are in accordance of swertisin reported data with structure shown in Figure 3(c).

3.2. Morphologic and Phenotypic Characteristics of Undifferentiated NIH3T3 Cells. At full confluence, NIH3T3 cells became homogenous and attained spindle shape, fibroblast-like morphology, and formed a monolayer (Figure 6(a)). Flow-cytometric data showed positive peaks for mesenchymal stem cell markers CD44, c-kit (CD117), CD49b, CD90, Sca-1, Vimentin, SMA, and a hematopoietic stem cell marker CD34 but negative for CD45 (Figure 4). We observed 99.8% cells positive for CD44, 99.5% cells positive for CD90, 80.56% cells positive for Sca-1, 50.1% cells positive for CD34, and ~5% cells positive for CD45. These cells also showed 39% staining for positive for both CD44 and 49b and ~35% cells showed dual staining for CD90 and 117 (ckit). Further more 96% cells stained positive for both Vimentin and smooth muscle actin. This result indicated stem/progenitor-like features in NIH3T3 cells similar to multipotent stem cells [21].

3.3. Swertisin Effectively Potentiates Islet-Cell Differentiation from NIH3T3 Cells. In order to identify most potent islet differentiating agent among various isolated compounds from

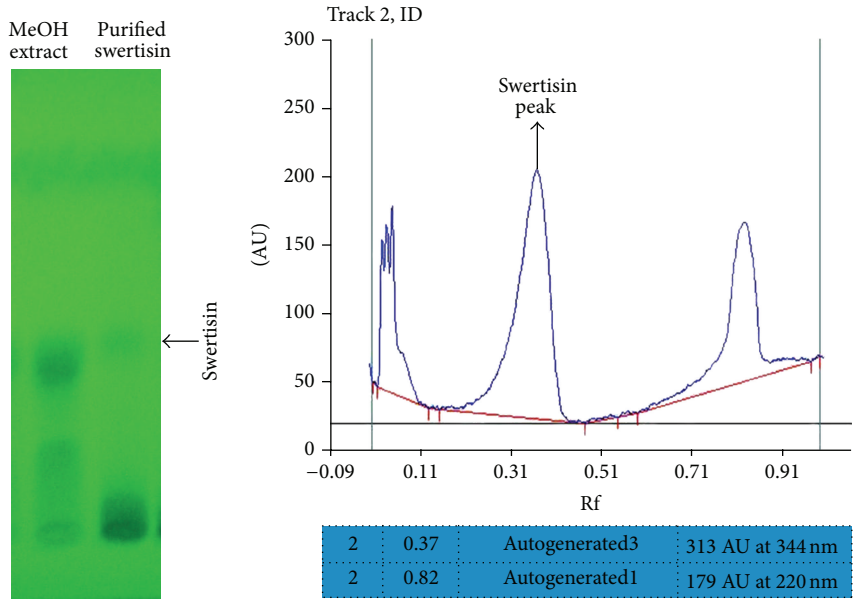

Figure 2: TLC and HPTLC profile of swertisin compound: (a) represents TLC plate scan at UV-265 nm showing methanolic extract and purified swertisin compound profile as a single band at Rf 0.32 (arrow marked), and (b) represents HPTLC curve showing single peak of swertisin at Rf 0.32 and UV-344 nm wavelength.

E. littorale, a comparative islet differentiation was carried out with various compounds using NIH3T3 cells as extra pancreatic progenitor cells for 8 days along with positive islet differentiation inducer agent activin A. After 8-day induction, cluster formation was observed microscopically where swertisin-induced group showed maximum zone of activation and cell aggregation followed by gentianine (Figure 7). ILCC yield and morphological features like average size and area were quantified. Swertisin-induced NIH3T3 cells showed the highest yield of ILCC with significant increase of 3.9-fold compared to SFM alone and activin A induced differentiation group (Figure 15). On calculating average area of ILCC generated amongst all groups, no significant difference was observed in all tested compounds, compared to control, indicating that clusters generated were more or less of similar area (Figure 16).

More pertinently, swertisin and gentianine both showed significantly higher number of ILCC categorized in two groups based on size, ranging from 150 to 300 μm (Figure 17) and from 300 to 3000 μm (Figure 18). On the other hand, activin A generated more numbers of ILCC ranging between 150 to 300 μm and less between 300 to 3000 μm. An increase of approximately 9.3-fold bigger size ILCC from swertisin compared to activin A eventually demonstrated generation of mature ILCC. These results represent the mean of three independent observations and differences were found to be statistically significant ($P < 0.01$ and 0.001). To further test the mode of islet differentiation between control SFM, activin A, and swertisin, microscopic observations were recorded in time-dependent manner and confirmed for insulin biogenesis. After induction with swertisin, the morphology of NIH3T3 cell gradually changed from a fibroblast-like shape to round cells within 2 days and large number of cells formed tight clusters (Figures 6(a), 6(b), and 6(c)). The differentiated NIH3T3 cells showed intense zone of activity and clustering in just 4 days with swertisin induction (Figures 6(c) and 6(d)). These clusters turned up to mature ILCC within 8 days and floated into the medium resembling to islet-like miniorganelles.

3.4. DTZ and C-Peptide Staining Depicts Islet Differentiation in NIH3T3 ILCC. We examined the clusters generated in each group, for insulin-producing cells, and stained them with zinc chelating agent DTZ which is a zinc-binding substance. Pancreatic islets are known to stain crimson red in color after DTZ incubation. Swertisin-induced NIH3T3 ILCC were distinctly stained crimson red by DTZ indicating a sequestration of zinc along with insulin granules (Figure 8). To further examine the new insulin biogenesis, we carried out C-peptide staining. C-peptide is a 3-4 kDa peptide released from insulin molecules within the beta cells. In accordance to our expectations, when swertisin-induced differentiated clusters were tested for C-peptide, along with SFM and activin A group, we found immense positive cytoplasmic staining of mouse C-peptide in swertisin generated ILCC whereas activin A mediated ILCC showed less staining with no staining in control SFM clusters (Figure 8, also see Figure SP-2 available online at http://dx.doi.org/10.1155/2013/280392).

3.5. Confocal Imaging Demonstrates Swertisin-Mediated ILCC Possess Proper Islet Architecture. To monitor the presence of various islet hormones and differentiation markers, immunocytochemical staining was performed in cell clusters generated with SFM + ITS, activin A, and swertisin induction

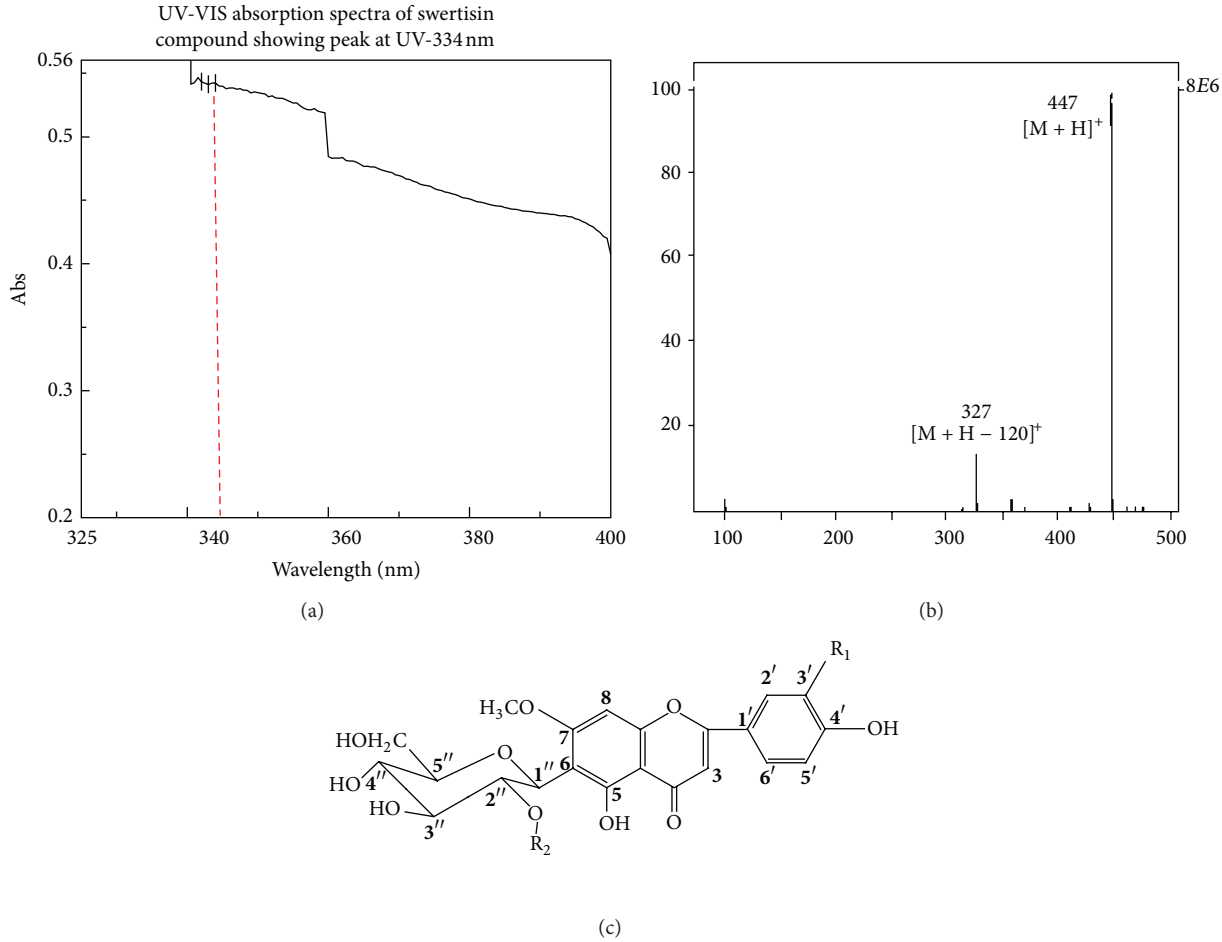

Figure 3: UV-Vis and mass fragmentation pattern of swertisin compound: (a) represents UV-Vis absorption spectra of swertisin compound. a base peak at height of 0.54 at 344 nm was seen purified swertisin (red line marked). (b) represents mass fragmentation curve showing two single peak 447 $[M + H]^+$ representing swertisin compound and 327 $[M + H - 120\ u]$ release of Glycosyl ring. (c) shows structure of swertisin based on mass fragmentation pattern.

and stained for PDX-1, insulin, C-peptide, and glucagon proteins. PDX-1 is considered as an important marker for initiation of islet differentiation. Pancreatic progenitor cells are known to be highly PDX-1 expressing. Many groups have also shown that ectopic expression of PDX-1 lead to islet cell differentiation [22]; moreover, it is also reported that even mature beta cells do express basal level of PDX-1 protein [23]. In our study, we observed that undifferentiated NIH3T3 cells were slightly PDX-1 positive (Figure 9) but negative for C-peptide staining, depicting potential to form ILCC similar to pancreatic progenitors. Upon differentiation with activin A, we observed NIH3T3 ILCC to show both insulin and C-peptide staining. Similarly, swertisin-induced ILCC also stained intensely positive for C-peptide, insulin, somatostatin, and glucagon hormones (Figure 9).

3.6. RT-PCR/Q-PCR Data Revealed Potent Islet Differentiation by Swertisin Induction. Using reverse transcriptase PCR and Q-PCR, mRNA expression of islet differentiation pathway genes and mature islets were quantified. In our study, mRNA transcripts were not detected in the undifferentiated NIH3T3 cells neither for pancreatic endocrine development genes nor for islet specific genes. However, undifferentiated NIH3T3 cells were found to be strongly expressing transcripts of stem/progenitor related genes like Nestin, Vimentin and smooth muscle actin(SMA). Upon differentiation, we found significant elevated expression of both differentiation pathway transcription factors like Nestin, PDX-1, Ngn-3, Pax-4, Nkx 6.1, and Reg-1 and endocrine genes like insulin and glucagon at the end in swertisin induced ILCC with significant reduction in expression for stem cell markers like Nestin, Vimentin, and SMA genes that was also noted (Figure 10(a)). The most peculiar thing about swertisin induction is that the flavonoid not only potentiates islet differentiation pathway, but also accelerates the pace of differentiation. This phenomenon is more obvious with significant reduction in Nestin, Vimentin, and SMA transcript and absence of Pax-4 and Nkx 6.1 expression in swertisin-induced clusters while the same is elevated in SFM alone and activin A induced ILCC even at 8th day. Expression of endogenous control gene Gapdh confirmed the uniformity of the experimental system and acted as input template control.

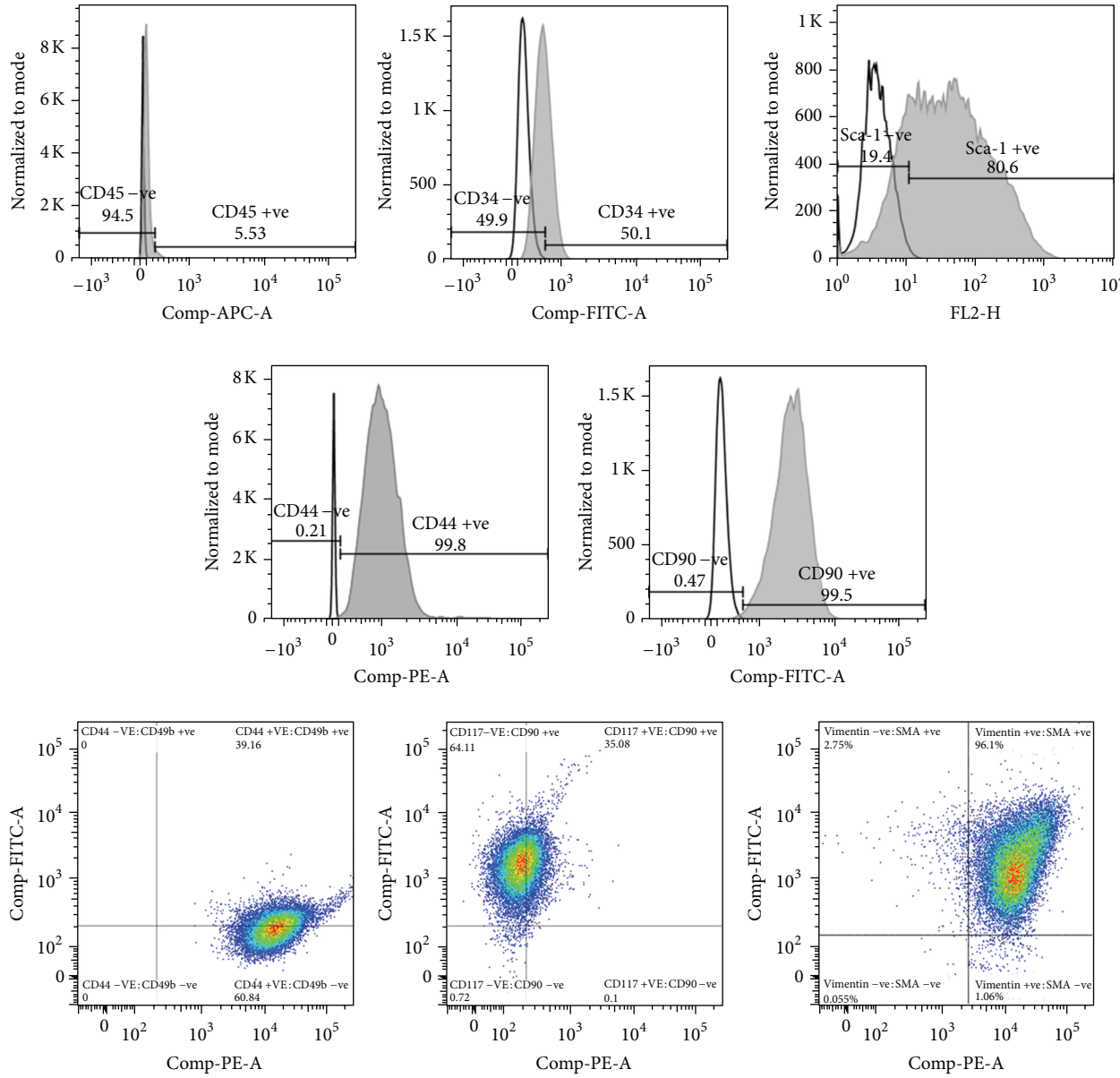

Figure 4: FACS dot plots of various stem cell markers in NIH3T3 cells. The figure depict histogram and dot plots of flow cytometry data analyzed for (1) CD34, (2) CD44, (3) CD45, (4) CD49b, (5) CD117, (6) CD90, (7) Sca-1, (8) Vimentin, (9) SMA.

Also the Q-PCR data demonstrated increase in PDX-1 transcripts at early timepoint and goes down again as the process of differentiation progress in time-dependent manner. Our results clearly showed that a steady increase in PDX-1 transcript in SFM with ITS control group initiated from day 2 and sustained till day 8, whereas when treated with activin A, the mRNA transcript first increased for the first 4 days and then declined significantly by day 6 and continued till day 8. Swertisin also showed increase in PDX-1 transcript right away from day 2 but declined by day 4 and 6 and further increased on day 8. The data showed early onset of differentiation in swertisin group with low levels in late differentiation phase hence not observed at day 10 or so (Figure 10(b)). We also performed real-time quantitative PCR for mouse insulin gene (INS-2 gene). Our data showed that both activin A and swertisin showed increased insulin transcript number while SFM + ITS fails to show the same on the 8th day (Figure 10(c)).

3.7. Immunoblot Confirmed Islet Differentiation Pathway Facilitation by Swertisin Induction. Protein immunoblotting was performed to monitor the initiation and fate of differentiation signaling pathway operated with SFM alone, activin A, and swertisin induction. Major transcription factors like Nestin, PDX-1, phospho-Smad-2, and Smad-7 (key signaling modulator for endocrine fate) for Ngn-3 up-regulation (a

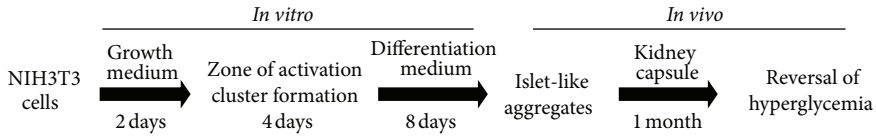

Figure 5: Schematic protocol for NIH3T3 cells normal growth and their differentiation to functional islet-like cells clusters (ILCC). The protocol involves 8-day *in vitro* differentiation into islet-like cell clusters that mature *in vivo* into functional organelle, following transplantation under the kidney capsules of BALB/c mice.

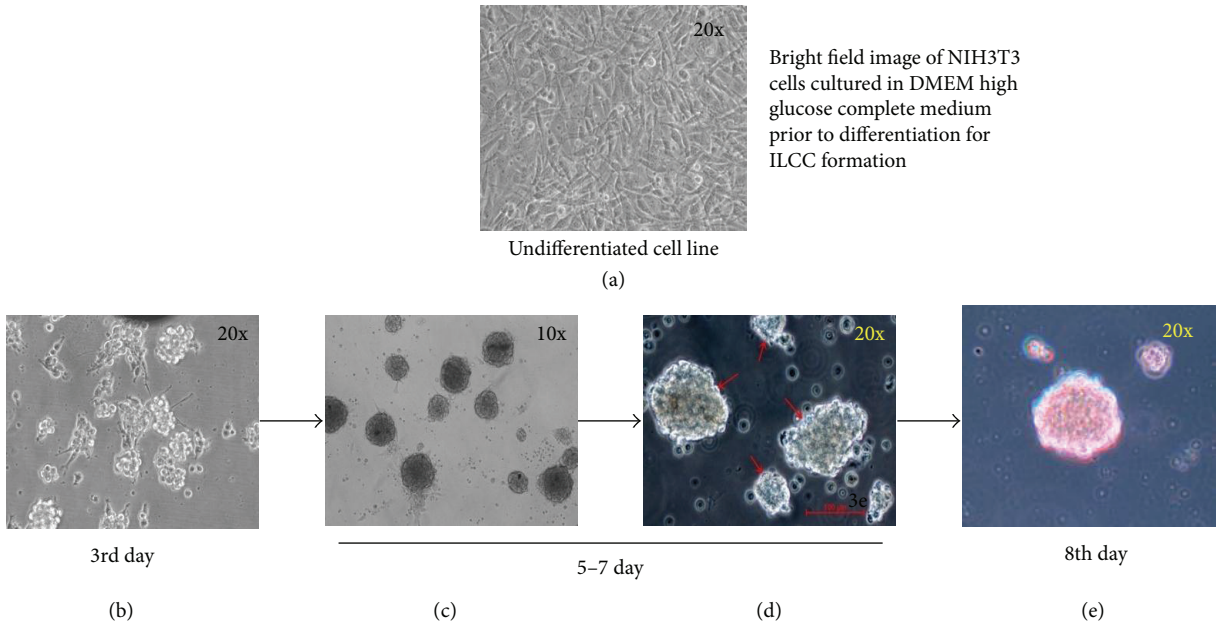

Figure 6: Schematic representation of event occurring in swertisin-induced ILCC formation. (a) represents basic fibroblastic morphology of NIH3T3 cells cultured in complete medium. (b)–(d) depicts event occurring during swertisin-induced differentiation process from the 3rd day to the 8th day. (e) represents DTZ staining in differentiated ILCC for primary confirmation of beta cell differentiation.

master regulator protein for islets) were analyzed in a time dependent manner from day zero to day eight. In this kinetic study, we observed that SFM-induced ILCC fail to downregulate stem/progenitor marker vimentin, resulting in no differentiation, whereas both activin A and swertisin showed decrease in vimentin from the 4th day onwards (Figure 11 and Figure SP-1). Compared to activin A, swertisin clusters showed more steep decrease in vimentin protein, which was completely lost by the 8th day.

Further, SFM alone clusters failed to show upregulation of Nestin, and PDX-1 proteins, which are crucial for initiation of differentiation (Figure 11), but the same was found to be elevated in both activin A and swertisin, and more prominently in swertisin-induced ILCC. Activin A induced ILCC showed an increase in Nestin protein expression (cleaved form 90 kDa) starting from day 2 which continued even till day 8 (Figure 11) suggesting a slower pace in differentiation process. On the contrary, swertisin generated ILCC showed upregulation of both forms of Nestin 170 kDa and 90 kDa which peaked at the 4th day, decreased significantly by the 6th day and completely lost by the 8th day, representing complete and proper differentiation of NIH3T3 cells with swertisin induction. Apart from above observations, swertisin mediated clusters also showed high expression of PDX-1 protein within 2 days and persisted even till the 8th day, whereas both SFM alone and activin A showed no similar trends (Figure 11).

Smad proteins are known to play a role in islet differentiation mediated by activin A. To purely understand the fate of islet differentiation by swertisin we also targeted Smad signaling by monitoring Smad2 and 7 proteins. In a day-wise study, we found that swertisin clusters showed dramatic decrease in Smad7 expression by day 2 with continuous increase in Smad2 phosphorylation till day 8, whereas activin A showed less Smad2 phosphorylation inspite of steep decrease in Smad7 expression (Figure 11). A similar pattern was seen with SFM alone group too, which failed to phosphorylate Smad2. It is important to note that Smad protein phosphorylation in swertisin clusters clearly demonstrates more immense potential for islet differentiation than activin A.

3.8. In Vitro Insulin/C-Peptide Release Assay Indicated Glucose Responsiveness of Swertisin-Generated ILCC. To further check the functional maturity of swertisin-induced ILCC,

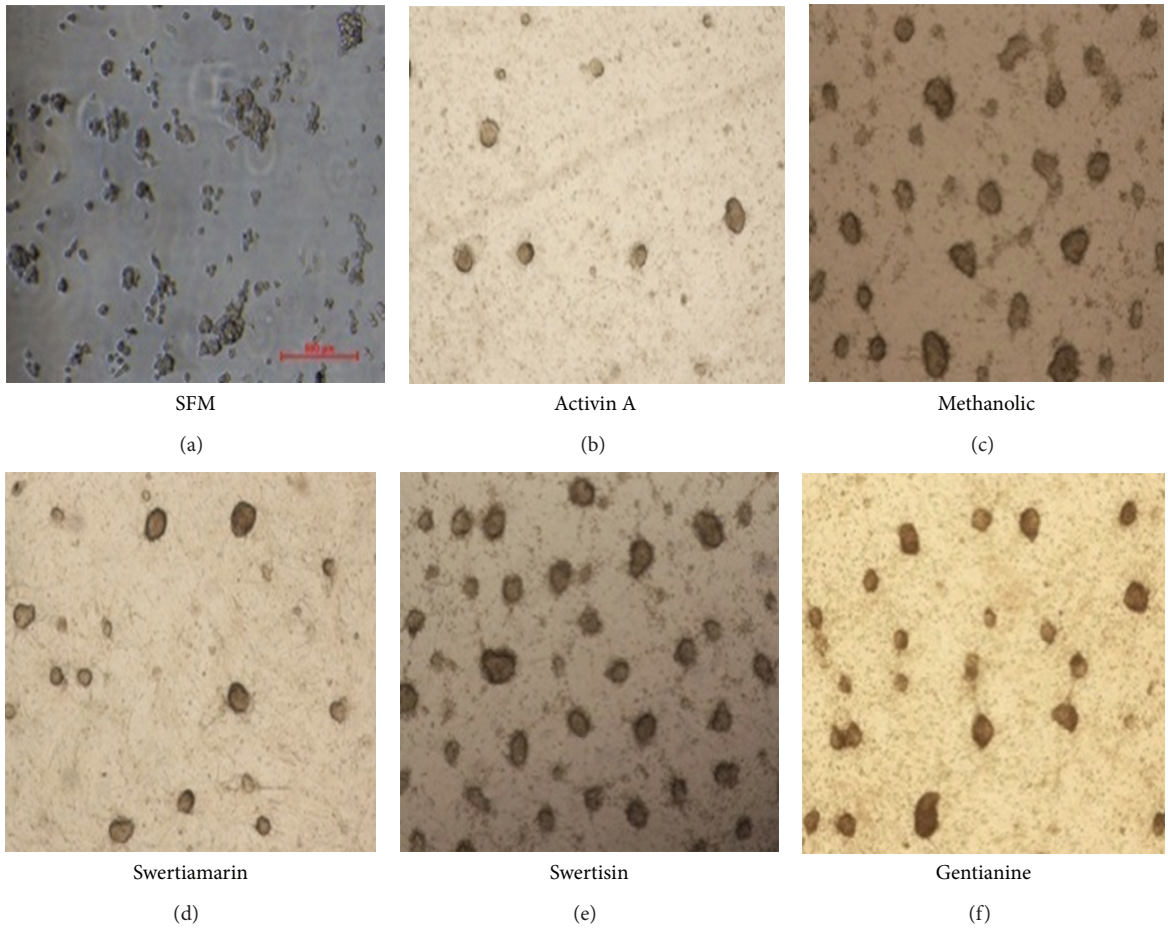

Figure 7: Pictorial representation of ILCC generation from control, activin A and various other *E. littorale* compounds: (a)–(f) demonstrate comparative pictorial representation of ILCC generation from NIH3T3 cells using SFM control, activin A and various EL compounds. (e) indicates maximum ILCC generation with swertisin induction.

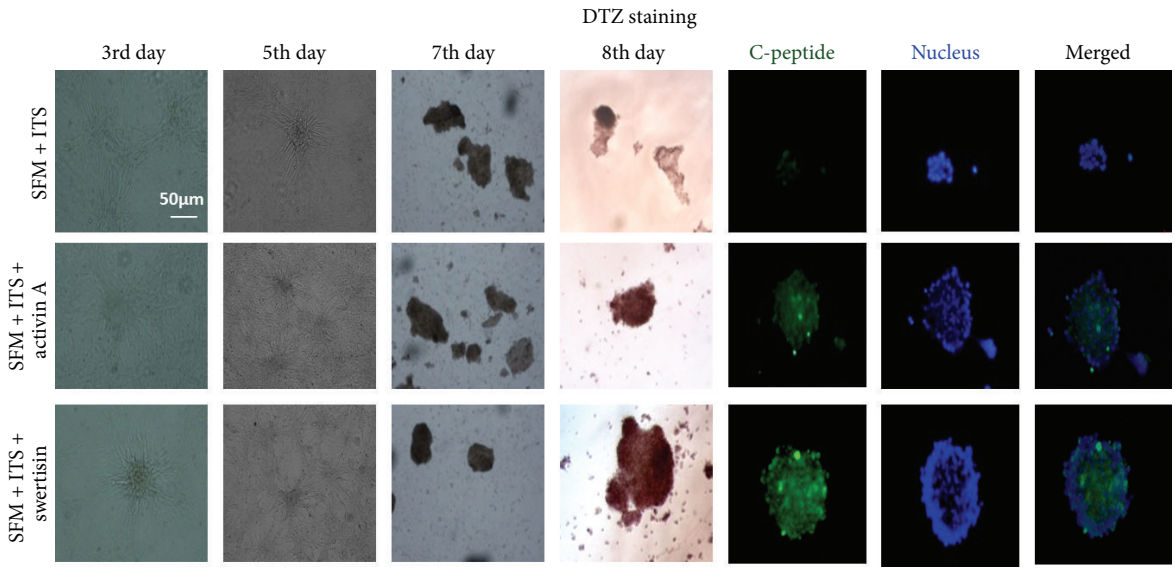

Figure 8: Comparative montage of morphological events occurring in 3–8 day time duration. The figure represents day-wise representation of morphological changes and differentiation event occurring with SFM, activin A and swertisin-mediated ILCC. The figure also shows DTZ staining in differentiated ILCC for primary confirmation of beta cell differentiation and C-peptide staining representing presence of insulin biogenesis and insulin granules.

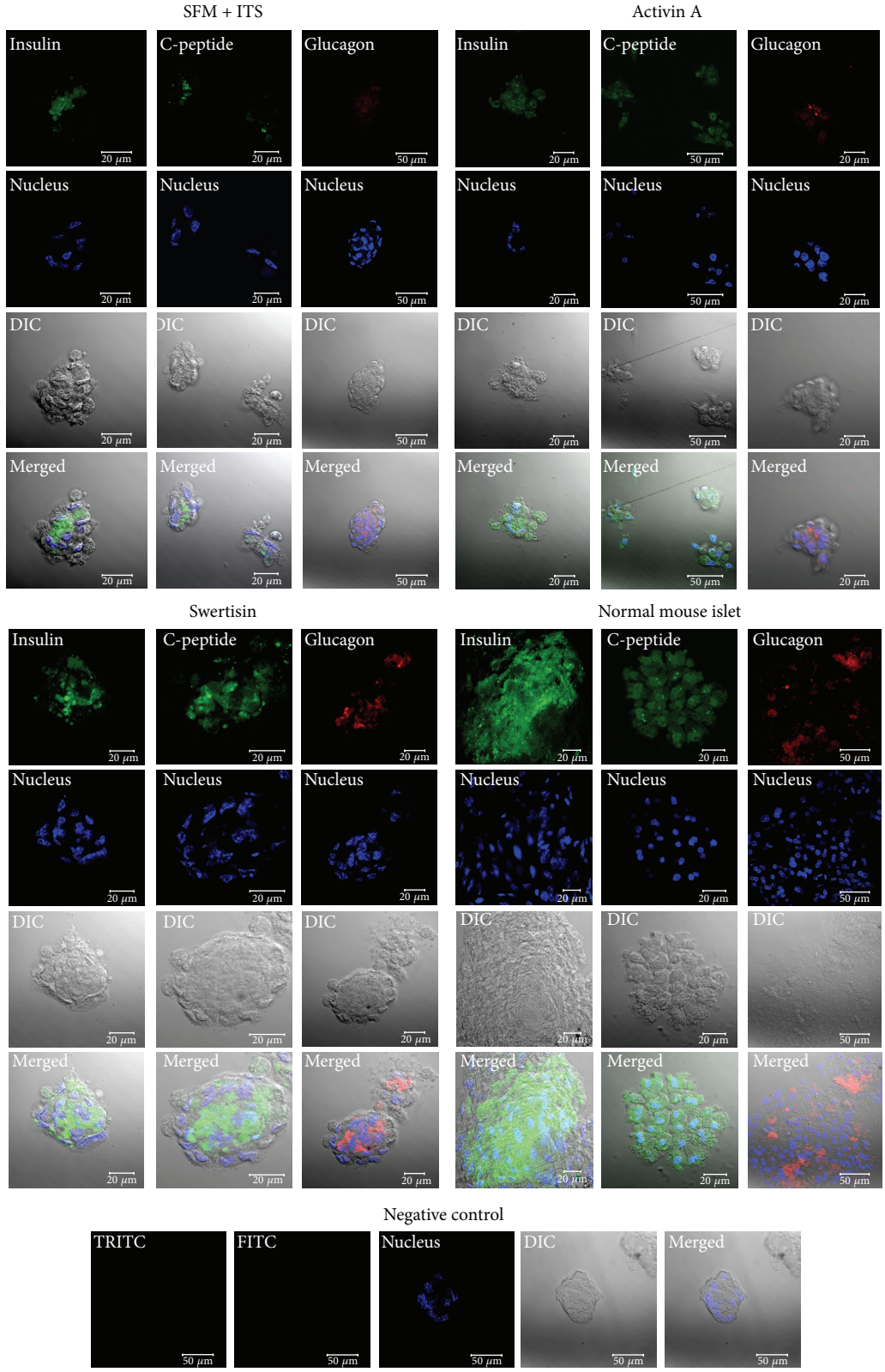

Figure 9: Confocal images of various islet markers in SFM + ITS, activin A, and swertisin induced ILCC. The figure depicts intense positive staining for insulin (green color), C-peptide (Green color), and glucagon (red color). Nucleus of all the clusters was stained with DAPI shown in blue color.

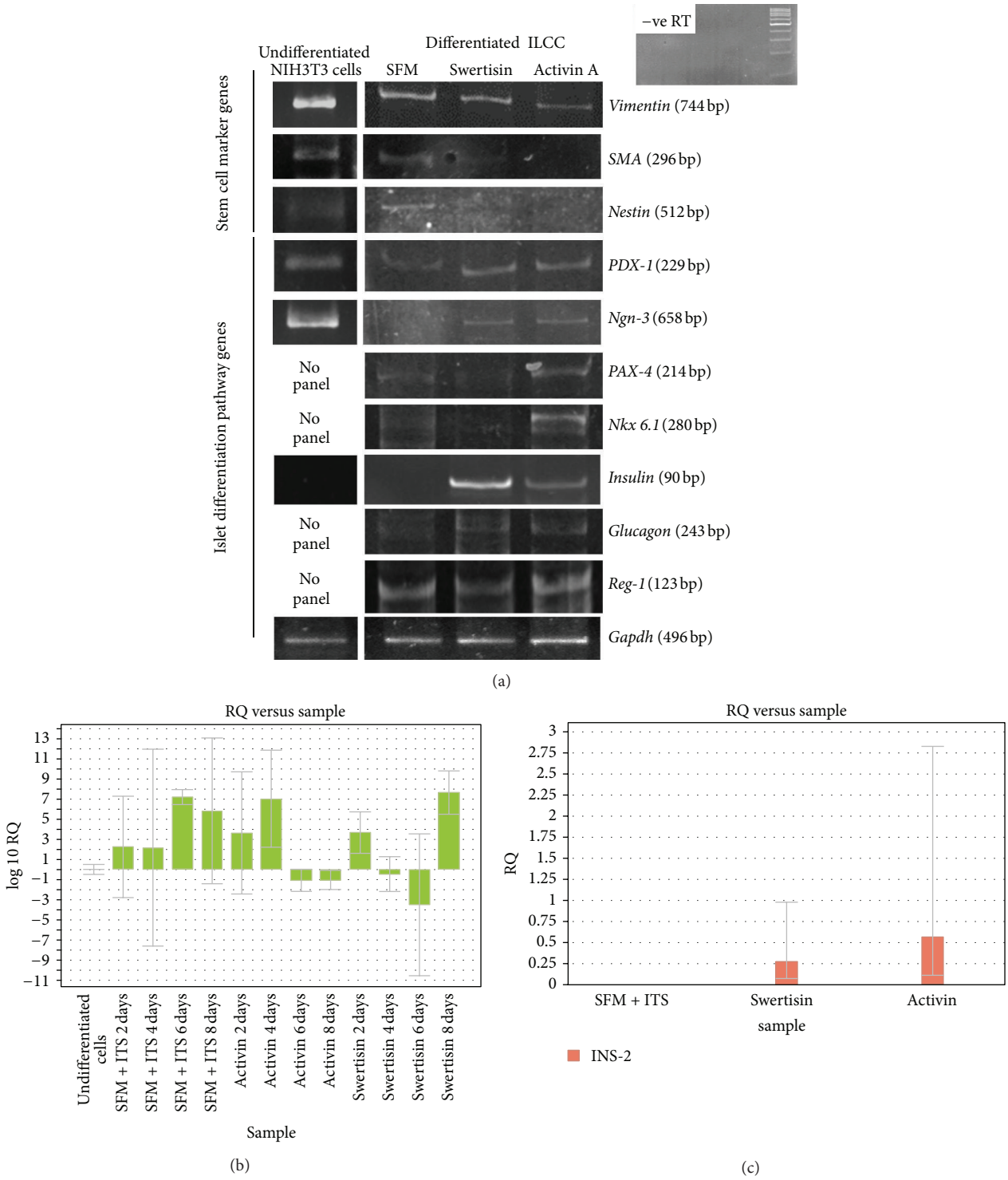

Figure 10: RT-PCR and Q-PCR expression data of various stem/progenitor and islet differentiation pathway genes. (a) shows mRNA expression of various stem/progenitor genes and key islet differentiation pathway transcription factor genes harvested at 10 days of differentiation protocol in SFM alone, activin A, and swertisin-mediated ILCC. (b) shows comparative PDX-1 transcript expression in differentiated clusters harvested at different timepoints. (c) represents insulin mRNA transcript levels in SFM + ITS, activin A, and swertisin-induced clusters at the 8th day.

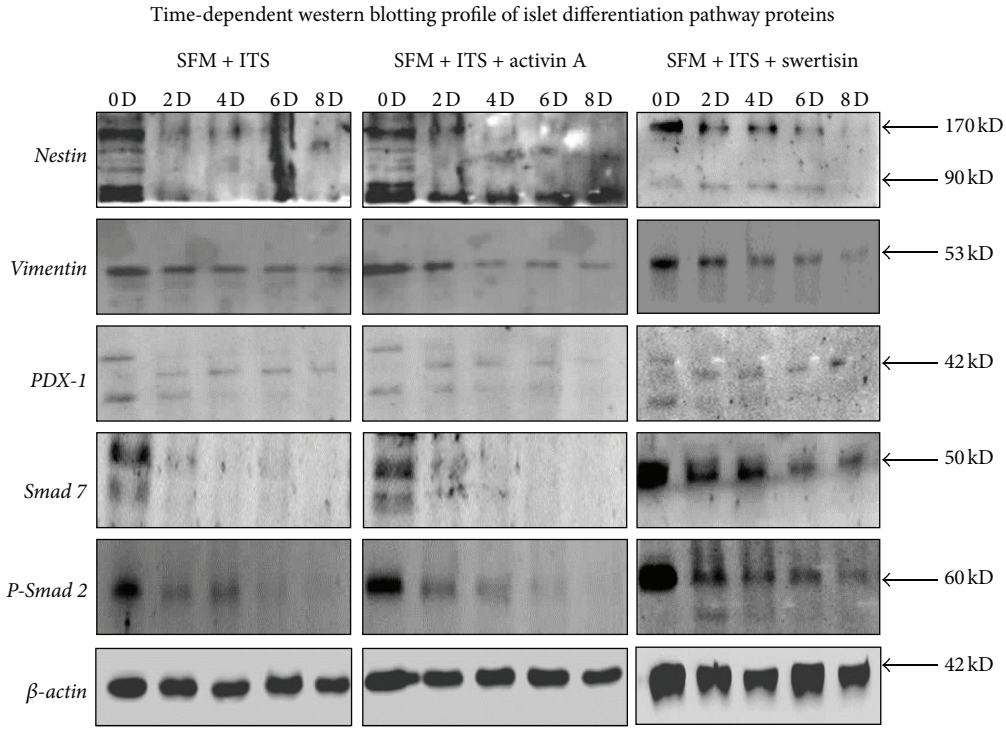

FIGURE 11: Time-dependent western blot study of various stem/progenitor and islet differentiation pathway protein. The figure shows time-dependent expression of various stem/progenitor markers and key islet differentiation pathway transcription factor during differentiation protocol ranging from day 0 to day 8.

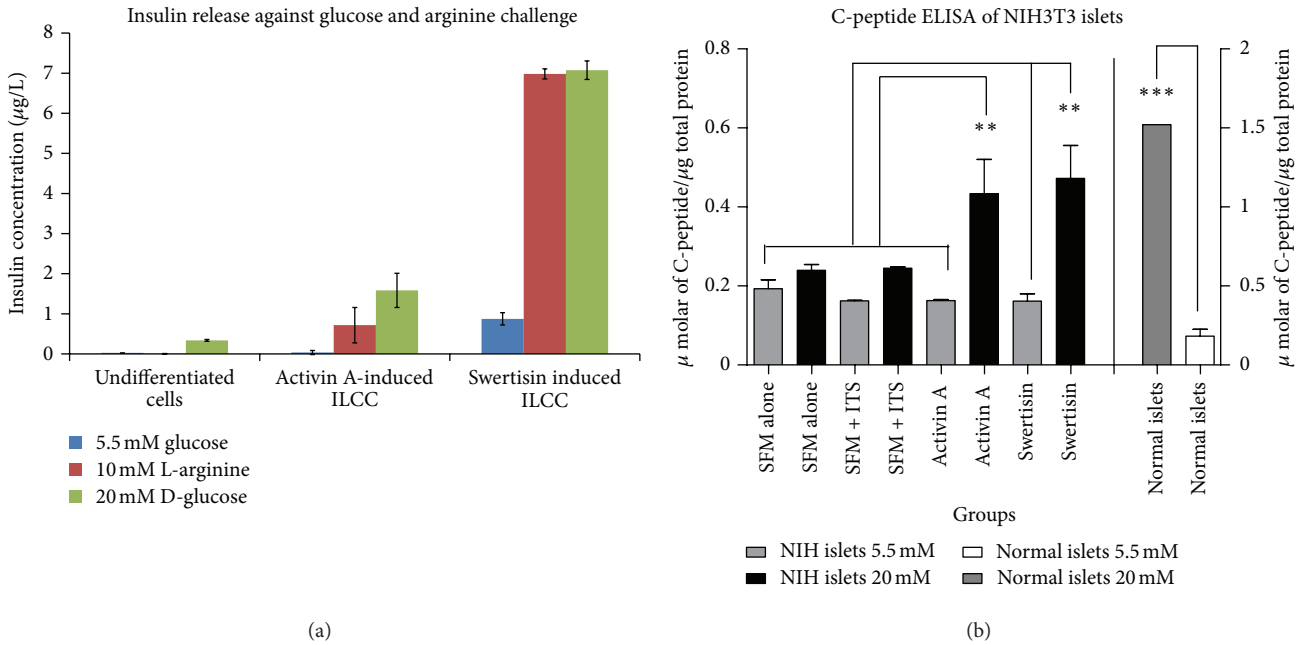

FIGURE 12: Glucose responsive insulin and C-peptide release assay. (a) represents glucose responsive insulin release from SFM, activin A and swertisin-mediated ILCC with increasing glucose and L-arginine concentration. Results are expressed as mean ± SEM of three independent observations, $N = 3$. (b) represents glucose responsive C-peptide release from SFM, SFM + ITS, activin A and swertisin-mediated ILCC. Results are expressed as mean ± SEM of three independent observations, $N = 3$.

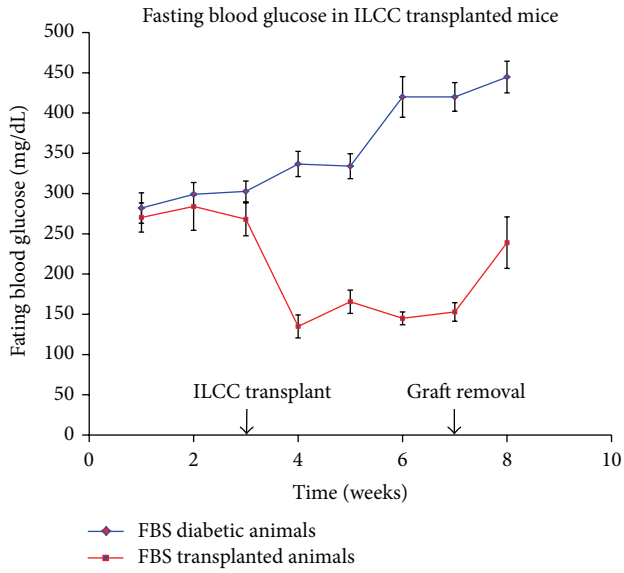

Figure 13: FBG in BALB/c mice after transplantation. The figure represents fasting blood glucose in time-dependent manner from swertisin-mediated transplanted ILCC and diabetic control mice. Glucose was monitored for 7 weeks, and 1 week after graft removal. Results are expressed as mean ± SEM of 5 transplanted animals.

in vitro glucose challenge was performed to monitor them for increasing amounts of insulin or C-peptide in a glucose-concentration-dependent fashion (Figure 12(a)). The mean insulin secretion by swertisin-mediated clusters (100) was 0.87 ± 0.022 μg/mL in response to 5.5 mM glucose, 6.98 ± 0.42 μg/mL in response to 10 mM L-Arginine, and 7.07 ± 0.23 μg/mL in response to 20 mM glucose concentration, which is significantly higher than SFM alone (0.025 ± 0.009 μg/mL in 5.5 mM, 0.004 ± 0.052 μg/mL in response to 10 mM L-Arginine, and 0.337 ± 0.15 μg/mL in 20 mM glucose concentration) and activin A (0.035 ± 0.003 μg/mL in 5.5 mM, 0.716 ± 0.442 μg/mL in response to 10 mM L-Arginine and 1.584 ± 0.12 μg/mL in 20 mM glucose concentration). Swertisin-differentiated ILCC showed 20.9, and 4.46 fold increase in insulin release compared to SFM and activin A induced ILCC, respectively, upon 20 mM glucose challenge. The result represents mean of three observations and differences were found to be statistically significant ($P < 0.01$). We also performed C-peptide release from the same ILCC in order to confirm new insulin synthesis as there is a possibility of uptake of insulin by endocytosis. By C-peptide ELISA we confirmed the presence of new insulin biogenesis as C-peptide release from both swertisin and activin A and was found to be significantly higher at 20 mM glucose induction compared to 5.5 mM induced clusters similar to normal mouse islets. When we compared SFM alone and SFM + ITS induced clusters, we could not find any significant release in C-peptide even upon high glucose challenge (Figure 12(b)).

3.9. STZ-Mediated Diabetes Induction and Islet Transplantation in Renal Capsule. To check the functionality of swertisin-induced NIH3T3 ILCC for insulin cell functionality *in vivo*, STZ-induced diabetic mice model was used. Multiple dose of STZ in BALB/c mice produced effective TD1 mice which greatly elevated blood glucose levels (Figure 13). The efficacy of glucose lowering tendency from swertisin-induced ILCC was monitored from transplanted renal grafts. All the mice receiving renal grafts showed significant reduction in FBG within one week of transplantation (Figure 13).

3.10. H&E Staining and Immunohistochemistry of Transplanted Renal Grafts Reconfirm In Vivo Functionality of Swertisin-Induced ILCC. To demonstrate the viability and functional integrity of transplanted islet grafts *in vivo* after stabilization period of four weeks, renal capsule having transplanted ILCC grafts was excised, sectioned, and histologically examined for intact islet architecture using H&E stain. Deep eosin cytoplasmic with less nuclear hematoxylin stain showed compact islet architecture. More of eosin staining resembles the graft staining similar to pancreatic islet section stain. Presence of C-peptide, insulin, glucagon, and somatostatin using immunohistochemistry was also assessed. Intense cytoplasmic staining for all the four hormones in excised tissue showed that transplanted ILCC graft was not only viable but also functional during transplantation period inside mouse kidney, whereas kidney cells did not stain for any islet hormone (Figure 14).

4. Discussion

The present study reframes a new arena to screen and identify the islet neogenic potential of an important compound from *E. littorale* plant to develop cheaper but readily available drug solution for diabetic patients. The literature in recent years has been repleted with a spectrum of experimental evidences that demonstrates the possibility of generating insulin producing cells from embryonic as well as adult stem cells with numerous growth factors [24–26]. Despite their promising potential for differentiation, it is still difficult to obtain sufficient islets for autologous islet transplantation [1]. One of the important obstacles is the lack of potent differentiating agents which can yield enormous amount of islet cells from starting pool of stem cells. Few differentiating agents like activin A, a member of TGF-β family, which converts embryonic stem cells to islet cells [27], glucagon like peptide-1 and keratinocyte growth factor are reported with tissue specific stem cell differentiation into islets [28]. Nicotinamide and exendin-4 are more such compounds which show islet neogenesis by inhibiting poly ADP-ribose synthesis [29], stimulating both β cell replication and neogenesis from ductal progenitor cells and inhibiting apoptosis of β cell [30, 31]. Above all the major impediment to it use as a therapeutic drug is the high cost which makes it unreachable to patients. To overcome this limitation, we screened islet differentiation potential of bioactive agents from medicinal herbal plants. Identification of such potent herbal active ingredient may revolutionize the therapeutic approach for achieving enormous islet yield for effective treatment of diabetic patients.

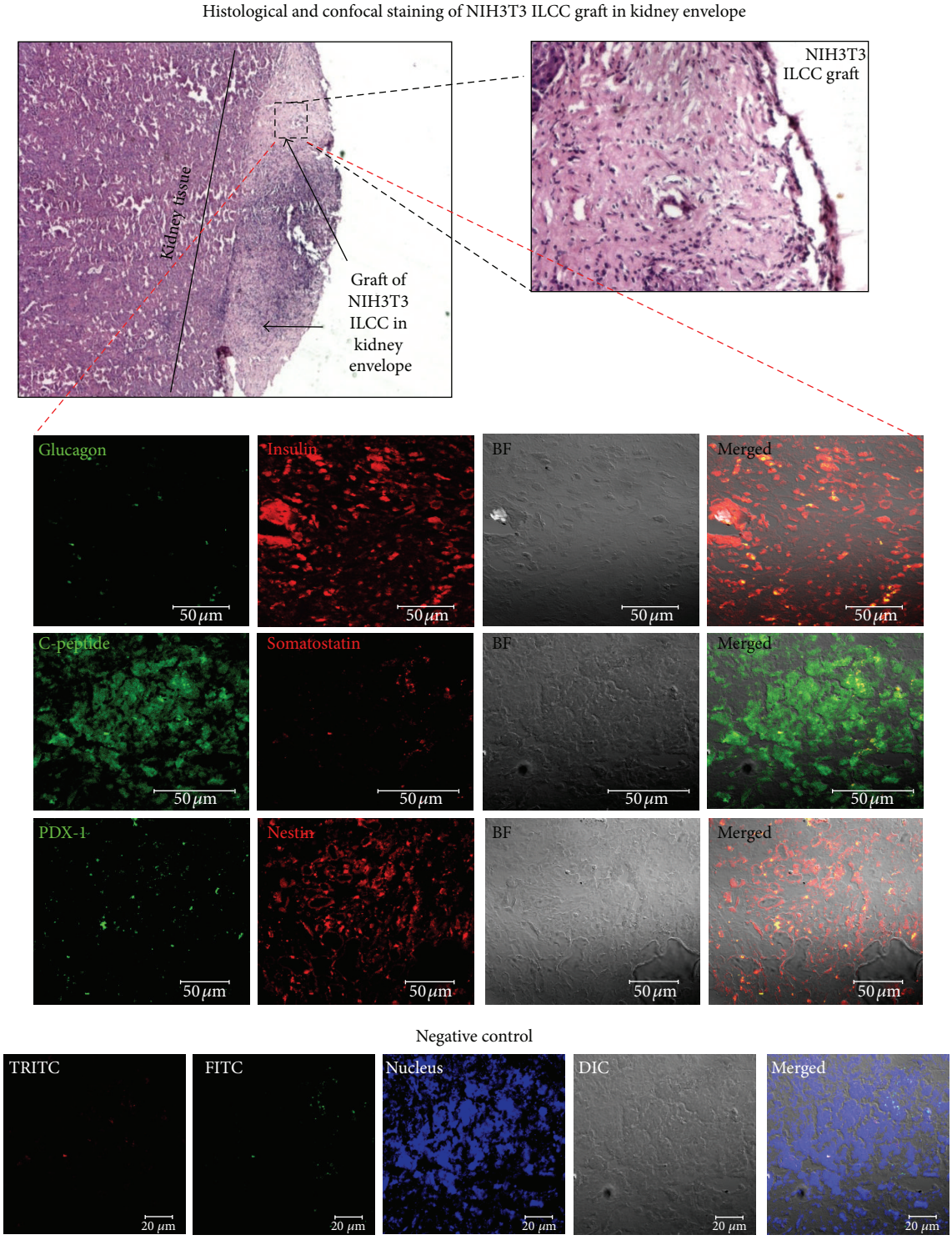

Figure 14: Histological and immunochemical staining of islet hormones in kidney-ILCC grafts. Top first two figure represents a histological observation of kidney graft from transplanted swertisin-mediated ILCC. Islet specific makers were also observed in these grafts, which show insulin (red), C-peptide (green), somatostatin (red), Pdx-1 (green), and Nestin (red).

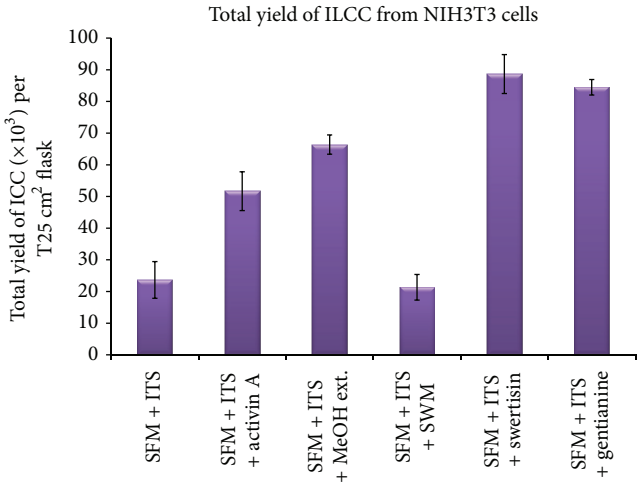

FIGURE 15: The figure depicts total yield of ILCC where swertisin showed significantly higher yield compared to SFM and activin A group.

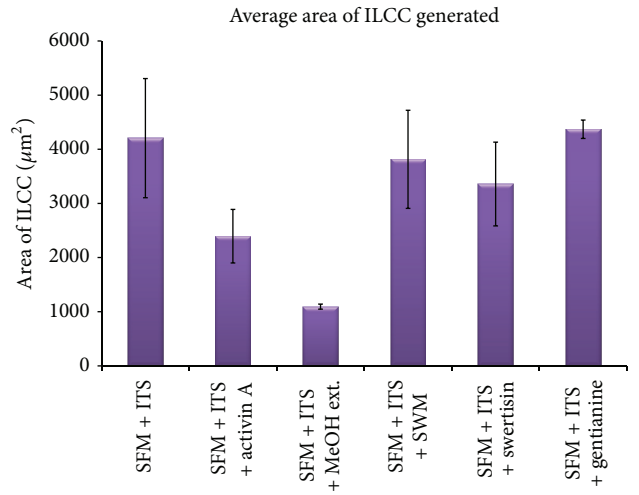

FIGURE 16: The figure shows comparative data of average area of ILCC generated with various compounds.

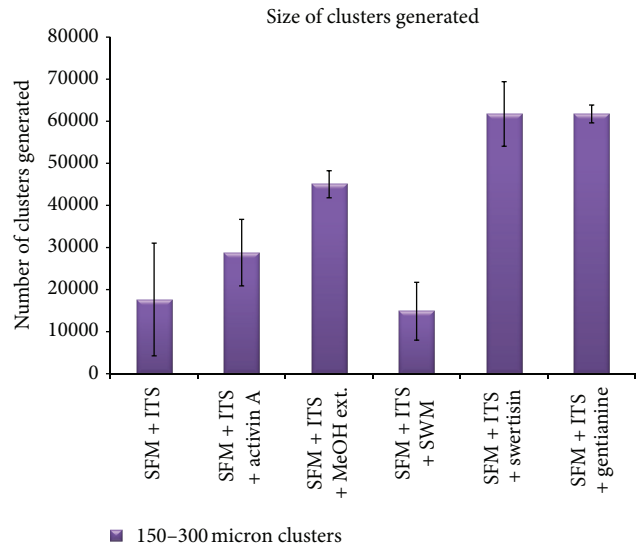

FIGURE 17: The figure represents size of ILCC clusters generated between range of 150–300.

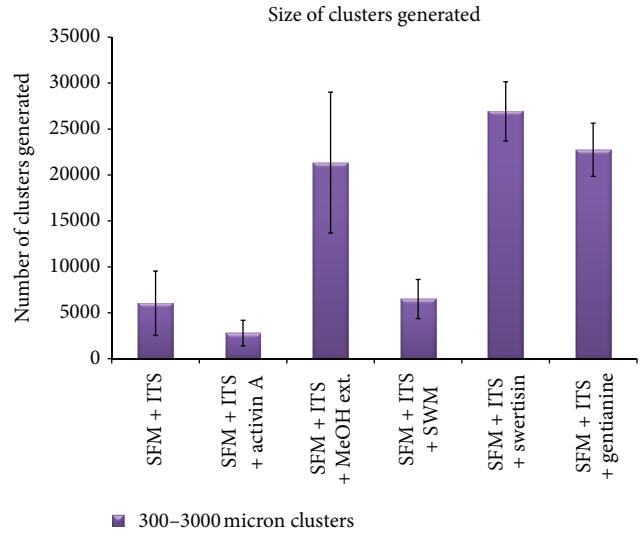

FIGURE 18: The figure represents size of ILCC clusters generated between range of 300–3000 μm.

Numerous plants and their products have been demonstrated for antidiabetic activity in both cellular and animal models [6–9]. The majority of them were targeted to emphasize the ameliorating effect of hyperglycemia either by increasing insulin secretion or by sensitizing downstream signaling [32, 33]. Fewer reports underwent extensive research to monitor the status of diabetic stressed beta cells within the pancreas upon such herbal treatments and mechanisms of plant products mediated beta cell replenishment. Few *in vitro* studies highlighted the role of certain herbal products in stem cell reprogramming into functional insulin-producing cells. In 2003,Kojima and Umezawa, have reported one such compound named conophylline showing AR42J cells conversion into insulin positive cells [10]. Another group, Ansarullah et al., has recently investigated the *in vivo* islet replenishing property of *Oreocnide integrifolia* in pancreatectomized mice [34].

In similar directions, the author's group has also reported islet differentiating activity from *E. littorale* plant earlier in 2010 [17]. Understanding and identifying the most potent islet neogenic active principle of this plant serves the aim of this study, which is the most essential criteria for developing the molecule as an islet cell replacement and therapeutic agent. In comparative screening for various extracts and isolated purified compounds from *E. littorale* methanolic extract, we observed islet cluster formation activity using NIH3T3 cells. It is significant to note that NIH3T3 cell differentiation to certain cell types have previously been reported by few groups, except islet differentiation property [35, 36]. *E. littorale* compounds induced effective differentiation of NIH3T3 cells similar to activin A. The rational for using

activin A as positive control was due to its reported ES cell to islet differentiation activity and NIH3T3 cells are also from embryonic in origin [27]. The screening of all other compounds in this fashion provided the most potent inducing agent which possesses the highest islet neogenic potential with the generation of a a large number of both smaller and bigger sized ILCC. This primary screening provided us a basis to identify this compound, which was further characterized for its structure and molecular differentiation pathway leading to insulin-producing cells.

For structure identification of an unknown compound, it is mandatory to first evaluate its primary characteristic-like UV absorption spectra, TLC pattern, HPTLC curve profile, and so forth [37].Colombo et al. have previously reported UV spectrum and HPTLC plots for various compounds from *E. littorale* [38]. We examined and compared our results with another report by Sawant et al. and found that our compound of interest shows identical Rf and absorption maxima values with swertisin as reported by these two groups [39]. Structural identification was further performed by ESI MS/MS spectroscopy. Mass fragmentation pattern of a base peak of m/z 447 [M + H] corresponded to swertisin structure, similar to earlier reports by Colombo et al. andSawant et al. [38, 39]. Therefore, we confirmed that the compound we isolated is indeed swertisin which has enormous islet neogenic potential.

One of the most logical modalities to confirm beta-cell differentiation is to monitor for zinc chelating agent dithizone (DTZ) staining in newly generated clusters [40]. Differentiation of NIH3T3 cells into islet cells in 8-day protocol generated large and mature clusters. Insulin production was confirmed by staining with DTZ. Some investigators argue that part of insulin detected may have been derived from insulin added to the culture media in certain protocols or insulin present in serum [41]. Therefore, it becomes essential to demonstrate new biogenesis of insulin molecule by C-peptide staining in newly differentiated clusters. Detection of C-peptide in newly generated clusters confirmed that insulin presence was the result of endogenous synthesis. Presence of insulin or C-peptide is not sufficient enough to regulate glucose, but presence of glucagon and somatostatin is also needed to maintain the homeostasis. Hence, islet transplant becomes a better strategy than that of β cells alone [42]. We observed the presence of other islet hormones in ILCCs generated by swertisin induction. The expression of glucagon and somatostatin in the ILCCs indicates that swertisin not only produces insulin positive cells, but it also facilitates the differentiation of glucagon and somatostatin positive cells to provide more or less a complete islet phenotype with mature islet architecture.

Some groups have reported that stem cell differentiation into islet cells follows embryonic ontology and their gene expression of differentiated clusters should be similar to that of pancreatic endocrine tissue [43]. Zhang et al. [44] reported that nestin positive precursor cells possess instructive signals that govern islet differentiation pathway. Ngn-3 is known to be the key master regulator of endocrine cell fate; initiation of islet differentiation signaling occurs by early induction of Ngn-3 protein [45]. The present study showed early induction of differentiation pathway genes like Nestin, Pdx-1, Ngn-3, Pax-4, Nkx 6.1, and Reg-1. The expression of insulin, Pdx1, Reg-1, and Nkx 6.1 is considered to be a specific functional characteristic of mature β cells and has previously been reported only *in vivo* [46].

Differentiation into insulin producing endocrine cells is induced by a cascade of signaling proteins controlled by several transcription factors such as Nestin, PDX-l, Neurogenin-3, and Smad-2 [47]. Abundant expression for Smad2 and Smad4 with low expression of Smad1, Smad3, and Smad7 was earlier reported by Zhang et al. [48] in developing pancreas. This Study showed that swertisin upregulates Ngn-3 in early days and goes down thereafter (data not shown). This in turn showed downstream repression of Smad7 while over expression of Smad2 leading to mature islet differentiation similar to activin A [47]. All these results were found to be in accordance with earlier reports by numerous groups [49]. Evidence has also been provided that swertisin induction showed significant high release of insulin upon glucose stimulation in a dose-dependent manner, suggesting that these cells possess the major functional capabilities of β cells, namely, insulin release in response to changes in extracellular glucose concentrations and the presence of insulin-containing secretory granules [50].

Progress towards the goal of direct assessment of graft integrity, viability, and function is mandatory for therapeutic application. Observing animal response for blood glucose under diabetic condition is a real functional challenge for differentiated clusters. Many studies previously demonstrated reversal of hyperglycemia upon transplantation of differentiated ILCC's under kidney envelope [29]. Kidney being a highly vascular organ supports survival and ideal niche for the functional maturity of newly generated ILCC. Glucose lowering effect of transplanted NIH3T3 ILCC under diabetic BALB/c mice kidney capsules confirmed that swertisin mediated clusters do possess functional maturity and are able to combat diabetic conditions. The molecular proof of this can be visualized by the fact that kidney graft isolated from transplanted animals showed the presence of viable islet architecture having immunopositivity for C-peptide, insulin, glucagon, and somatostatin along with basal expression of nestin and PDX-1. Furthermore, a fasting blood glucose response showed that the blood glucose levels of transplanted diabetic mice exhibited similar kinetics to those of normal control mice [29], representing a recovery of insulin secretory ability in the swertisin-mediated NIH3T3 ILCC transplanted mice. Taken together, these results suggest that these induced cells have a similar function to normal physiological islets both *in vitro* and *in vivo*.

Therefore, in conclusion, we state that swertisin is a novel molecule which can serve as an efficient differentiating agent generating islet-like cell types, with enormous yield and mature functional status. Despite these encouraging observations, in the authors' opinion, the issue of generating islet-cell mass *in vitro* and surviving *in vivo* using less expensive and readily available differentiating agents still remains a challenge unless we attempt to explore more medicinal herbal products with potential stem cell conversion for miraculous therapeutic activities like islet neogenesis.

Abbreviations

E. littorale:	*Enicostemma littorale*
ILCC:	Islet-like cell clusters
SFM:	Serum free media
ITS:	Insulin transferrin selenite cocktail
DTZ:	Dithizone
STZ:	Streptozotocin
FBG:	Fasting blood glucose
T1D:	Type1 diabetes
T2D:	Type2 diabetes
HGF:	Hepatocyte growth factor
SMA:	Smooth muscle actin
NaF:	Sodium fluoride
ESI-MS/MS:	Electron stimulated ionization mode for mass spectroscopy
INGAP:	Insulin-like neurogenic growth peptide
NIDDM:	Noninsulin-dependent diabetes mellitus.

Conflict of Interests

The authors declare that there is no conflict of interests associated with this paper.

Author's Contribution

Sarita Gupta, and N. Dadheech conceived and designed the experiments; S. Soni and N. Dadheech carried out the isolation and characterization of compounds; N. Dadheech, A. Srivastava, S. Dadheech, Shivika Gupta, and R. Gopurappilly performed other experiments; N. Dadheech, R. R. Bhonde, Sarita Gupta—Analyzed the data; N. Dadheech, and Sarita Gupta wrote the paper.

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Research Article

Rhinacanthus nasutus Ameliorates Cytosolic and Mitochondrial Enzyme Levels in Streptozotocin-Induced Diabetic Rats

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The present study was conducted to evaluate the therapeutic efficacy of *Rhinacanthus nasutus* (*R. nasutus*) on mitochondrial and cytosolic enzymes in streptozotocin-induced diabetic rats. The rats were divided into five groups with 6 rats in each group. The methanolic extract of *R. nasutus* was orally administered at a dose of 200 mg/kg/day, and glibenclamide was administered at a dose of 50 mg/kg/day. All animals were treated for 30 days and were sacrificed. The activities of both intra- and extramitochondrial enzymes including glucose-6-phosphate dehydrogenase (G6PDH), succinate dehydrogenase (SDH), glutamate dehydrogenase (GDH), and lactate dehydrogenase (LDH) were measured in the livers of the animals. The levels of G6PDH, SDH, and GDH were significantly reduced in the diabetic rats but were significantly increased after 30 days of *R. nasutus* treatment. The increased LDH level in diabetic rats exhibited a significant reduction after treatment with *R. nasutus*. These results indicate that the administration of *R. nasutus* altered the activities of oxidative enzymes in a positive manner, indicating that *R. nasutus* improves mitochondrial energy production. Our data suggest that *R. nasutus* should be further explored for its role in the treatment of diabetes mellitus.

1. Introduction

Type 1 diabetes mellitus (T1DM) is caused by the destruction of insulin-producing β cells in the pancreas and is usually the result of an autoimmune disease. T1DM leads to uncontrolled blood glucose levels, which are associated with long-term damage, including retinopathy, neuropathy, nephropathy, and damage to the heart and blood vessels and with multiple organ failures [1]. To date, various types of herbs and other plant materials from all over the world have been used for the treatment of diabetes mellitus. Medicinal plants are a good source of compounds with hypoglycemic effects and are important for the development of new drugs and as adjuncts to existing therapies [2]. Some of the medicinal plants such as *Panax ginseng* and *Piper longum* possess significant antihyperglycemic and antihyperlipidemic actions which have been reported to have the potential to alleviate impaired oxidative stress in diabetic rats [3, 4]. Generally, herbal products are gaining popularity due to their natural origins, the lower incidence of side effects, and their relatively lower costs relative to synthetic drugs [5], although their efficacies may still be inferior when compared to some current available treatments such as insulin and meformin.

Rhinacanthus nasutus (Linn) is a flowering plant that belongs to the *Acanthaceae* family and is well recognized for its remedial uses. This plant is commonly known as *Nagamalli* in Telugu, *Doddapatika* in Kannada, *Kaligai* and *Anichi* in Tamil, *Yuthikaparni* in Sanskrit, *Jupani* in Hindi, and *Gajakarni* in Marathi [6]. *R. nasutus* is also commonly known as Snake Jasmine due to the shape of its flowers. In addition, the root of this plant has been reported to be used in traditional medicine to counter the effects of snake venom [7]. Different parts of this plant have also been traditionally used for the treatment of various diseases

such as diabetes, eczema, pulmonary tuberculosis, herpes, hypertension, hepatitis, and several types of skin diseases. In Thailand, *R. nasutus* has been traditionally used for the treatment of various cancers such as colon [8], cervical, and liver cancers [9].

Previously, we reported that *R. nasutus* possesses antimicrobial properties and can kill a variety of infecting organisms, in addition to exhibiting antidiabetic effects, hypolipidemic activity [10], and significant *in vitro* and *in vivo* antioxidant activities [11]. In this study, we hypothesized that *R. nasutus* may exert antidiabetic effects by ameliorating cytosolic and mitochondrial enzymes levels.

2. Materials and Methods

2.1. Collection of Plant Material. The fresh leaves of *R. nasutus* were collected from Tirumala Hills, Tirupati, and Chittoor district of Andhra Pradesh, in the period of July–October 2009. Seshachala and Tirumala Hills (Rayalaseema region, Andhra Pradesh, India), areas that are geographically located in the South Eastern Ghats, are recognized for their rich flora and fauna [12]. The plant specimen was verified to be of the correct species by Dr. Madhava Setty, a botanist from the Department of Botany, S. V. University, Tirupati.

2.2. Preparation of the Extract. Fresh leaves of *R. nasutus* (500 g) were shade-dried and milled into fine powder using a mechanical grinder (TTK Prestige, Chennai, India). The powdered plant material was macerated and shaken in methanol using a bath shaker (Thermo Scientific, Mumbai, India) for 48 h. The extract was then filtered with filter paper (Whatman No. 1) and evaporated to dryness under vacuum and reduced pressure using a rotary evaporator at 40°C. The concentrate was then placed on aluminum foil before freeze drying. The residual extract was dissolved in sterile water (1 mL) before use.

2.3. Chemicals. Streptozotocin (STZ) was purchased from Sigma (USA). All other chemicals and reagents used in this study were of analytical grade. Glibenclamide (Sugatrol, Hyderabad, India) was purchased from a local drug store.

2.4. Experimental Design. Adult male Wistar rats weighing between 150 and 180 g were obtained from Sri Venkateswara Enterprises, Bangalore. They were individually housed in clean, sterile polypropylene cages under standard conditions (12 h light/dark cycles) with free access to standard chow (Hindustan Lever Ltd., Bangalore, India) and water *ad libitum*. For one week prior to the start of the experiments, the animals were acclimatized in the laboratory. The animal experiments were designed and performed in accordance with the ethical norms approved by the local Ministry of Social Justices and Empowerment, Government of India, and the Institutional Animal Ethics Committee Guidelines (Resolution no. 05/(i)/a/CPCSEA/IAEC/SVU/MDN-PVR/dt.13.09.2010).

2.5. Induction of Experimental Diabetes. The rats were divided into five groups of six animals each:

Group I. Normal rats (controls: animals receiving only buffer).

Group II. *R. nasutus*-treated normal rats (200 mg/kg/day).

Group III. Diabetic rats (untreated).

Group IV. *R. nasutus*-treated diabetic rats (200 mg/kg/day).

Group V. Glibenclamide-treated diabetic rats (50 mg/kg/day).

The dose of 200 mg/kg was selected based on our previous study [13] where we found that 200 mg/kg dose gave similar effects to 250 mg/kg dose.

The toxicity of the extract has been tested. There were no toxic effects observed. There are some previous studies where it has been reported that the plant extract has no toxic effect when used in animals at higher doses also 500 mg/kg dose [8].

Diabetes was induced by a single intraperitoneal injection of a freshly prepared STZ solution (Sigma, no. 242-646-8) (50 mg/kg in citrate buffer 0.01 M, pH 4.5) to overnight-fasted rats. Diabetes was confirmed by the presence of polydipsia and polyurea and by measuring the nonfasting plasma glucose levels 48 h after injection of STZ. Only animals that were confirmed to have blood glucose levels of greater than 250 mg/dL [1] were included. All of the animals were allowed free access to tap water and pellet show per the guidelines of the Institute Animal Ethics committee.

2.6. Phytochemical Analysis. Qualitative tests were conducted on the crude extract using various solvents such as hexane, ethyl acetate, methanol, and water for the different phytochemical constituents present in the plant extract based on the previous method described by Harborne [14].

2.7. Test for Glycosides (Keller-Kiliani Test). The test for glycosides was conducted based on the method published by Kokate et al. [15]. Briefly, the extracts were dissolved in glacial acetic acid, and two drops of ferric chloride solution (5%, w/v in 90% alcohol) were added. The mixture was then transferred to a test tube containing 2 mL of concentrated sulfuric acid. The presence of a reddish brown ring between the two layers confirmed the presence of glycosides.

2.8. Test for Flavonoids. The test for flavonoids (lead acetate test) was conducted based on the method published by Peach and Tracey [16]. Briefly, several drops of lead acetate solution (10%) were added to the alcoholic solution of the extract and a yellow precipitate was formed.

2.9. Test for Phenols. The presence of phenols (ferric chloride test) was confirmed using the method published by Trease and Evans [17]. Briefly, several drops of neutral ferric chloride solution (5%, w/v in 90% alcohol) were added to the extract. A blackish green color indicated the presence of a phenolic group, indicating that the extract contained phenolic substances, which are antioxidants.

2.10. Biochemical Measurements. All animals were sacrificed by cervical dislocation at the end of the experiment on day 30. The liver tissues were excised at 4°C. The tissues were washed with ice-cold saline and were immediately immersed in liquid nitrogen and stored at −80°C for further biochemical analysis. The activities of selected cytosolic enzymes were then assayed.

The activity of lactate dehydrogenase (LDH) was measured using the method adapted from Prameelamma and Swami [18] with slight modifications. The activity of glucose-6-phosphate dehydrogenase (G6PDH) was measured using the method established by Lohr and Waller [19]. The levels of mitochondrial enzymes including succinate dehydrogenase (SDH) were assayed using a modified method published by Nachlas et al. [20]. The activity of glutamate dehydrogenase GDH) was determined by the method established by Lee and Lardy [21]. All enzymatic assays in this study were performed using crude liver homogenate.

2.11. Statistical Analysis. The results were expressed as the mean ± SD ($n = 6$). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Phytochemical Analysis of Different Extracts of R. Nasutus. R. nasutus extracts produced using different solvents were screened for their phytochemical contents. Several compounds were confirmed to be present in the various types of extract (Table 1). Steroids were present in all of the extracts, whereas triterpenes and saponins were present only in the hexane and ethyl acetate extracts. Flavonoids, tannins, and carbohydrates were present in the aqueous extract. Glycosides were not detected in any of the extracts. Because the methanolic extract contained the highest concentrations of compounds, it was selected for further analysis.

3.2. The Effects of R. nasutus Extract on Cytosolic and Mitochondrial Enzymes. The oral administration of the *R. nasutus* extract to diabetic rats significantly reduced the LDH activity relative to that of diabetic control rats. This reduction was also observed for diabetic rats treated with glibenclamide, indicating that the activity of *R. nasutus* is similar to that of glibenclamide. However, no difference in LDH activity was observed in normal rats treated with *R. nasutus* (Figure 1), indicating that the effects of *R. nasutus* reverse the changes in LDH activity only in diabetic rats.

The activity of the mitochondrial marker enzyme SDH was significantly decreased in the diabetic rats. In this study, we demonstrated that the decrease in SDH activity among the diabetic rats was ameliorated by treatment with the *R. nasutus* extract. The increase in SDH activity in *R. nasutus* extract-treated diabetic rats was similar to the augmentation of the SDH activity by glibenclamide (Figure 2), indicating that *R. nasutus* has protective effects on SDH activity similar to those of glibenclamide.

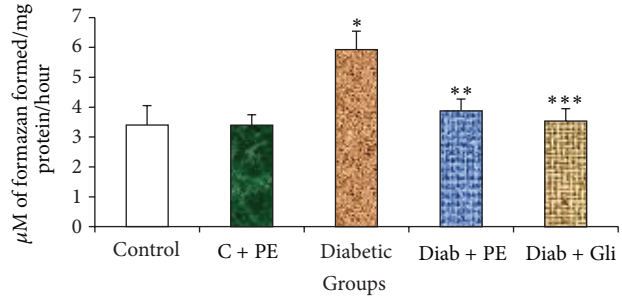

Figure 1: Changes in LDH activity in the liver tissue of experimental rats. Bars with the same superscripts do not differ significantly at $P < 0.05$. C = control, PE = plant extract, Diab = diabetic, Gli = glibenclamide.

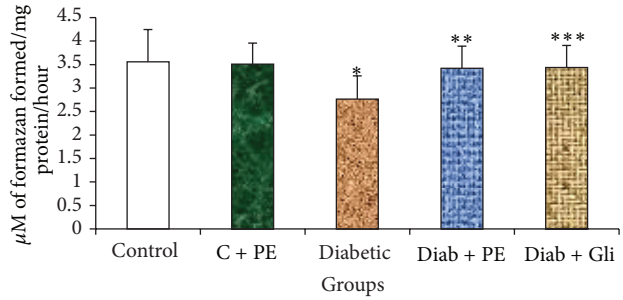

Figure 2: Changes in the SDH level in the liver tissue of experimental rats. Bars with the same superscripts do not differ significantly at $P < 0.05$. C = control, PE = plant extract, Diab = diabetic, Gli = glibenclamide.

STZ injection resulted in a significant decrease in GDH activity among animals in the diabetic group, a change that was not observed in normal control rats. Interestingly, we found a higher level of GDH activity in *R. nasutus*-treated diabetic rats compared with diabetic control rats. Treatment with the *R. nasutus* extract resulted in an improvement in the GDH activity that was equal to the improvement observed in the glibenclamide-treated diabetic rats (Figure 3), again indicating that the extract has protective effects on GDH activity similar to those of glibenclamide.

The G6PDH activity in diabetic rats was significantly decreased compared with that in the normal control rats. Conversely, diabetic rats treated with *R. nasutus* for 30 days exhibited a noticeable increase in G6PDH activity that was almost equal to that resulting from glibenclamide treatment (Figure 4), indicating that *R. nasutus* has positive effects on diabetes.

4. Discussion

Increased LDH activity in diabetic rats has been reported by various researchers [22–24]. In earlier reports, Singh et al. [25] reported that the LDH levels of diabetic rats were higher than those of the control rats and that the elevated LDH levels were associated with decreased insulin secretion. In diabetic animals, the extreme accumulation of

Table 1: Phytochemical analysis of various extracts of *R. nasutus*.

Contents	Hexane	Ethyl acetate	Methanol	Water
Steroids	+	+	+	+
Triterpenes	+	+	−	−
Saponins	+	+	−	−
Flavonoids	−	−	+	+
Phenolic compounds	−	−	+	−
Tannins	−	−	+	+
Carbohydrates	−	−	+	+
Glycosides	−	−	−	−

+: present
−: absent.

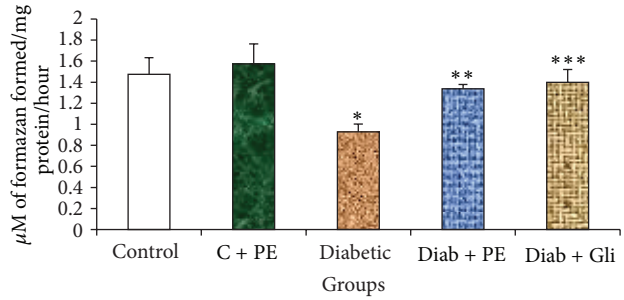

Figure 3: Changes in the GDH level in the liver tissue of experimental rats. Bars with the same superscripts do not differ significantly at $P < 0.05$. C = control, PE = plant extract, Diab = diabetic, Gli = glibenclamide.

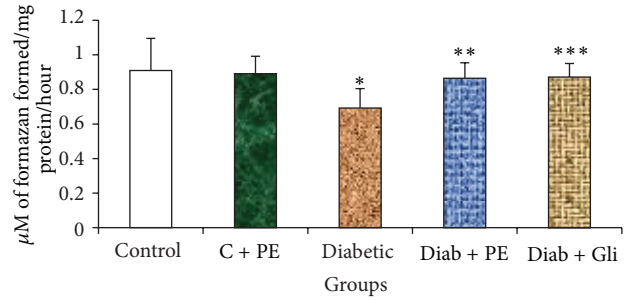

Figure 4: Changes in G6PDH in the liver tissue of experimental rats. Bars with the same superscripts do not differ significantly at $P < 0.05$. C = control, PE = plant extract, Diab = diabetic, Gli = glibenclamide.

pyruvate may lead to higher LDH activity. In the presence of LDH, excessive pyruvate is converted into lactate, leading to increased LDH activity, which could be attributed to the reduced insulin levels in diabetic individuals. Elevated LDH levels were observed in the STZ-induced diabetic rats and are associated with impaired glucose-stimulated insulin secretion [26]. Previous reports also suggest that other herbs such as *Murraya koenigii* and *Ocimum sanctum* can reduce LDH levels in diabetic rats [27]. However, the mechanism is still unclear.

The continuous administration of *R. nasutus* extract for 30 days reduced the LDH activities in diabetic rats in a manner parallel to that induced by glibenclamide. Glibenclamide is an oral antihyperglycemic drug that is used to treat diabetes due to its fast action, relatively low cost, and easy availability. Glibenclamide binds to the surface receptor present on the β cells of the pancreas thereby reduces the conductance of the ATP-sensitive potassium channels. The reduction in potassium efflux causes membrane depolarization and the influx of calcium through calcium channels, which eventually causes insulin secretion [28]. The protective role of *R. nasutus* against these effects indicates that this plant is able to prevent the harmful effects of high LDH levels observed in diabetes.

SDH is one of the most important marker enzymes for mitochondria. Its activity is generally higher than that of other enzymes in both developing and adult animals. As reported by Satav and Katyare [29], the hepatic SDH activity was significantly decreased in STZ-induced diabetic rats. Diminished SDH activity in diabetic rats affects the succinate-fumarate conversion, contributing to depressed oxidative metabolism in mitochondria. It has been suggested that the diabetogenicity of STZ is due to the inhibition of the activities of citric acid cycle enzymes, such as SDH. In the present study, the SDH activity was ameliorated by *R. nasutus* treatment in diabetic rats. The presence of antioxidants, such as phenolic compounds, may have also contributed to this effect, as various antioxidants were confirmed to be present in this plant extract in our study. Further studies to investigate this association and to determine the exact composition of this extract will be useful. It has been reported that the increased SDH activity in diabetic rats treated with plant extracts is indicative of better energy utilization due to the production of intermediates in the tricarboxylic acid cycle [25]. Thus, our findings suggest that there is increased mitochondrial oxidative potential and energy synthesis when diabetic rats are treated with the methanolic extract of *R. nasutus*.

The regulation of ammonia levels in hepatic tissue is impaired in diabetic animals and humans [30]. In this study, the activity of GDH was significantly decreased in the livers of diabetic rats. This decrease in GDH activity may have been due to the instability of energy metabolism, the impairment of glutamate transport, or the activation of lipid peroxidation in the liver. In contrast, diabetic rats treated with *R. nasutus* extract exhibited improvements in GDH activity. The increased levels may be due to the synchronization of energy metabolism and the elevation of glutamate levels in the cells. The ameliorated activities of mitochondrial enzymes and GDH by treatment with a plant extract again suggest that this plant extract has a protective role against diabetes complications because a similar improvement in GDH activity was observed with the continuous administration of a standard antidiabetic drug, glibenclamide, for 30 days. Therefore, *R. nasutus* should be investigated further as a potential antidiabetic herb.

The extramitochondrial enzyme G6PDH is highly specific for NADP as an electron acceptor. The present results showed a dramatic decrease in the levels of G6PDH in the liver tissue of STZ-induced diabetic rats. These results were similar to those of previous studies that also demonstrated

lower G6PDH activity in diabetic tissues [31–33]. It has been reported that hyperglycemia decreases the activities of hexose monophosphate shunt enzymes in diabetic animals and decreases G6PDH activity in diabetic rats. The reduced activity of G6PDH affects the NADPH concentrations in cells, thus contributing to oxidative stress, which can lead to diabetic complications [34]. In the present study, the elevated G6PDH activity observed with the continuous administration of *R. nasutus* to diabetic rats may help reduce diabetes-associated complications. The recovery of G6PDH activity in the plant-extract-treated diabetic rats may be due to the antioxidants present in the leaves [35]; these antioxidants include phenolic groups, the presence of which we confirmed through phytochemical analysis. Further studies to identify the exact composition of the extract will be useful in the future.

STZ results in the irreversible destruction of β cells and has been widely used to induce type 1 diabetes in experimental animal models. The considerable destruction of β cells after STZ injection is purported to be due to the inhibition of free radical scavenging enzymes, thus encouraging the production of various free radicals [36, 37]. This destruction of β cells accounts for the marked decrease in the amount of insulin produced by the β cells of the pancreas, which in turn affects glucose metabolism. In the present study, the observed significant increase in the blood glucose level in diabetic rats could be due to the destruction of pancreatic β cells by STZ, strengthening the hypothesis that STZ induces diabetes via the generation of free radicals. Because the liver performs most of the reactions involved in the synthesis and utilization of glucose, it is plausible that the elevation of the glucose level in STZ-treated rats can be attributed by the oxidative stress produced in the pancreas due to single-strand breaks in the DNA of the pancreatic islets [38].

Some of the active constituents of *R. nasutus* have previously been reported. In a study conducted by Sendl et al. [39], rhinacanthin C and D were found to have antiviral activity, with effects comparable to those of ganciclovir and acyclovir. In another study, the *in vitro* antiproliferative activity of rhinacanthin C was reported to be comparable with or slightly weaker than that of the anticancer agent 5-fluorouracil [40]. The researchers also confirmed that the *in vitro* antiproliferative activity of the ethanolic extract of *R. nasutus* roots was due to rhinacanthin C, whereas the activity of the aqueous extract of the leaves of *R. nasutus* was due to compounds other than rhinacanthin C that are still unknown. Both the aqueous and ethanolicextracts of *R. nasutus* showed *in vivo* antiproliferative activity after daily oral administration once for only 14 days. To our knowledge, no studies have investigated the possible constituents of *R. nasutus* that contribute to the changes in the cytosolic and mitochondrial enzymes levels and can help control diabetes.

5. Conclusion

The continuous administration of *R. nasutus* for 30 days resulted in significant improvements in cytosolic and mitochondrial enzymes levels and activities, and these effects may contribute to the antidiabetic effects of this plant.

Conflicts of Interests

The authors declare that they have no conflicts of interests concerning this paper.

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Research Article

An Aqueous Extract of Radix Astragali, *Angelica sinensis*, and *Panax notoginseng* Is Effective in Preventing Diabetic Retinopathy

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Diabetic retinopathy (DR), in which inflammation has been implicated playing important roles, is one of the most common diabetes complications. Dang Gui Bu Xue Tang (DBT), an aqueous extract of Radix Astragali and Radix *Angelica sinensis*, is a classical prescription in Traditional Chinese Medicine for treating inflammation and ischemic diseases. Here, we investigated the effects of a modified recipe of DBT, with addition of *Panax notoginseng*, in treating diabetic retinopathy. An aqueous extract of Radix Astragali, Radix *Angelica sinensis*, and *Panax notoginseng* (RRP) was given to Goto-Kakizaki (GK) rats and streptozotocin-induced Sprague-Dawley (SD) rats. Leukostasis, vascular leakage, and acellular capillaries in retinal vasculature of animals were determined. Expression of retinal inflammatory biomarkers was assessed. We found that RRP reduced leukostasis, acellular capillaries, and vascular leakage compared to diabetic control rats. We also found that RRP decreased the expression of inflammatory factors including IL-1β, IL-6, TNF-α, NF-κB, MCP-1, ICAM-1, or VCAM-1 in the retinas of GK rats and reversed high glucose-induced inhibition of endothelial cell migration and proliferation in vitro. We conclude that RRP has a potent effect in preventing the pathogenesis and/or progression of DR and thus may serve as a promising nontoxic therapeutic approach of DR.

1. Introduction

Diabetic retinopathy (DR) is a major cause of blindness among working-age adults in developed countries. Recent studies have identified inflammation as an important molecular mechanism in the development of DR [1]. During diabetes, hyperglycemia induces increased oxidative stress [2], inflammation [3], and vascular dysfunction through multiple cellular pathways and results in increased leukostasis [4, 5], vascular permeability [6], and formation of acellular capillaries [7]. Proinflammatory cytokines and chemokines activate the endothelium to increase expression of adhesion molecules and chemokines, by which leukocytes are mediated to attach to the vascular wall and transmigrate through the endothelium. Leukostasis has been found significantly increased in retinas of diabetic animals and might contribute to retinal vascular permeability and capillary nonperfusion in DR [8].

The Goto-Kakizaki (GK) rat, generated from glucose-intolerant Wistar rats, is considered to be a nonobese model of spontaneous type 2 diabetes with mild elevated glucose levels [9–11]. Regarding diabetic retinopathy, previous studies have reported that increased endothelial/pericyte ratio [12], subretinal accumulation of activated microglia/macrophages [13], increased vascular endothelial growth factor (VEGF) production in certain ocular tissue [14], and increased number of acellular capillaries [15], known as early histological changes of diabetic retinopathy, were confirmed in GK rats.

An aqueous extract of Radix *Angelica sinensis* and Radix Astragali, named Dang Gui Bu Xue Tang (DBT) and commonly used in treating ischemic ailments in traditional Chinese medicine, was modified here by addition of

Panax notoginseng (the modified DBT recipe is expressed as RRP in this paper). In Chinese pin yin, the herb *Angelica sinensis* is pronounced as Dang Gui, Radix Astragali is pronounced as Huang Qi, and *Panax notoginseng* is pronounced as San Qi. Bu Xue refers to hematopoietic effects, and Tang refers to aqueous solution. Radix Astragali extract has been shown to have potent anti-inflammatory activity [16–22]. Radix *Angelica sinensis* has been used alone, or in combination with others, in the treatment of various inflammatory diseases [23–27]. *Panax notoginseng* saponins extracted from the roots of *Panax Notoginseng* are free radical-scavenger, with an antioxidant and anti-inflammatory property [28–36].

Diabetic retinopathy signs are broadly divided into nonproliferative and proliferative retinopathy. There is no clinical symptom in nonproliferative stage, while vision impairment appears when it progresses into proliferative stage. There is currently no effective intervention in preventing DR occurrence and progression; thus the present study was undertaken to investigate the effects of RRP in preventing and/or treating diabetic retinopathy in an animal model of type 2 diabetes.

2. Materials and Methods

2.1. Standardization of RRP. RRP was provided by the Department of Pharmacy of the First Affiliated Hospital of Xiamen University, China. Identification of the major compounds in RRP was determined by high performance liquid chromatography (HPLC, Agilent 1200 HPLC system, Agilent, CA, USA). A Spherex C-18 analytical column (250 × 4.6 mm, 5.0 μm, Phenomenex, CA, USA) was used with the mobile phase which consisted of 0.2% formic acid in water (A) and methanol (B). The mobile phase gradient elution was programmed as follows: 95–80% A (0–40 minutes), 65% B (40–50 minutes), 100% B (50–55 minutes), and 95% A (55–60 minutes). The column temperature was maintained at 35°C, the flow rate was set at 1 mL/minutes, and the sample injection volume was set at 10 μL.

2.2. Animals and Treatments. Male Goto-Kakizaki (GK) rats and Wistar counterparts were purchased from Shanghai Experimental Animal center, Chinese Academy of Sciences (Shanghai, China). The rats were housed in temperature and humidity-controlled room, kept on a 12 h light/dark cycle and provided with unrestricted amount of rodent chow and water. At the age of 28 weeks, the rats were randomly allocated into four groups: normal vehicle treated control Wistar rats (Control), GK rats treated with vehicle (Diabetic, water was used as the vehicle in this study), GK rats treated with RRP at the dose of 4 g/kg body weight/d (RRP), and GK rats treated with Calcium Dobesilate at the dose of 200 mg/kg body weight/d (CD). Calcium Dobesilate, a therapeutic agent for prevention of diabetic retinopathy [37], was chosen here as a positive control. Vehicle, RRP, and CD were administered orally between 9 and 10 am once daily by gavage for 12 weeks. Body weight and blood glucose measurements were performed twice weekly. Glucose measurements were taken from the tail vein and measured using OneTouch Ultra Blood Glucose Meter (LifeScan, USA). Triglyceride (TG) and total cholesterol (TC) were assayed by enzymatic methods with automatic multichannel chemical analyzer (Hitachi 7450, Hitachi Corp., Tokyo, Japan). Type 1 diabetes was induced by a single intraperitoneal injection of streptozotocin (STZ) (60 mg/kg) in 8-week-old male Sprague-Dawley (SD) rats. Age-matched control rats received an equal volume of vehicle (0.01 M citrate buffer, pH 4.5). 48 h after STZ injection, the blood glucose level was measured from the tail vein. Rats with a blood glucose level over 20 mmol/L were considered to be diabetic. The animals were then divided into three groups: (1) normal SD rats (control, $n = 10$), (2) diabetic rats (Diabetic, $n = 10$), and (3) diabetic rats with RRP treatment (4 g/kg body weight/d) (RRP, $n = 10$). All animal experiments were approved by Xiamen University Animal Care and Use Committee.

2.3. Measurement of Retinal Endothelial Permeability. Retinal endothelial permeability was measured using the Evans blue (EB) dye injection method as previously described [38] with minor modifications. Briefly, EB (Sigma, St. Louis, MO) was dissolved in saline (30 mg/mL), filtered, and injected through the tail vein at a dosage of 45 mg/kg within 10 seconds. After the dye had circulated for 2 hours, the rats were anesthetized with pentobarbital (40 mg/kg body weight), the chest cavity was opened, and cardiac perfusion was performed via the left ventricle with 1% paraformaldehyde in citrate buffer (0.05 M, pH 3.5) under a constant pressure of 120 mm Hg. Immediately after perfusion, the retinas were carefully dissected under an operating microscope. After retinas were fully dried at 4°C then the weights of them were measured, EB dye was extracted by incubating each sample in 150 μL formamide for 18 hours at 70°C. The extract was centrifuged at 14,000 rpm for 60 minutes at 25°C. Absorbance was measured using 100 μL of the supernatant at 620 nm and 740 nm. The concentration of EB (ng/mg protein) in the extracts was calculated from a standard curve and normalized by the weight of the dry retina (mg).

2.4. Visualization and Quantification of Retinal Vascular Leakage Using FITC-Labeled BSA. Anesthetized SD rats received tail vein injections of fluorescein isothiocyanate-BSA (FITC-BSA, 100 mg/kg) (Sigma-Aldrich). After 20 min, rats were sacrificed and eyes were removed and immediately fixed in 4% paraformaldehyde for 30 min. Retinas were dissected, flatly mounted onto a glass slide, and imaged by fluorescent microscopy (IX71, Olympus, Tokyo, Japan). A computer-assisted method was used to quantify leakage using Image Pro 6.0 software. In this procedure, the intensity of fluorescence in nonleakage areas was used as background fluorescence. After deduction of background signals, the total intensity of fluorescence contributed by the leaked FITC-BSA was used to represent the leakage.

2.5. Quantification of Retinal Acellular Capillaries. To analyze the retinal vasculature for acellular capillaries, retinal vasculature was isolated using the trypsin digest method [39] with slight modifications. Briefly, 10% buffered formalin-fixed retinas were dissected after enucleation, washed in PBS

overnight, and incubated with 3% trypsin (Invitrogen-Gibco, Grand Island, NY) at 37°C for 2-3 h with gentle shaking. Retinal tissues were brushed away in water, and the isolated vasculature was mounted on glass slides and stained with periodic acid-Schiff (Sigma-Aldrich, St. Louis, MO) and hematoxylin. Acellular capillaries were counted in six field areas from each quadrant of the retina using 40x objective lenses and expressed as the total number of acellular vessels per square millimeter of retina area.

2.6. Quantification of Retinal Leukostasis. Quantification of leukostasis was performed using fluorescein-isothiocyanate-(FITC-) conjugated concanavalin A (ConA) as described previously [4]. The chest cavity of each deeply anesthetized rat was carefully opened and a perfusion needle was inserted into the left ventricle. After cutting the right atrium, the animals were immediately perfused with 500 mL of PBS per kg body weight and heparin (0.1 mg/mL) to wash out nonadherent blood cells. FITC-conjugated ConA (20 μg/mL in PBS; pH 7.4; 5 mg/kg body weight; Sigma-Aldrich, St. Louis, MO) was then perfused to label adherent leukocytes and vascular endothelial cells. Residual unbound ConA was flushed by PBS perfusion. Eyes were removed and fixed in 4% paraformaldehyde for 1 h. The retinas were carefully dissected and flat mounted. Images of retinas were observed by confocal microscopy (Leica TCS SP5, Leica Microsystems, Wetzlar, Germany), and the total number of adherent leukocytes within the vessels of each retina was counted.

2.7. ELISA Assay. Activation of the transcription factor NF-κB was determined in the retina by ELISA kit from Active Motif (Carlsbad, CA) based on the principle that only the active form of NF-κB in the sample binds to oligonucleotide containing the NF-κB consensus site (5′-GGG ACT TTC C-3′) which is immobilized on the microtiter plate. The primary antibody against the p65 subunit of NF-κB was used in the assay and the secondary antibody used is conjugated to horseradish peroxidase. Retina was homogenized in the lysis buffer (as provided by the manufacturer), and after removing the cell debris, 10 μg protein was used for the ELISA [40]. Retinal VEGF levels were quantified by ELISA using a kit from R&D Systems, MN. The samples were incubated for 2 hours and washed out, and then the plate was incubated with rat VEGF conjugate. This assay was sensitive to the concentration of VEGF as low as 15 ng/mL.

2.8. Western Blot Analysis. Retinas were dissected and sonicated in a lysis buffer containing 50 mmol/L Tris (pH 7.6), 150 mmol/L NaCl, 5 mmol/L EDTA, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate, and a protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN). The lysate was centrifuged at 14,000 rpm for 10 min at 4°C. Supernatants were collected and determined the protein concentrations using the Bradford method. Retinal proteins were then separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membrane. After blocking, membranes were blotted overnight at 4°C with anti-VEGF (1 : 500), antioccludin (1 : 500) (Santa Cruz, CA), and anti-β-actin (1 : 1000) antibodies (Sigma-Aldrich). Horseradish peroxidase-linked anti-rabbit or mouse (1 : 10,000) (Santa Cruz, CA) was used for secondary detection. Immunoreactivity was visualized using an ECL kit (Amersham Pharmacia Biotech, Piscataway, NJ) and Kodak X-OMAT film (Eastman Kodak Co., Rochester, NY). Band intensities were quantified using a gel documentation system and Quantity One software (Bio-Rad).

2.9. Cell Culture and Treatment. EA.hy926, an endothelial cell line, were starved in DMEM containing 0.5% FBS for 24 h and then cultured in DMEM culture medium with either 5.5 (N), 25 mM d-glucose (H), or 25 mM d-glucose with 1 h RRP before treatment for 24 h. This medium was supplemented with 10% FBS, 1% l-glutamine, 2% HAT, and 1% penicillin/streptomycin in a humidified atmosphere of 5% CO_2 at 37°C.

2.10. Cell Viability and Migration Assay. CCK8 assay was used to measure cell viability. In brief, EA.hy926 cells were seeded into 96-well plates at a density of 1000 cells/well in 100 μL DMEM culture medium (5.5 mM d-glucose) and allowed to adhere overnight and then cultured in culture medium with either 5.5 mM (N), 25 mM (H), or 25 mM d-glucose pretreated with RRP for 24 hours. After that, 10 μL of CCK8 was added to each well for another 2 h at 37°C and the absorbance measured at 450 nm.

The role of high glucose and/or RRP on the migration of EA.hy926 cells was analyzed by cell scratch assay. The cells were plated at 70,000 cells/well in a 12-well plate with DMEM (5.5 mM d-glucose) till cells become 80% confluent. The cells was scratched once using a sterile 1 mL pipette tip and washed twice with complete medium. DMEM with either 5.5 mM (N), 25 mM (H), or 25 mM d-glucose pretreated with RRP was then added in complete medium, and the incubation continued at 37°C. An image was made at 0 and 24 h after the addition of high glucose and/or RRP using a microscopy (IX71, Olympus, Tokyo, Japan). The width of the gap after 24 h was measured and the extent of migration was calculated.

2.11. Real-Time Polymerase Chain Reaction (PCR). Total RNA was extracted using RNA simple Total RNA Kit (Tiangen Biotech, China). Reverse transcription of total RNA to cDNA was carried out with primeScript RT reagent kit with gDNA eraser (TaKaRa, China) in a MyCycler Thermal Cycler (Bio-Rad, USA) following the manufacturer's instruction. Real-time quantitative PCR was performed with the SYBR Premix Ex Taq real-time PCR kit (Takara, Dalian, China) in a Light Cycler 480 System (Roche). The following primers were used in this study: Tumor necrosis factor-α (TNF-α) sense, 5′-ACA CCA TGA GCA CGG AAA GC-3′; antisense, 5′-CCG CCA CGA GCA GGA A-3′; Interleukin-1β (IL-1β) sense, 5′-AAT GGA CAG AAC ATA AGC CAA CA-3′; antisense, 5′-CCC AAG GCC ACA GGG AT-3′; Interleukin-6 (IL-6) sense, 5′-GTT GCC TTC TTG GGA CTG ATG-3′; antisense, 5′-ATA CTG GTC TGT TGT GGG TGG T-3′; nuclear transcription

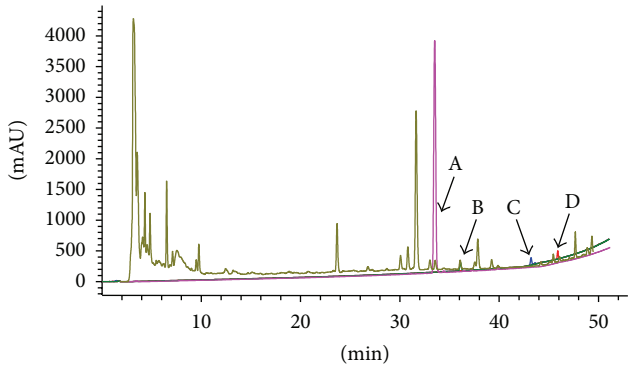

Figure 1: Chemical standardization of RRP by HPLC fingerprint analysis. In the HPLC fingerprint at an absorbance of 203 nm, the peaks corresponding to calycosin (A), ginsenoside Rg1 (B), ligustilide (C), and ginsenoside Rb1 (D) were identified.

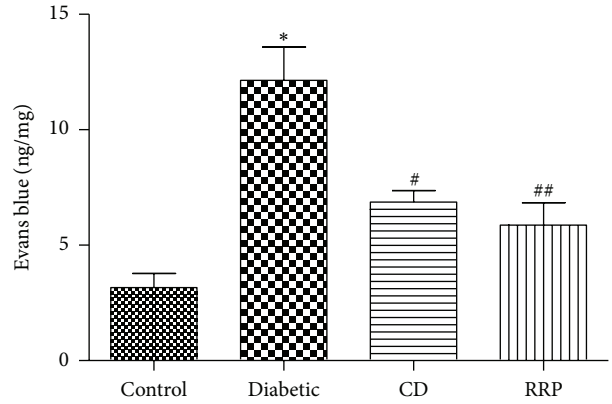

Figure 2: Vascular permeability in the retinas from normal Wistar rats (Control), GK rats treated with vehicle (Diabetic), GK rats treated with CD (CD), and GK rats treated with RRP (RRP) was measured with Evans blue as a tracer. Evans blue was normalized by total protein concentration in the tissue. Permeability is expressed as ng of dye per mg of protein in the tissue. All data were expressed as mean ± SD ($n = 6$). $^{*}P < 0.05$ versus Control group; $^{\#}P < 0.05$ versus Diabetic group; $^{\#\#}P < 0.01$ versus Diabetic group.

factor-κB (NF-κB)/p65 sense, 5′-AGA AGC GAG ACC TGG AGC AA-3′; antisense, 5′-CGG ACC GCA TTC AAG TCA TAG-3′; VEGF sense, 5′-ACA GGG AAG ACA ATG GGA TGA-3′; antisense, 5′-GGG CCA GGG ATG GGT TT-3′; monocyte chemotactic proteins-1 (MCP-1) sense, 5′-AAT GGG TCC AGA AGT ACA TTA GAA A-3′; antisense, 5′-GGT GCT GAA GTC CTT AGG GTT G-3′; intercellular adhesion molecule-1 (ICAM-1) sense, 5′-CGG GTT TGG GCT TCT CC-3′; antisense, 5′-GCC ACT GCT CGT CCA CAT AG-3′; vascular cell adhesion molecule-1 (VCAM-1) sense, 5′-ATC TTC GGA GCC TCA ACG G-3′; antisense, 5′-CCA ATC TGA GCG AGC GTT T-3′; β-actin sense, 5′-CCC ATC TAT GAG GGT TAC GC-3′; antisense, 5′-TTT AAT GTC ACG CAC GAT TTC-3′. To determine the relative expression levels, the threshold cycle (Ct) values of target genes were normalized with β-actin of the same sample and expressed as relative to controls.

2.12. Statistical Analysis. All results were presented as the mean ± SD. Statistical evaluation of the results was performed using a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test using GraphPad Prism 5.0 software (GraphPad, CA, USA). Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. HPLC Analysis of RRP. In order to standardize the herbal extract chemically, we performed HPLC analysis. Figure 1 shows a typical HPLC fingerprint of RRP, in which major peaks were identified by comparing both the retention times of RRP and reference standards; 4 compounds (A: calycosin; B: ginsenoside Rg1; C: ligustilide; D: ginsenoside Rb1) in RRP were well identified.

3.2. RRP Has No Effect on Body Weight, Blood Glucose, and TG and TC Levels. After 12-week treatment, body weight and TG showed no difference among groups. As shown in Table 1, all GK rats developed hyperglycemia and hypercholesterolemia (TC) compared to the normal control Wistar rats ($P < 0.05$), but no significant differences were found in all four GK rats groups, suggesting that neither CD nor RRP has any effect in restoring the disordered glucose and lipid metabolism in GK rats.

3.3. RRP Attenuated Retinal Vascular Permeability. Blood-retinal barrier (BRB) breakdown is a hallmark of diabetic retinopathy, evidenced by increased blood vessel permeability. The retinal blood vessel permeability in Diabetic group significantly increased (12.1 ± 4.5 ng/mg) compared with that in control group (3.2 ± 1.4 ng/mg) ($P < 0.001$) showing an impaired BRB in diabetes. CD treatment significantly decreased retinal vascular permeability (6.9 ± 1.0 ng/mg, $P < 0.05$), whereas RRP reversed the retinal vascular permeability to a further extent (5.9 ± 2.4 ng/mg, $P < 0.01$) (Figure 2).

To further investigate the breakdown of the blood-retinal barrier, we measured vascular leakage in a group of STZ-induced diabetic rats using FITC-labeled albumin. Vascular leakage was visualized and quantified in diabetic SD rats 3 weeks after inducing diabetes (Figures 3(a)–3(d)). Similar to GK rats, compared with the control SD rats, diabetic SD rats had more leaked FITC-labeled albumin (Figure 3(b)) in their retina, and RRP treatment significantly decreased the retinal vascular leakage (Figure 3(c)). Computer-assisted quantitative analysis demonstrated a similar level of FITC-labeled albumin leakage in RRP treated diabetic rats and control SD rats controls (Figure 3(d)), whereas the FITC-labeled albumin leakage in diabetic rats was significantly higher.

To determine the mechanism of RRP effect in diabetes-induced vascular leakage, we examined the protein expression of VEGF and occludin (important tight junction protein). Western blot analysis of retinas from control SD rats showed significantly increased VEGF level and decreased

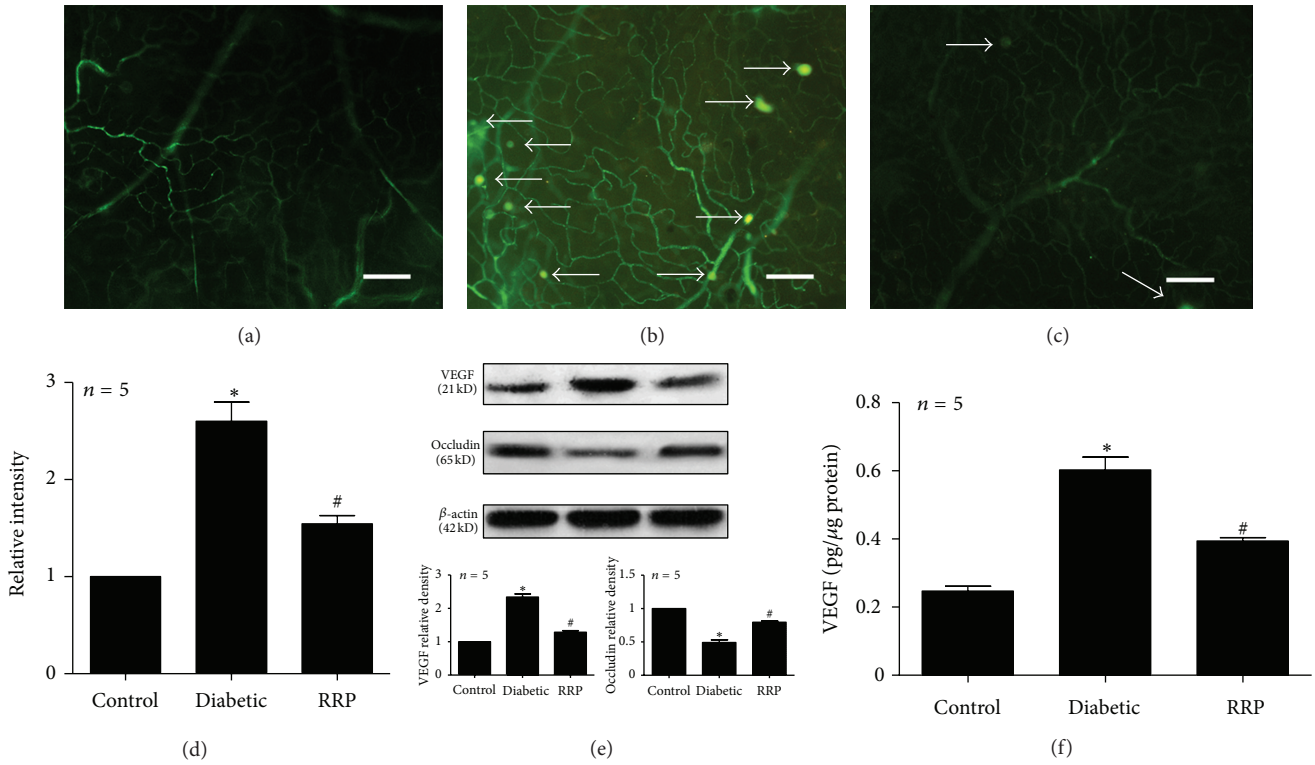

Figure 3: (a–d) Analysis of retinal vascular leakage in the retinas from (a) normal control rats (Control), (b) STZ-induced diabetic rats (Diabetic), and (c) STZ-induced diabetic rats treated with RRP (RRP) 3 weeks after the onset of diabetes using FITC-labeled albumin. Microscopic images of retinal flat mounts (a–c) showing fluorescently labeled albumin (arrowheads) in retinas of SD rats. (d) Quantification of the FITC-labeled albumin from (a), (b), and (c). Scale bars represent 100 μm. (e) Western blot analysis of retinal VEGF and occludin expression in Control, Diabetic, and RRP groups. (f) VEGF concentrations were measured in the retina of rats in Control, Diabetic, and RRP groups using an ELISA kit from R&D Systems. All data were expressed as mean ± SD ($n = 5$). $^*P < 0.05$ versus normal SD rats; $^\#P < 0.05$ versus Diabetic SD rats.

Table 1: Metabolic and physical parameters.

Group	Body weight (g)	Blood glucose (mmol/L)	Cholesterol (mmol/L)	Triglyceride (mmol/L)
Control	409.0 ± 31.2	6.272 ± 0.856	1.486 ± 0.143	0.953 ± 0.242
Diabetic	411.0 ± 47.3	19.718 ± 3.897*	2.527 ± 0.214*	1.003 ± 0.445
CD	409.8 ± 32.1	21.798 ± 2.976*	2.693 ± 0.260*	0.910 ± 0.454
RRP	411.2 ± 33.0	18.510 ± 7.319*	2.422 ± 0.108*	0.982 ± 0.353

All data were expressed as mean ± SD. $^*P < 0.05$ versus Control group.

occludin level in diabetic SD rats' retinas, and RRP treatment greatly reversed both these changes (Figure 3(e)). Retinal VEGF protein level was confirmed by ELISA assay (Figure 3(f)) which also showed that the level of retinal VEGF protein increased in diabetic rats group compared to control, and was reduced by RRP treatment.

Given the fact that there was no change of blood glucose levels, the restored retinal vascular permeability by RRP should be attributed to the improved local microenvironment.

3.4. RRP Treatment Prevented Retinal Capillary Vasoregression. To further assess the effect of RRP on retinal vascular lesions in diabetic GK rats, we quantified the number of acellular capillaries, a sign of retinal capillary vasoregression, by trypsin digestion assay. The number of retinal acellular capillaries of diabetic rats was significantly increased about 6-fold compared to the control rats (Figures 4(a) and 4(b)) ($P < 0.001$), suggesting a damaged and degenerated microcirculation surroundings. RRP treatment largely reduced this vascular lesion by 52.6% ($P < 0.001$) (Figures 4(c) and 4(d)).

3.5. RRP Decreased Retinal Leukostasis. It has been shown that diabetic retinal vascular leakage and nonperfusion are temporally and spatially associated with retinal leukocyte stasis (leukostasis) in the rat model of streptozotocin-induced diabetes [41]. Leukostasis, a major parameter for inflammation and early pathologic changes in DR, was labeled with FITC-conjugated ConA. The number of adherent leukocytes in the retinal microvasculature of Control Wistar rats was

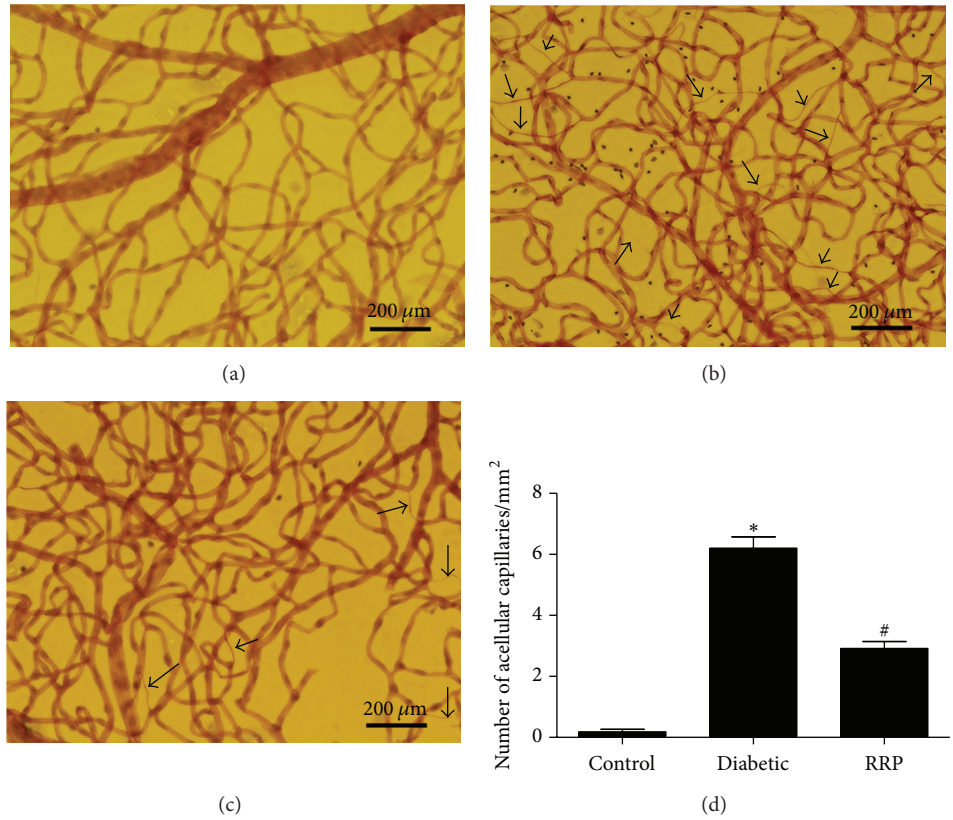

Figure 4: Analysis of acellular capillaries (arrowheads) in retinas from (a) normal Wistar rats (Control), (b) GK rats treated with vehicle (Diabetic), and (c) GK rats treated with RRP (RRP). (d) Quantification of the number of acellular capillaries from (a), (b), and (c). Scale bar represents 200 μm. All data were expressed as mean ± SD ($n = 3$). $^{*}P < 0.001$ versus Control rats; $^{\#}P < 0.001$ versus Diabetic rats.

negligible (Figure 5(a)), while diabetes caused a significant increase of leukocytes adhesion to the endothelia cells (Figure 4(b)) ($P < 0.001$). Twelve-week administration of RRP significantly decreased retinal leukostasis by 44.8% compared to Diabetic group ($P < 0.001$) (Figures 5(c) and 5(d)).

3.6. RRP Decreased the Gene Expression of Inflammatory Cytokines In Vivo and In Vitro. To verify the effect of RRP on diabetic retinal inflammation, we examined the gene expression of proinflammatory markers, adhesion molecules, and chemokines. All inflammatory mediators were expressed at very low levels in the retina of control rats (Figure 6). In Diabetic group, retinal mRNA levels of IL-1β, IL-6, TNF-α, NF-κB p65, VEGF, MCP-1, ICAM-1, and VCAM-1 were significantly increased (1.5- to 35-fold) compared to Control rats ($P < 0.05$) (Figures 6(a) and 6(b)). These increases were restored to control values in rats treated with RRP. The activation of NF-κB in Diabetic group was significantly increased compared to Control group ($P < 0.001$) (Figure 6(c)) and decreased significantly by RRP treatment ($P < 0.01$). RRP inhibition of inflammatory cytokines was reproduced in vitro in cultured endothelial cell line—EA.hy926 cells. The gene expression of inflammatory cytokines and adhesion molecules increased dramatically after the cells were incubated in high glucose (25 mm/L) medium for 24 h, while RRP treatment significantly blocked this deleterious effect (Figure 6(d)).

3.7. RRP Reverses High Glucose-Induced Inhibition of Endothelial Cell Migration and Proliferation. In wound-healing assays in vitro, high glucose markedly attenuated EA.hy926 cells migration, and this effect was reversed by RRP addition (Figures 7(a)–7(c)). High glucose decreased the viability of EA.hy926 cells as measured by CCK8 assay and this effect was reversed by RRP (Figure 7(d)).

4. Discussion

Dang Gui Bu Xue Tang, an aqueous extracts of Radix Astragali and Radix *Angelica sinensis*, a commonly used prescription in treating ischemic ailments in traditional Chinese medicine (TCM), was modified here by addition of *Panax notoginseng* (RRP) and was investigated in treating diabetic retinopathy. All these herbal medicines have anti-inflammatory properties and are frequently used for the treatment of diabetes and diabetic complications in TCM [26, 42]. We showed here that RRP treatment prevented leukocyte adherent to the vascular wall, attenuated vascular leakage, inhibited formation of acellular capillaries, the three early signature pathologies of diabetic retinopathy, thus ameliorated the retina damage and prevented DR

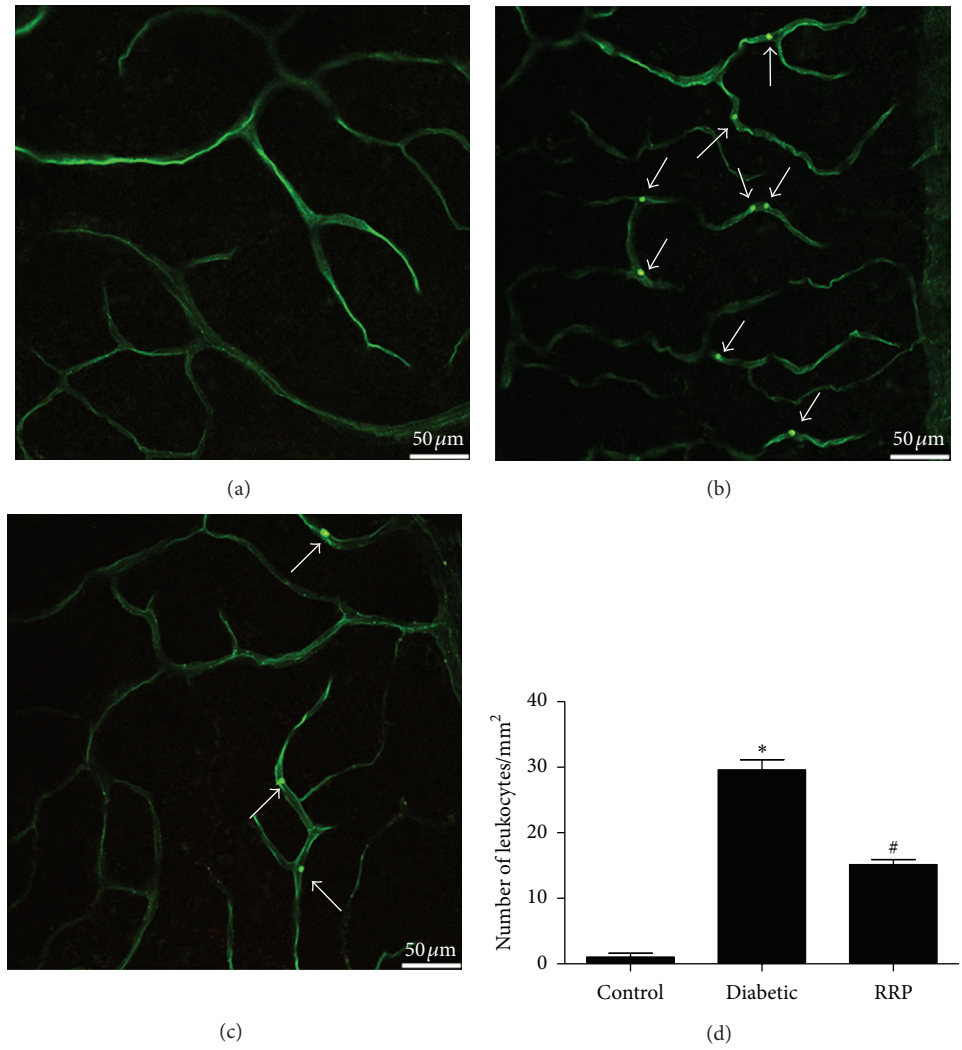

Figure 5: Analysis of leukocyte adhesion (arrowheads) in retinas from (a) normal Wistar rats (Control), (b) GK rats treated with vehicle (Diabetic), and (c) GK rats treated with RRP (RRP). (d) Quantification of the number of leukocytes from (a), (b), and (c). Scale bar represents 50 μm. All data were expressed as mean ± SD ($n = 3$). $^{*}P < 0.001$ versus Control group; $^{\#}P < 0.001$ versus Diabetic group.

progression in rat model of diabetes. RRP protection from diabetic retinopathy may be via inhibition of inflammatory response and improvement of microcirculation in the retina.

DR is a low-grade chronic inflammatory condition [43]. Previous studies have shown that anti-inflammatory drugs can prevent early DR [44, 45]. Radix Astragali, Radix *Angelica sinensis*, and *Panax notoginseng* have been shown inhibit proinflammatory factors or chemokines expression such as IL-1β, IL-6, TNF-α, VEGF, MCP-1, ICAM-1 or VCAM-1 [18, 23–25, 35, 36]. Here we confirmed that RRP suppressed the gene expression of a series of proinflammatory cytokines which may consequently result in decreased inflammation in retina of diabetic rats (Figure 6). Proinflammatory cytokines (such as TNF-α and interleukins) and chemokines (such as MCP-1) activate endothelium to increase expression of adhesion molecules (such as ICAM-1, VCAM-1) and chemokines, by which leukocytes/monocytes were mediated to attach to the vessel wall and transmigrate through the endothelium [1]. ICAM-1 blockade prevents diabetic retinal leukostasis and vascular leakage [41]. We had speculated that RRP may protect retina from leukostasis via blockade of the inflammation and subsequent activation of leukocyte/monocyte adhesion process. After 12-week administration, we observed that RRP suppressed inflammatory gene expression and NF-κB activation and efficaciously reversed diabetes-induced elevation of ICAM-1 and VCAM-1 levels. Increased leukostasis is an early event in DR [5] and is proposed lead to BRB breakdown, endothelial cell lesion, and capillary loss [46]. In this study, we observed that the number of leukocytes/monocytes which adhered to the retinal vascular endothelium was increased in diabetic GK rats; accordingly, vascular permeability and the number of acellular capillaries increased dramatically in diabetic GK rats. RRP treatment inhibited the leukostasis in retinal vasculature (Figure 5), which may be benefited from the suppressed expression of ICAM-1 and VCAM-1

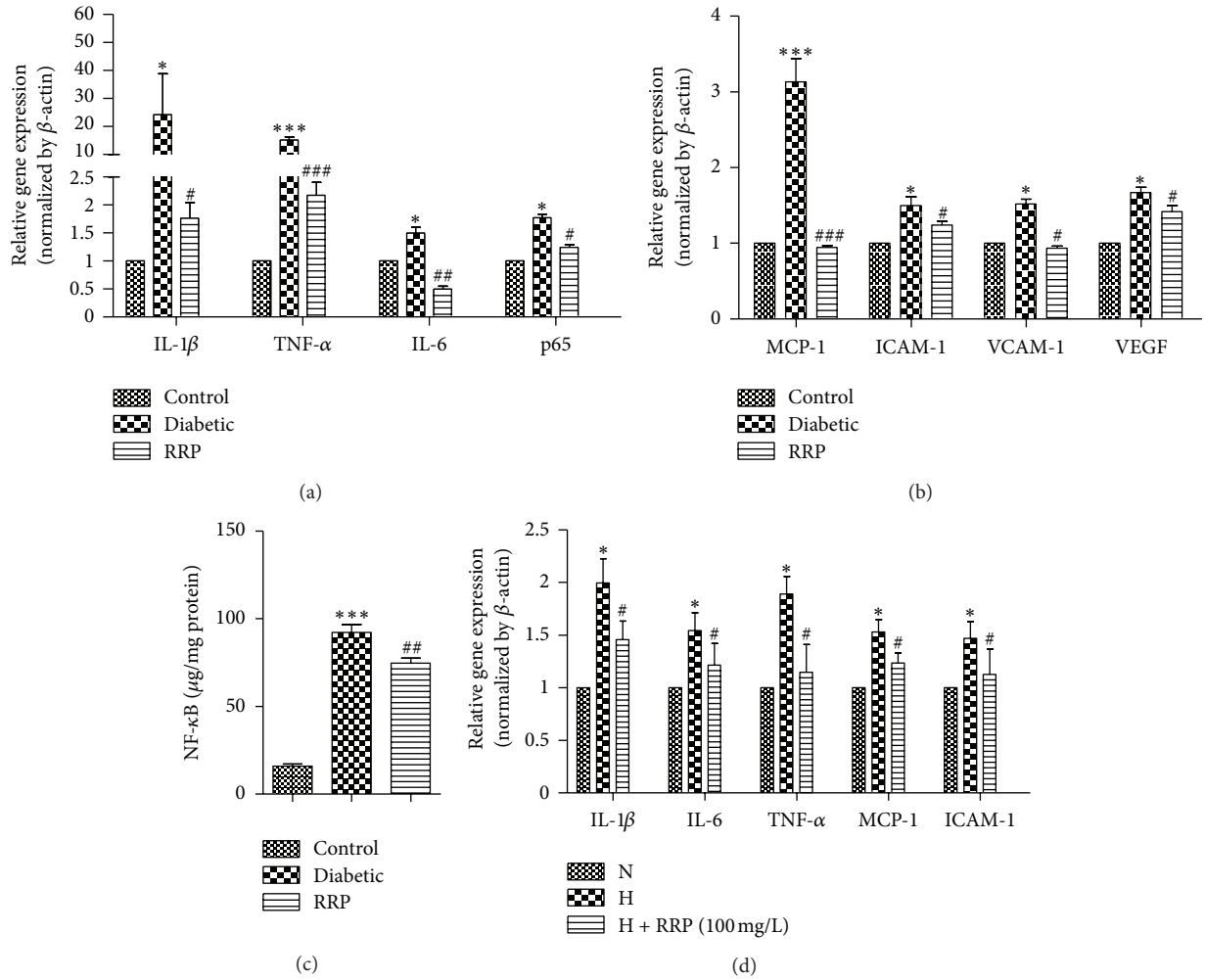

Figure 6: (a, b) Real-time PCR analysis of gene expression of pro-inflammatory markers and (c) ELISA assay detection of NF-κB activation in the retinas from normal Wistar rat (Control), GK rats treated with vehicle (Diabetic), and GK rat treated with RRP (RRP) ($n = 3$). $^{*}P < 0.05$, and $^{***}P < 0.001$ versus Control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus Diabetic group. (d) Real-time PCR analysis of gene expression of pro-inflammatory markers in EA.hy926 cells cultured in DMEM medium with either 5.5 (N), 25 mM glucose (H), or 25 mM glucose before treatment with RRP (dissolved in dimethyl sulfoxide) 1 h ago for 24 h after starving in DMEM containing 5.5 mM glucose for 24 h. $^{*}P < 0.05$ versus N group; $^{\#}P < 0.05$ versus H group ($n = 5$). All data were expressed as mean ± SD.

molecules. Alteration of the inner BRB is the hallmark of DR and breakdown of the BRB appears early in the progression of retinopathy in GK rats [47], as well as in diabetic patients [48, 49]. In consistent with the attenuated leukostasis, we found that, compared with untreated Diabetic group, 12 weeks application of RRP significantly reduced the retinal vascular permeability of diabetic GK rats (Figure 2) and inhibited the formation of acellular capillaries (Figure 4). Furthermore, 3-week application of RRP greatly reduced the retinal vascular leakage in STZ-induced diabetic rats (Figure 3), suggesting an overall protection of RRP in preventing retina damage in diabetes. We did not see obvious effect of RRP on body weight, blood glucose, and TG or TC levels (Table 1) while the improvement of retinopathy was clear, implying that the hyperglycemia-induced local environment change especially inflammation plays more important roles in the pathogenesis of retinopathy than the systemic hyperglycemia. It has been shown retinal inflammatory mediators are principal in the resistance of retinopathy to arrest after cessation of hyperglycemia, and proinflammatory cytokines and adhesion molecules remained high after good glycemic control for at least six months that has followed six months of poor glycemic control [50]. Reversal of hyperglycemia fails to provide any significant effect on the development of histopathology that is characteristic of DR [51]. Various studies have demonstrated that inhibition of pro-inflammatory cytokines (such as IL-1β, TNF-α, and VEGF), chemokines (such as MCP-1), or adhesion molecules (such as ICAM-1) can reduce diabetes-induced leukostasis [3, 41, 44], degeneration of retinal capillaries [52], or BRB breakdown [53]. Thus, our results suggest that the antiretinopathy effect of RRP may be unrelated to lowering blood glucose, but it is due to

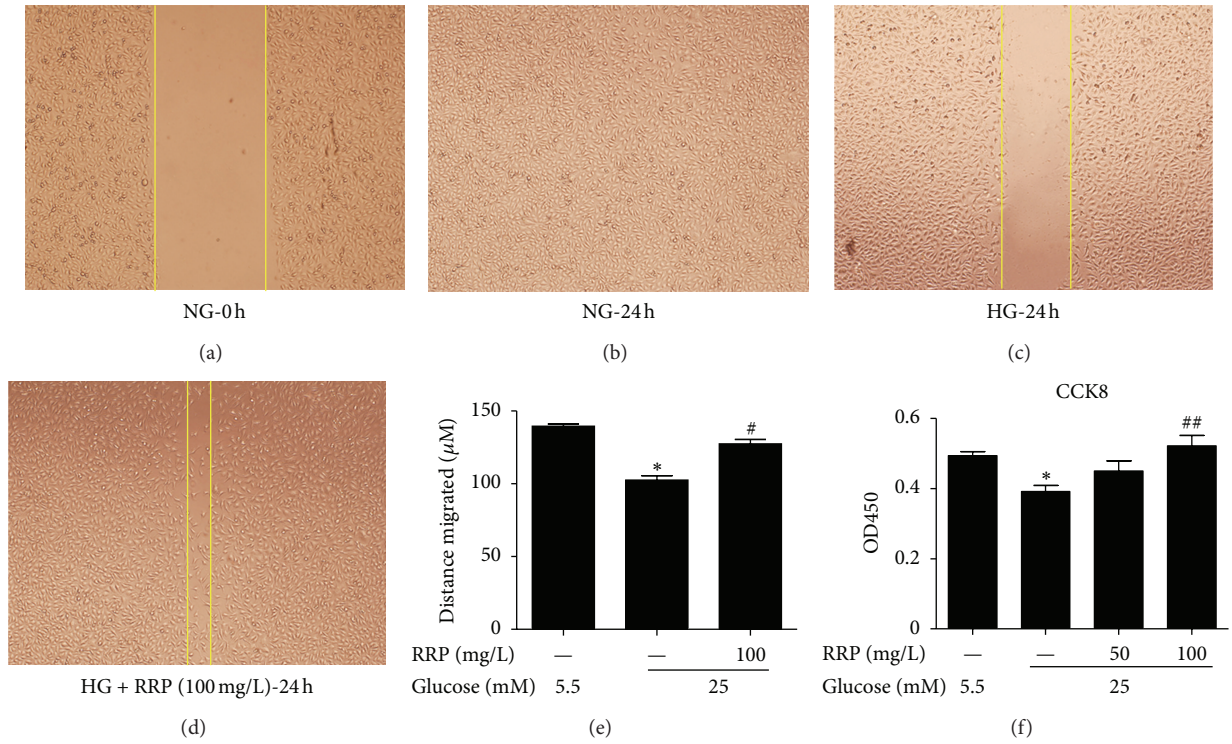

Figure 7: EA.hy926 cells were incubated with 5.5 mM D-glucose (NG), 25 mM D-glucose (HG), and HG treated with RRP for 24 h. (a–e) RRP reversed high glucose induced suppression of EA.hy926 cells migration. The width of the gap after 24 h was measured and subtracted from that at 0 h to quantify the distance the cells migrated. Yellow lines indicate distance between EA.hy926 cells on both sides of wound. (f) RRP reversed high glucose induced suppression of EA.hy926 cells proliferation. Measurements were made by CCK8 assay. All data were expressed as mean ± SD ($n = 5$). $^{*}P < 0.05$ versus normal glucose group; $^{\#}P < 0.05$ versus high glucose group.

the inhibition of inflammation in the retinas of GK rats. In addition to the in vivo study in diabetic GK rats, we also confirmed the RRP inhibition effect of inflammatory cytokines in cultured endothelial cells.

Endothelial cell loss via apoptosis was the early pathological change in DR; we showed here that RRP reversed the inhibited cell migration and decreased cell death induced by high glucose (Figure 7) in cultured EA.hy926 cells, suggesting a protective effect of RRP in preventing early endothelial cell death [43].

In summary, our results demonstrated that RRP had an inhibitive effect in the development and/or progression of DR in GK rats. RRP prevention of retinopathy may be associated with the inhibition of leukostasis and inflammation. Diabetic patients usually go to see the ophthalmologist only after the visual complications have already occurred, when there is no much ideal intervention available to reverse or eliminate the damage. With strong effects in ameliorating feature characteristics of DR-increased permeability, leukostasis, and acellular capillaries, RRP may serve as a prevention and/or intervention strategies in managing DR.

Conflict of Interests

There are no potential conflict of interests and financial activities related to the present paper to disclose.

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Review Article

Herbal Therapies for Type 2 Diabetes Mellitus: Chemistry, Biology, and Potential Application of Selected Plants and Compounds

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Diabetes mellitus has been recognized since antiquity. It currently affects as many as 285 million people worldwide and results in heavy personal and national economic burdens. Considerable progress has been made in orthodox antidiabetic drugs. However, new remedies are still in great demand because of the limited efficacy and undesirable side effects of current orthodox drugs. Nature is an extraordinary source of antidiabetic medicines. To date, more than 1200 flowering plants have been claimed to have antidiabetic properties. Among them, one-third have been scientifically studied and documented in around 460 publications. In this review, we select and discuss blood glucose-lowering medicinal herbs that have the ability to modulate one or more of the pathways that regulate insulin resistance, β-cell function, GLP-1 homeostasis, and glucose (re)absorption. Emphasis is placed on phytochemistry, anti-diabetic bioactivities, and likely mechanism(s). Recent progress in the understanding of the biological actions, mechanisms, and therapeutic potential of compounds and extracts of plant origin in type 2 diabetes is summarized. This review provides a source of up-to-date information for further basic and clinical research into herbal therapy for type 2 diabetes. Emerging views on therapeutic strategies for type 2 diabetes are also discussed.

1. Impact and Pathogenesis of Type 2 Diabetes

Diabetes mellitus is a chronic metabolic disease with life-threatening complications. The International Diabetes Federation (IDF) estimates that 285 million people, 6.4% of the world population, suffered from diabetes in 2010 and this prevalence will increase to 439 million people, 7.7% of the world population by 2030 [1]. Over 90% percent of diabetic patients are diagnosed with type 2 diabetes (T2D) [2, 3]. The cost of health care associated with diabetes continues to grow and is a huge economic burden for afflicted patients and countries. In 2007, approximately 17.5 million adults were

reported to be receiving treatment for diabetes in the USA, where the estimated cost of diabetes was US 174 billion dollars [4].

Despite considerable progress in scientific studies on T2D and research and development of antidiabetic agents, the cause of T2D is not yet fully understood. Mounting evidence from epidemiological studies suggests that genetic and environmental factors are primary causes of T2D. Both factors contribute to insulin resistance and loss of β-cell function that result in impairment in insulin action, insulin production, or both. This impairment results in the development of hyperglycemia, a major pathological feature of T2D [5]. Such hyperglycemia is detrimental to β cells and peripheral tissues, a condition termed glucotoxicity, which is clinically relevant as a cause of diabetes-related complications such as cardiovascular disease, nephropathy, retinal blindness, neuropathy, and peripheral gangrene [6]. Therefore, maintenance of glycemic homeostasis is the most common therapeutic aim for patients with T2D. Moreover, aberrant lipid metabolism in adipose and other tissues can cause lipotoxicity, which can further worsen diabetic complications. The β cells in the pancreas are the key players in glycemic homeostasis. Glucotoxicity, lipotoxicity, endoplasmic reticulum (ER)/oxidative stress, inflammatory mediators, and incretin were reported to modulate β-cell function and survival [7]. The relationship between the causes and pathogenesis of T2D is illustrated in Figure 1(a).

Insulin is a protein hormone that regulates the metabolism of glucose, fat, and protein in the body [8]. Any defect in insulin production and action leads to serious metabolic problems. Pancreatic β cells are the only endocrine cells known to produce insulin. In the development of T2D, β-cell function in the presence of increasing hyperglycemia and insulin resistance declines. This decline may begin early in the disease and accelerates after compensatory overwork that drives the progression of T2D. Targeting β-cell failure early in disease progression has evolved as a new approach to treat T2D [9]. Currently, no antidiabetic drugs have been proven clinically effective for the prevention of β-cell atrophy although thiazolidinediones (TZDs) and glucagon-like peptide-1 (GLP-1) analogues have reported to be effective in animals [10–12]. Therefore, maintenance and enhancement of β-cell function have the potential to stabilize, delay, and even reverse T2D [7]. Specific growth factors, cell cycle mediators, and nuclear factors have been proposed to regulate β-cell homeostasis [13, 14]. New therapeutic classes of diabetes medications that act to regulate β-cell function could be clinically potent in reversing the disease.

Insulin resistance is a condition whereby the body's cells become resistant to the action of insulin. Insulin resistance usually emerges many years before the onset of T2D as a result of interplay between genetic and environmental factors [15]. Metabolic hormones (e.g., leptin, adiponectin, and glucagon), nutrient excess, systemic free fatty acids, ER stress/oxidative stress, adipose hypoxia, adipose inflammation, and so on account for the generation of insulin resistance [16]. At the molecular level, fatty acid derivatives, inflammatory mediators, and free radicals trigger a negative regulation of IRS-1, PI3K, Akt, GSK-3β, JNK, and other mediators downstream of insulin receptors.

Loss of glycemic control in T2D patients is an undesirable and detrimental consequence. Therefore, normalization of blood glucose by reducing glucose absorption from the gut and kidney is effective to control glycemic homeostasis in diabetic patients. Reduction of dietary saccharides or inhibition of glucose from oligosaccharide degradation by α-glucosidases in intestines helps to diminish blood glucose level. Contrarily, the renal tubule can recover 90% of urine glucose, contributing to glucose homeostasis in the body. Since sodium glucose cotransporter 2 (Sglt 2) is primarily expressed in kidney tubules, an Sglt 2 inhibitor was developed as antihyperglycemic agent. α-Glucosidase inhibitors and Sglt 2 inhibitors have been demonstrated to be clinically effective against hyperglycemia. Nonetheless, Sglt 2 inhibitor was not approved by the US Food and Drug Administration (FDA) because of safety concerns [17].

Incretin consists of glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), which are generated by enteroendocrine K-cells and L-cells, respectively [18, 19]. GLP-1 plays a dominant role in modulating β-cell function (insulin production and β-cell proliferation/protection), reducing glucagon secretion, attenuating gastric emptying, and decreasing appetite/weight gain [20]. Accordingly, the action of incretin is impaired in T2D [21–23]. GLP-1 has a short half life (<2 min) due to its fast cleavage by dipeptidyl peptidase-4 (DPP-4) [24, 25]. Therefore, GLP-1 mimetics and DPP-4 inhibitors have been approved by the FDA as new classes of antidiabetic drugs [26, 27].

More information on the molecular mechanisms underlying the pathogenesis of T2D is required for further clinical success.

2. Pharmacotherapies for T2D and the Related Challenges

T2D arises from a defect in insulin secretion, insulin action, and/or both. Hence, T2D therapy has evolved from monotherapy using insulin, insulin secretagogues, or sensitizers alone to combination therapy using insulin/insulin secretagogues plus sensitizers and/or incretin-based drugs. The mechanisms of action implicated in these remedies include insulin production, sensitization of the insulin receptor pathway, and/or GLP-1 secretion. The more pathways the drugs target, the better the clinical outcome and therapy seem to be. More recently, manipulation of β cells *per se* or in combination with other antidiabetic therapeutics has emerged as a new strategy to ameliorate and, particularly, cure T2D [7].

By far the most popular approach to treating T2D is glycemic control in an attempt to reduce complications and death. When pharmacological methods are used to interfere with these mechanisms, the percentage of glycosylated hemoglobulin A_{1c} (HbA_{1c}), an indicator of long-term glycemic control, in medicated T2D patients is suggested to be below 7%. Since hyperglycemia is implicated in diabetic complications and death in patients, better regulation of

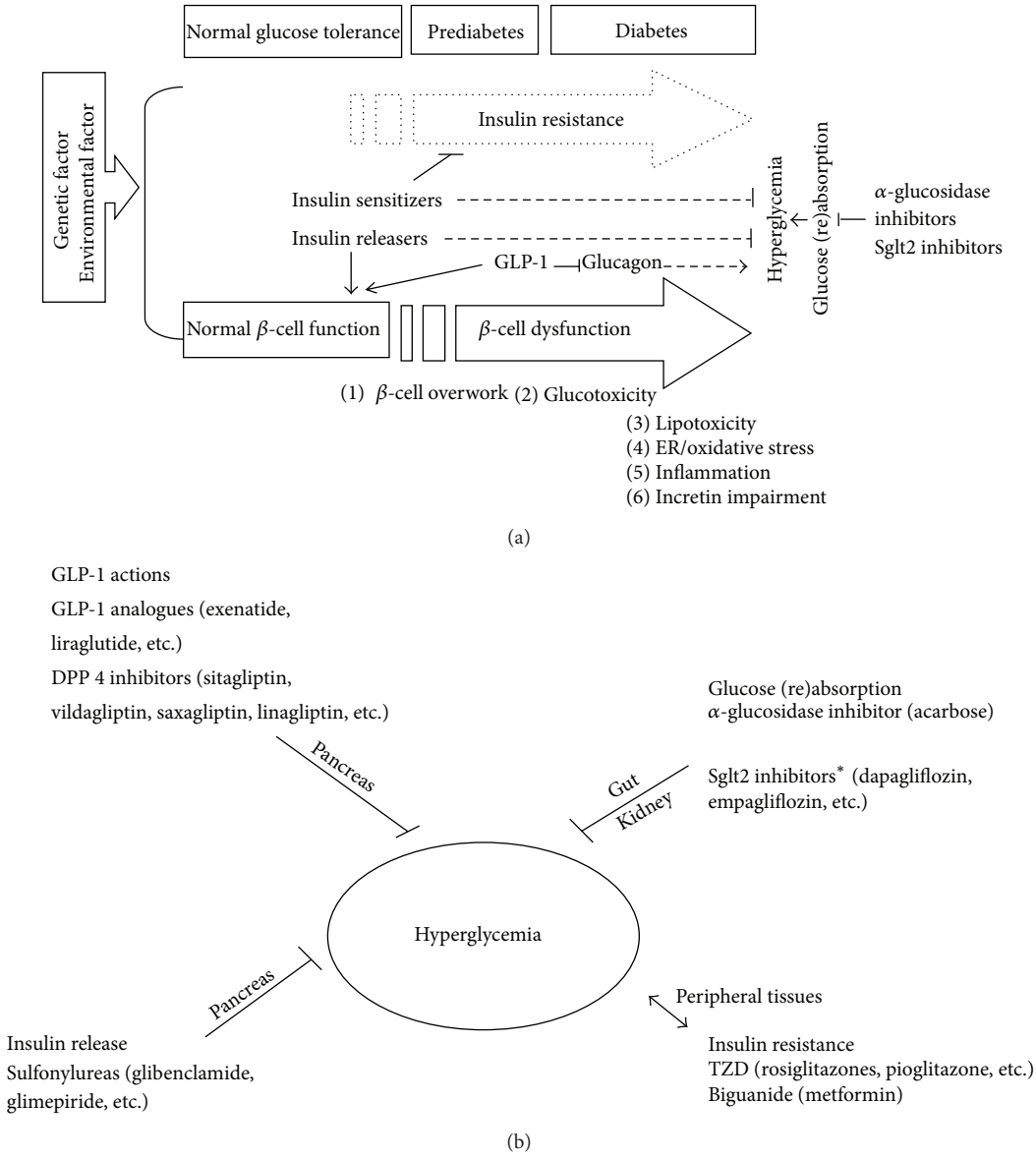


Figure 1: Etiology, development, and current therapies for T2D. (a) Genetic and environmental factors are the main contributors to the development of insulin resistance and impaired glucose tolerance. Under normal glucose tolerance conditions, β cells secrete insulin in response to a surge in glucose after a meal. At the initial stage, β cells overwork to compensate for the development of insulin resistance. Later on, β cells are no longer able to secrete enough insulin to overcome insulin resistance. As a result, glucose tolerance is impaired and the disease progresses from prediabetes to diabetes. Diabetes is characterized as a loss of blood glucose homeostasis, a condition termed hyperglycemia, in the patients. Glucotoxicity, lipotoxicity, ER/oxidative stress, inflammation, and incretin impairment are risk factors for β-cell dysfunction. Besides insulin, insulin releasers, insulin sensitizers, GLP-1 analogues/DDP-4 inhibitors, and a-glucosidase inhibitors and Sglt 2 inhibitors are common antidiabetic drugs. (b) Insulin releasers (e.g., sulfonylureas such as glibenclamide and glimepiride) can stimulate pancreatic β cells to secrete insulin. Insulin sensitizers (TZDs (e.g., rosiglitazone and pioglitazone) and biguanide (metformin)) reduce insulin resistance in the peripheral tissues. GLP-1 has multiple direct actions on pancreas (insulin and glucagon production) and gastric emptying. Injection of exogenous GLP-1 (e.g., exenatide and liraglutide) or inhibition of endogenous GLP-1 degradation by DPP-4 inhibitors (e.g., sitagliptin, vildagliptin, saxagliptin, and linagliptin) can maintain GLP-1 levels. Inhibitors of α-glucosidases (acarbose) and Sglt 2 (e.g., dapagliflozin and empagliflozin) reduce glucose absorption in guts and glucose reabsorption in kidney, respectively. All the drugs can diminish hyperglycemia. *Sglt 2 inhibitors were disproved by the FDA because of a safety issue.

glycemic maintenance ameliorates progression and severity of T2D. In the past, several drugs for T2D including oral antidiabetic agents (OAAs), insulin, and incretin-based drugs have been developed to control homeostasis of blood sugar via different mechanisms (Figures 1(a) and 1(b)) [28]. Among OAAs, insulin releasers such as sulfonylurea-type drugs (e.g., glibenclamide and glimepiride) can directly stimulate pancreatic β cells to secrete insulin, leading to

lower blood glucose. However, these secretagogues cannot rescue β-cell atrophy. In contrast, insulin sensitizers such as TZDs (e.g., rosiglitazone and pioglitazone) and a biguanide, metformin, can directly lower insulin resistance and, subsequently, blood glucose. Glucose (re)absorption is viewed as an alternative way to lower blood glucose level. Inhibitors of Sglt 2, dapagliflozin, empagliflozin, and α-glucosidase inhibitor, acarbose, inhibit the activity of sodium-glucose cotransporter-2 and α-glucosidases, respectively, leading to a decrease in glucose (re)absorption via the renal tubules and the intestine, respectively [17, 29]. Another new class of diabetes therapeutics is the GLP-1 analogues (exenatide and liraglutide) and DPP-4 inhibitors (e.g., sitagliptin, vildagliptin, saxagliptin, and linagliptin). This class leverages multiple actions of GLP-1 to lower blood glucose, including increasing insulin, reducing glucagon, and gastric emptying [30]. Notably, insulin is still an effective drug for T2D. Aside from medication, the importance of diet and lifestyle management in preventing and alleviating T2D should not be neglected.

But current antidiabetic agents lack efficacy and also have undesirable side effects [31]. For instance, insulin secretagogues are frequently linked with weight gain, hypoglycemia, and inability to protect β cells from death [28, 32]. TZDs and biguanides result in weight gain and kidney toxicity, respectively. Acarbose usually causes gastrointestinal upset such as diarrhea and flatulence. Additionally, a clinical trial of Sglt 2 recently failed due to safety concerns [17]. Despite the multiple benefits of incretin-based drugs, these drugs are still accompanied by severe gastrointestinal problems such as sour stomach, belching, nausea, vomiting, indigestion, and diarrhea [29]. Even in well-managed patients, daily injection of insulin cannot match the natural precise timing and dosing of insulin secretion from the pancreas in response to hyperglycemia, resulting in severe complications.

Moreover, in addition to lack of efficacy and undesirable adverse effects, all current antidiabetic agents have a major shortcoming in that they are only designed to alleviate T2D and not to cure it. Evidence suggests that targeting one or two metabolic pathways is insufficient to cure T2D. Drugs with the ability to target more metabolic pathways seem to show more encouraging results than those that target a single pathway, as evidenced by GLP-1. But it should also be noted that drugs that are effective in targeting metabolic pathway(s) are often associated with adverse effects [33]. To ensure patients' welfare, there is still an obvious need to develop antidiabetic medicines with satisfactory efficacy and no severe adverse effects.

3. Herbal Therapy for T2D

Long before the birth of orthodox Western medicine, medicinal herbs were applied to treat a wide range of disease categories [34]. Due to emphasis on scientism and other complicated reasons, Western medicine now prevails over "traditional" forms of medicine including herbal medicine systems. Although herbal medicine systems are sometimes misinterpreted as being unscientific and anachronistic, their long-term existence proves they are able to compete with Western drugs at some level. The use of a medicinal herb, alone or in combination with other herbs, can be thought of as a type of combination therapy because of the complexity of the phytochemicals and bioactivities in the plant. Thus, a single antidiabetic herb with thousands of phytochemicals may have multiple benefits by targeting several metabolic pathways and essentially "killing several birds with one stone." One study supported this principle by demonstrating that a combination therapy of orthodox medicine and herbal medicine exhibited a better (synergistic) effect than either medicine alone [35]. Therefore, herbal medicine can complement orthodox therapy in T2D and provides hope for a cure.

Medicinal herbs have never become obsolete and still play a prominent role in human health care. Among them, over 1200 plants have been claimed to be remedies for diabetes [36, 37]. Over 400 plants as well as 700 recipes and compounds have been scientifically evaluated for T2D treatment [38]. Metformin was developed based on a biguanide compound from the antidiabetic herb, French lilac, and is now a first-line drug for T2D [39]. Medicinal herbs contain diverse bioactive compounds and can have multiple actions on insulin action, insulin production, or both. In the present review, we focus on scientific studies of selected glucose-lowering herbs and phytocompounds and their ability to target insulin resistance, β-cell function, incretin-related pathways, and glucose (re)absorption (Figures 2(a) and 2(b)). Phytochemistry, antidiabetic bioactivities, and likely modes of action of the selected plants and compounds are discussed.

4. Selected Medicinal Herbs and Compounds for T2D

More than 400 plants and compounds have shown antidiabetic activities *in vitro* and/or *in vivo*. Instead of listing each extract/compound, here, we select some plant chemicals and/or extracts with the ability to control blood glucose as well as to modulate at least one of the following mechanisms involved in insulin resistance: β-cell function, incretin-related pathways, and glucose (re)absorption. Chemical structure, antidiabetic activity and action in cells, animal models, and the results of administration of the plant extracts and compounds to patients of T2D are discussed. The chemical and biological properties of the compounds discussed in this section are summarized in Table 1.

4.1. Herbs and Compounds That Regulate Insulin Resistance

4.1.1. Amorfrutins and Licorice. Licorice, the common name for the plants that comprise the genus *Glycyrrhiza*, is utilized as herbal medicine for a wide range of diseases. The ethanol extract of *G. uralensis* was found to reduce blood glucose, fat weight, and blood pressure in rodent models [128]. This extract also has PPAR-γ activity [128]. Further, amorfrutins isolated from the licorice, *G. foetida*, were found to bind to and activate peroxide proliferator-activated receptor-γ (PPAR-γ), a central player in glucose and lipid metabolism [40]. These compounds lowered blood glucose, fat weight,

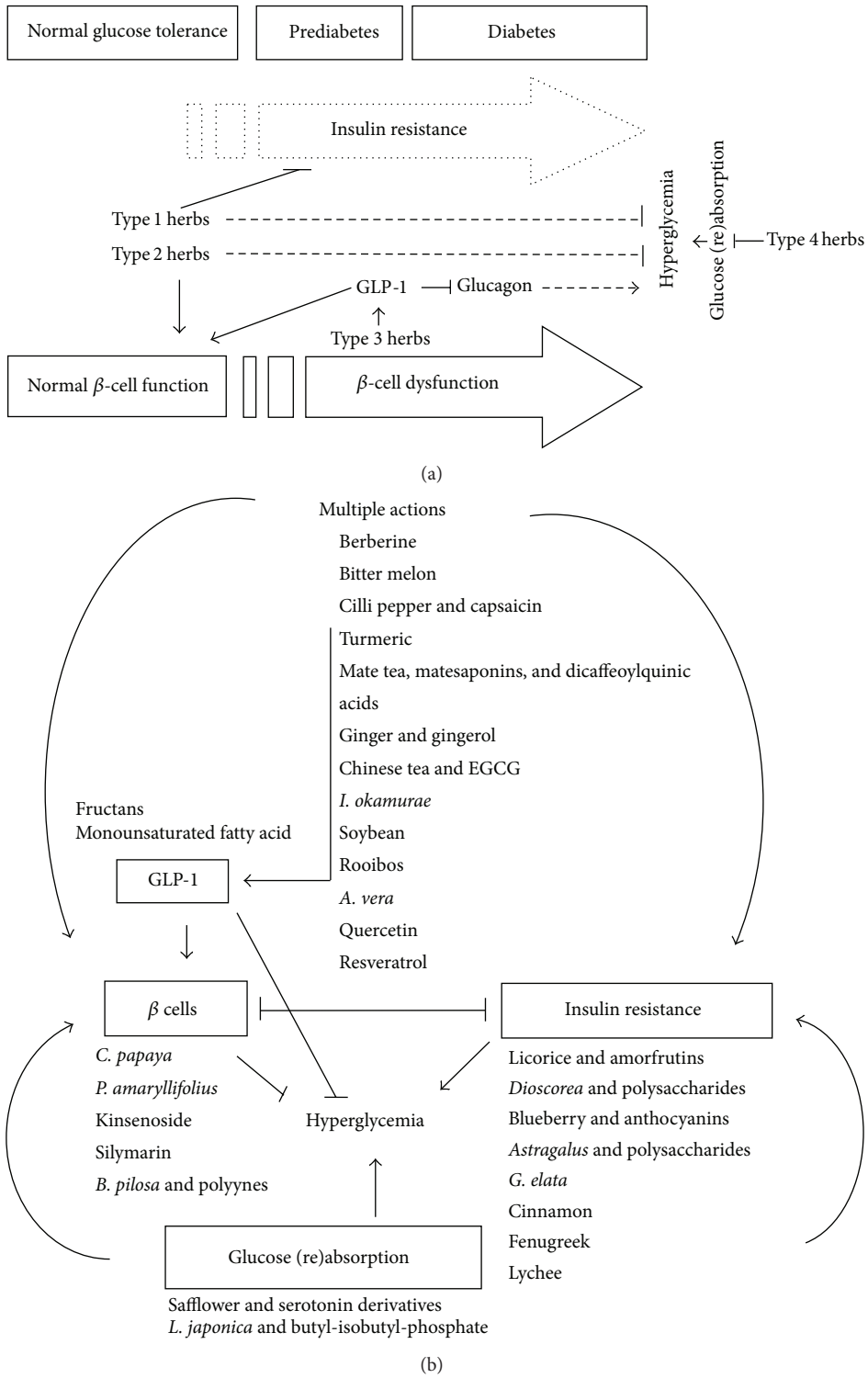


Figure 2: Mechanisms underlying herbal therapies using antidiabetic plants and phytocompounds. (a) Different types of medicinal herbs can be classified based on their modes of action such as insulin resistance (type 1 herbs), β-cell function (type 2 herbs), and GLP-1 (type 3 herbs) and glucose (re)absorption (type 4 herbs). (b) The selected plants and compounds exert their antihyperglycemic effect through targeting one single mechanism (insulin resistance (type 1 herbs), β-cell function (type 2 herbs), GLP-1 (type 3 herbs), or glucose (re)absorption (type 4 herbs)) or multiple mechanisms.

Table 1: Active compounds and biological actions of antidiabetic herbs.

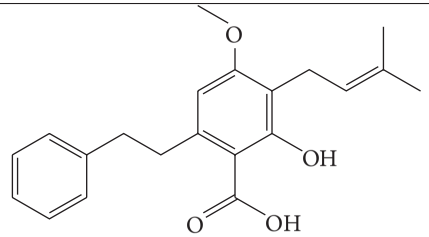
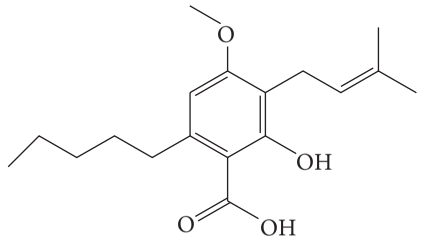
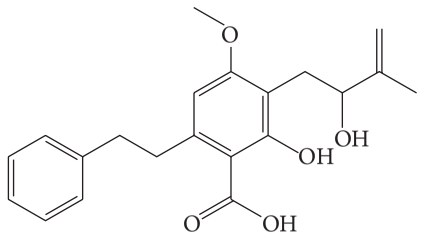
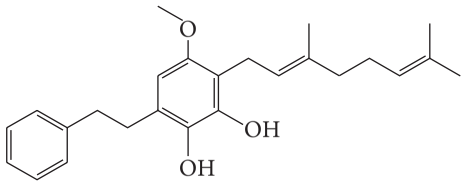
Herb	Compound name	Chemical structure	Antidiabetic mechanism(s)	References
G. uralensis	Amorfrutin 1		Regulate insulin resistance	[40]
	Amorfrutin 2			
	Amorfrutin 3			
	Amorfrutin 4			
D. rhizome	*Dioscorea* polysaccharides	N.A.	Regulate insulin resistance	[41–44]
V. spp.	Phenolics and Anthocyanins	N.A.	Regulate insulin resistance	[45–47]
A. membranaceus	*Astragalus* polysaccharides	N.A.	Regulate insulin resistance	[48–51]

Table 1: Continued.

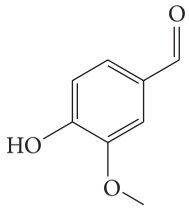
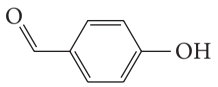
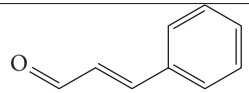
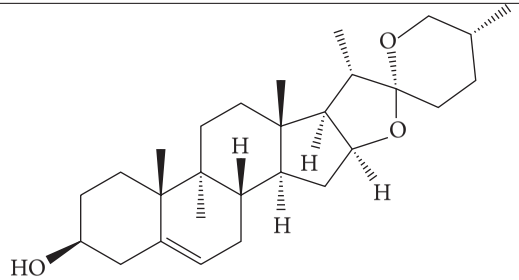
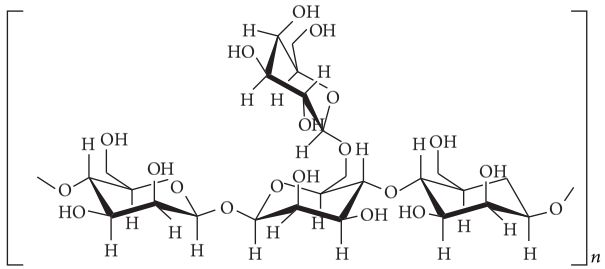
Herb	Compound name	Chemical structure	Antidiabetic mechanism(s)	References
G. elata	Vanillin		Reduces insulin resistance	[52]
	4-hydroxybenzaldehyde			
C. verum *C. zeylanicum* *C. aromaticum*	Cinnamaldehyde		Regulate insulin resistance	[53]
T. foenum-graecum	Diosgenin		Reduces insulin resistance.	[54, 55]
	Galactomannan		Reduces insulin resistance	[55]

Table 1: Continued.

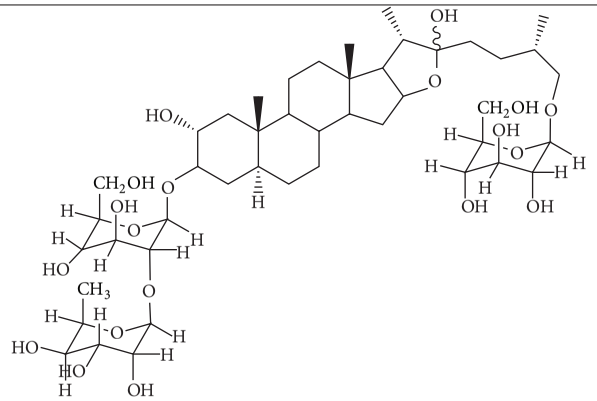
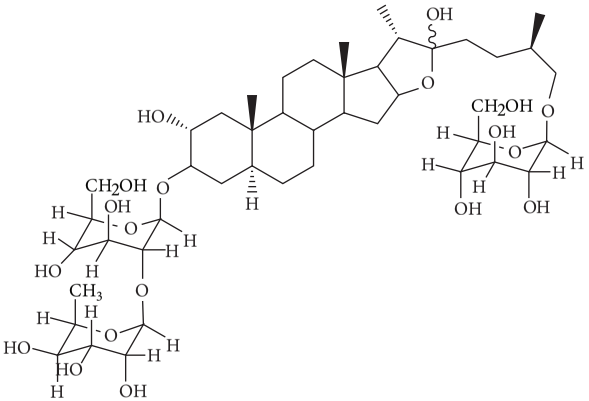
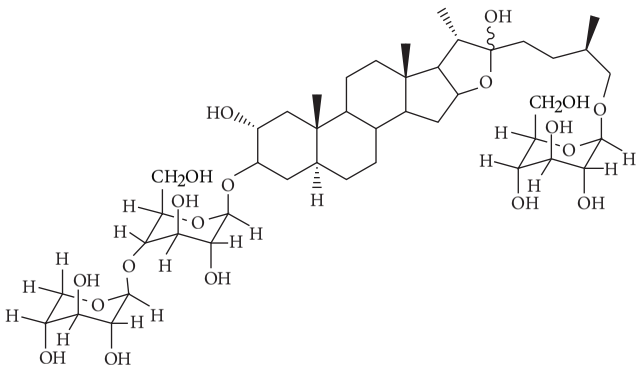
Herb	Compound name	Chemical structure	Antidiabetic mechanism(s)	References
	Trigoneoside Xa			
	Trigoneoside Xb			
	Trigoneoside XIb			

Table 1: Continued.

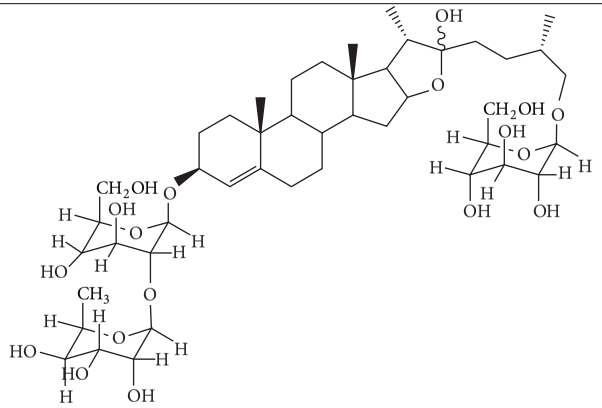
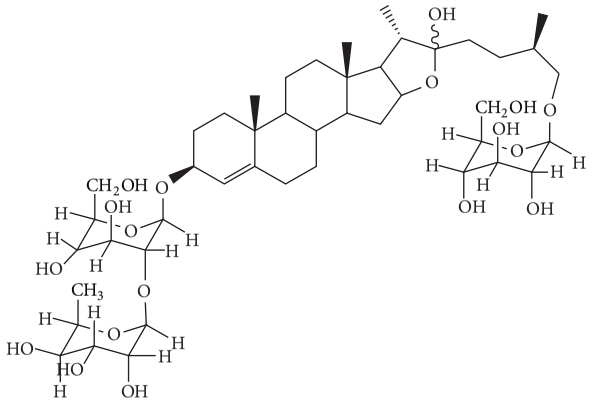
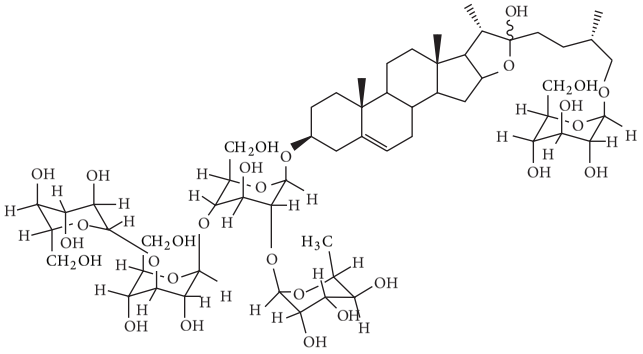
Herb	Compound name	Chemical structure	Antidiabetic mechanism(s)	References
	Trigoneoside XIIa			
	Trigoneoside XIIb			
	Trigoneoside XIIIa			

Table 1: Continued.

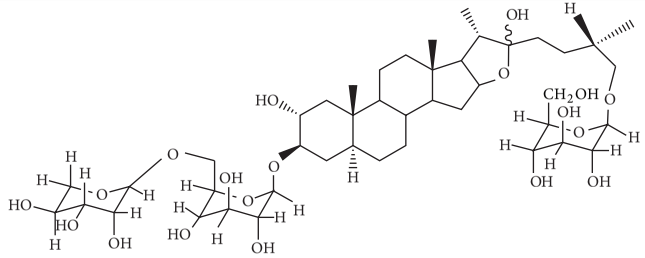
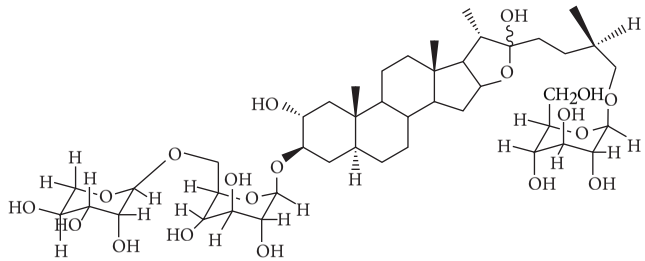
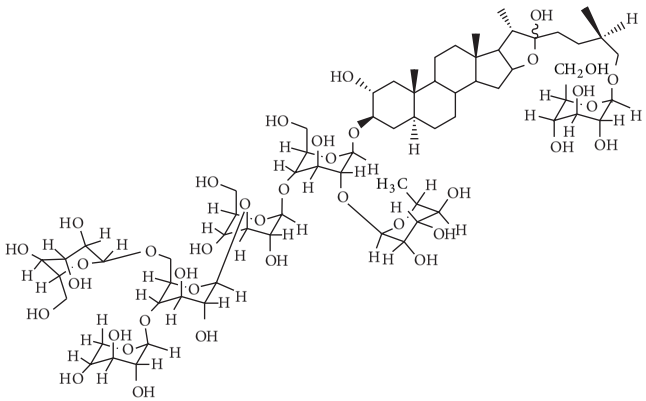
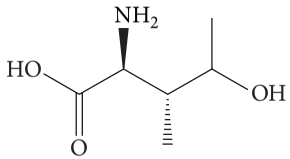
Herb	Compound name	Chemical structure	Antidiabetic mechanism(s)	References
	Trigoneoside Ia			
	Trigoneoside Ib			
	Trigoneoside Va			
	4-hydroxyisoleucine			

Table 1: Continued.

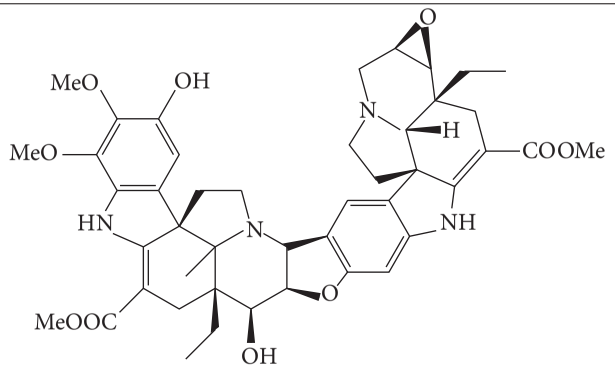
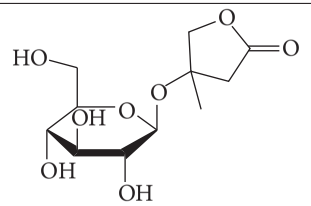
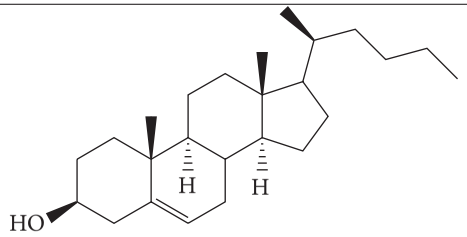
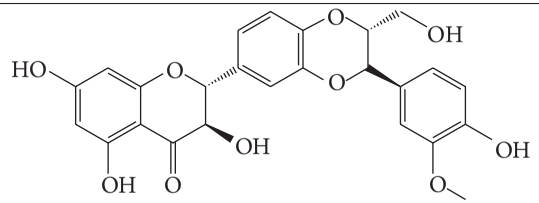
Herb	Compound name	Chemical structure	Antidiabetic mechanism(s)	References
L. chinensis	Oligonol (mixture of compounds)	N.A.	Regulate insulin resistance	[56–58]
C. papaya *P. amaryllifolius*	Flavonoids, alkaloids, saponin, and tannins	N.A.	Regulate β-cell function	[59]
T. divaricate *E. microphylla*	Conophylline		Regulate β-cell function	[60–63]
A. roxburghii	Kinsenoside		Regulate β-cell function	[64]
N. stellata	Nymphayol		Regulate β-cell function	[65]
S. marianum	Silybin		Regulate β-cell function	[66–74]

Table 1: Continued.

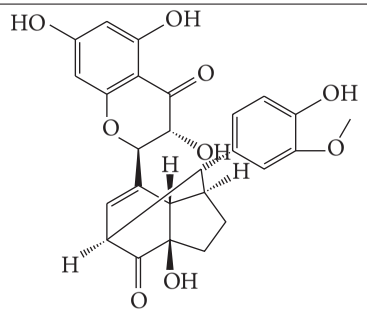
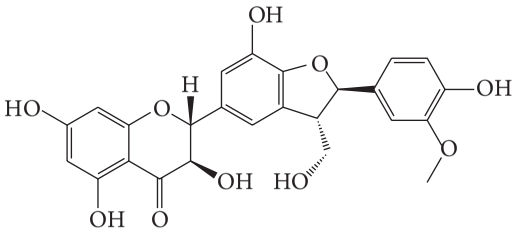
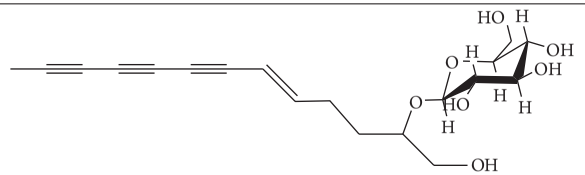
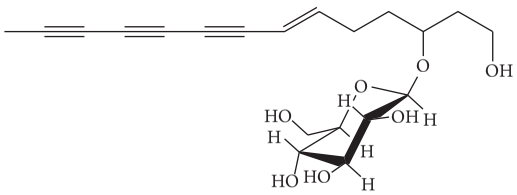
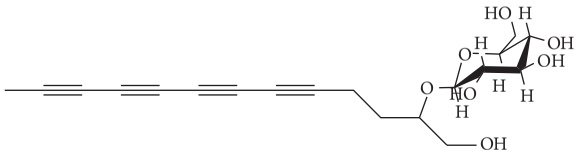
Herb	Compound name	Chemical structure	Antidiabetic mechanism(s)	References
	Silydianin			
	Silychristin			
B. pilosa	3-β-D-glucopyranosyl-1-hydroxy-6(*E*)-tetradecene-8,10,12-triyne		Regulate β-cell function	[75, 76]
	2-β-D-glucopyranosyloxy-1-hydroxy-5(*E*)-tridecene-7,9,11-triyne			
	2-β-D-glucopyranosyloxy-1-hydroxytrideca-5,7,9,11-tetrayne (cytopiloyne)		Regulates β-cell function	[53]

Table 1: Continued.

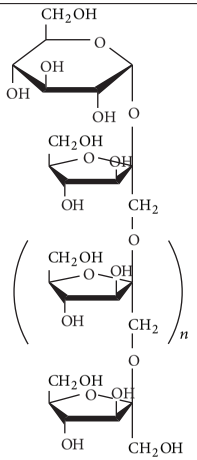
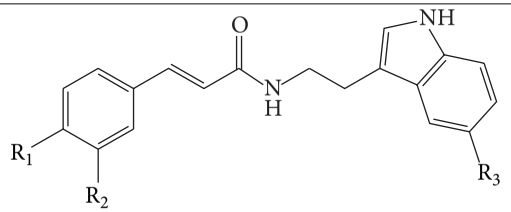
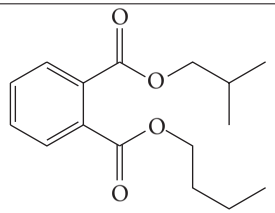
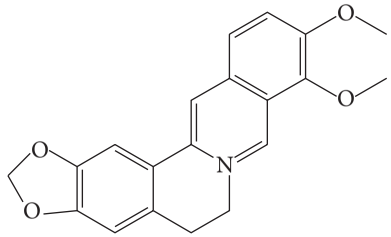
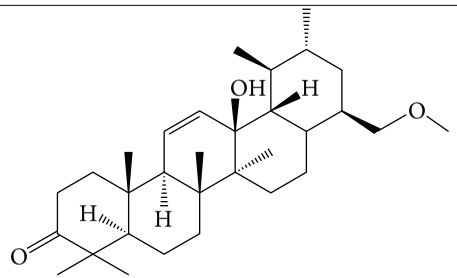
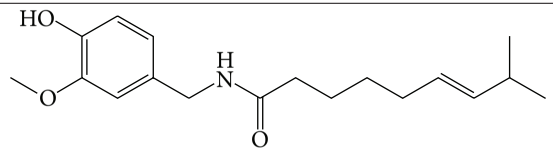
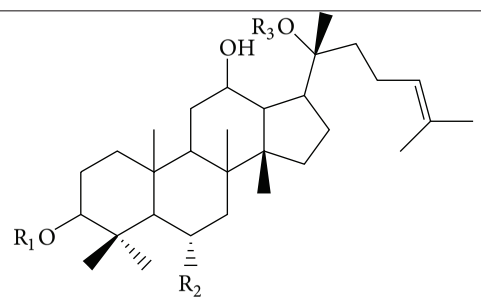
Herb	Compound name	Chemical structure	Antidiabetic mechanism(s)	References
G. sylvestre	No reported active compounds	N.A.	Regulate β-cell function	[77–79]
Dietary fibers from the roots of *A. tequilana*, *Dasylirion* spp. and so forth	Inulin/Raftilose		Regulate GLP-1 homeostasis	[80–82]
Olive oil	Monounsaturated fatty acids	N.A.	Regulate GLP-1 homeostasis	[83–86]
C. tinctorius	Serotonin derivatives	 1 R_1 = OH; R_2 = H; R_3 = OH 2 R_1 = OH; R_2 = OMe; R_3 = OH	Regulate glucose absorption in the guts	[87]
L. japonica	Butyl-isobutyl-phthalate		Regulate glucose absorption in the guts	[88]

Table 1: Continued.

Herb	Compound name	Chemical structure	Antidiabetic mechanism(s)	References
B. vulgaris	Berberine		Regulate two or more pathways (lower hyperglycemia, increase insulin resistance, pancreatic β-cell regeneration, and decrease lipid peroxidation)	[89–94]
M. charantia	Momordicin		Regulate two or more pathways (lower blood glucose due to their insulin-like chemical structures)	[38]
Capsicum plants	Capsaicin		Regulate two or more pathways (regulation of insulin resistance and probably β cells)	[95, 96]
P. ginseng	Ginsenoside Rb1 Ginsenoside Rb2 Ginsenoside Rc Ginsenoside Rd Ginsenoside Re Ginsenoside Rf Ginsenoside Rg1		Regulate two or more pathways (regulate β-cell function, improvement of insulin resistance)	[97, 98]

Ginsenosides	R_1	R_2	R_3
Rb1	Glc-^{2}Glc	H	Glc-^{6}Glc
Rb2	Glc-^{2}Glc	H	Ara(pyr)-^{6}Glc
Rc	Glc-^{2}Glc	H	Ara(fur)-^{6}Glc
Rd	Glc-^{2}Glc	H	Glc-
Re	II	Rha-^{2}Glc-O-	Glc-
Rf	H	Glc-^{2}Glc-O-	H
Rg1	H	Glc-O-	Glc-

Table 1: Continued.

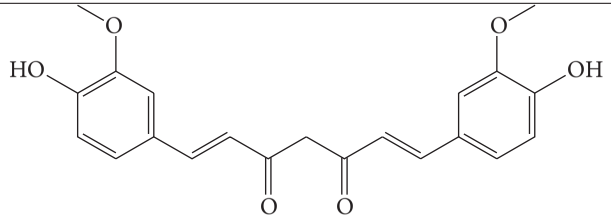
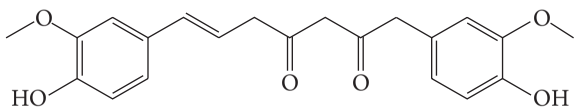
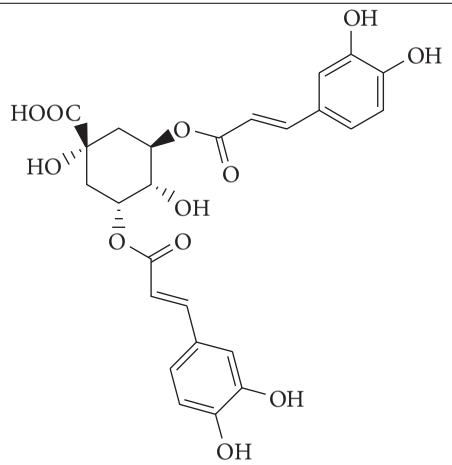
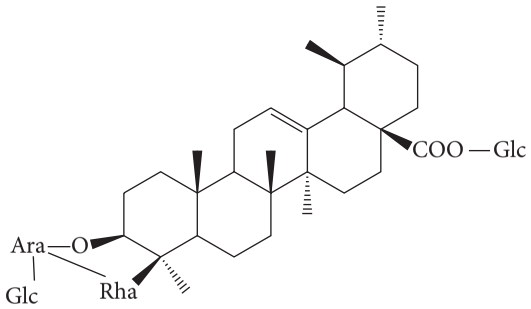
Herb	Compound name	Chemical structure	Antidiabetic mechanism(s)	References
C. longa	Curcumin		Regulate two or more pathways (regulation of insulin resistance and β-cell function)	[99]
	Turmerin			[100]
I. paraguariensis	3,5-*O*-dicaffeoylquinic acid		Regulate two or more pathways (augmentation of GLP-1 production)	[101]
	Matesaponin 2			

Table 1: Continued.

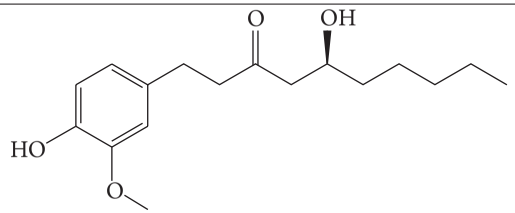
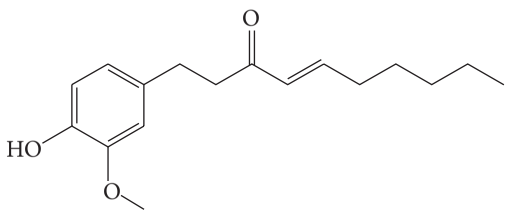
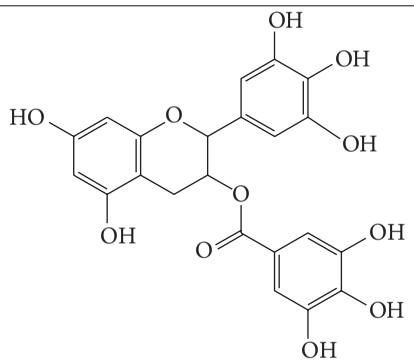
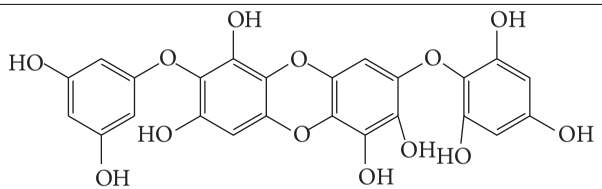
Herb	Compound name	Chemical structure	Antidiabetic mechanism(s)	References
Z. officinale	Gingerol		Regulate two or more pathways (islet cell protection and increased insulin receptor signaling)	[102]
	Shogaol		Unclear but can elevate glucose uptake in response to insulin in muscles and adipose cells	[103]
C. sinensis	Epigallocatechin 3-gallate (EGCG)		Regulate two or more pathways (islet protection, increase in insulin secretion, decrease in insulin tolerance, decrease in gluconeogenesis and insulin-mimetic action)	[104–106]
I. okamurae	Diphlorethohydroxycarmalol		Regulate two or more pathways (α-glucosidase and α-amylase inhibitor)	[107]

Table 1: Continued.

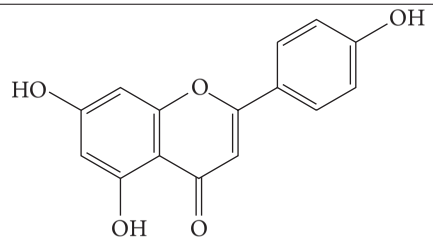
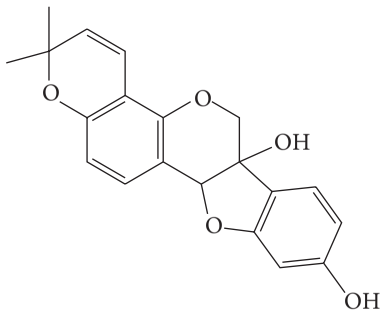
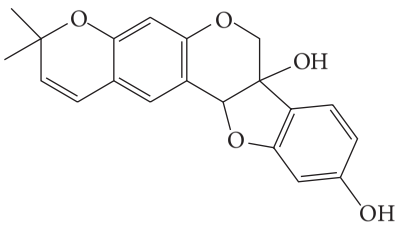
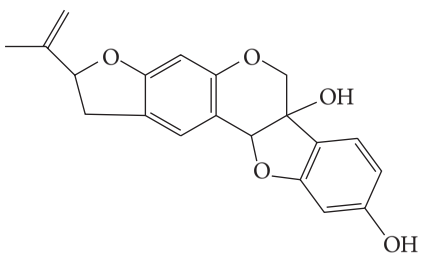
Herb	Compound name	Chemical structure	Antidiabetic mechanism(s)	References
G. max	Genistein		Regulate two or more pathways (preserves islet mass, activates protein kinase A (PKA) and extracellular-signal-regulated kinases (ERK) 1/2, activates AMPK, and reduces insulin sensitivity)	[95, 108–111]
	Glyceollin I			
	Glyceollin II		Regulate two or more pathways (enhance GLP-1 secretion, improve insulin secretion, regulate β-cell function)	[112]
	Glyceollin III			

Table 1: Continued.

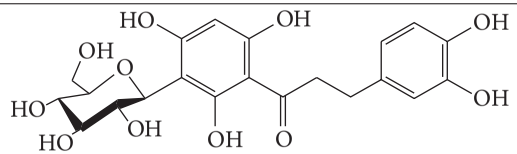
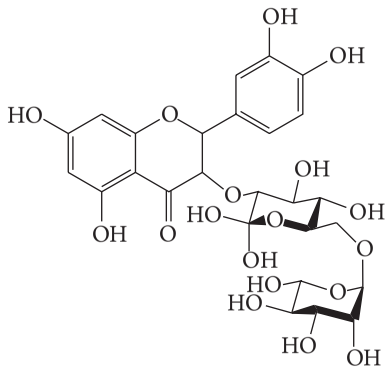
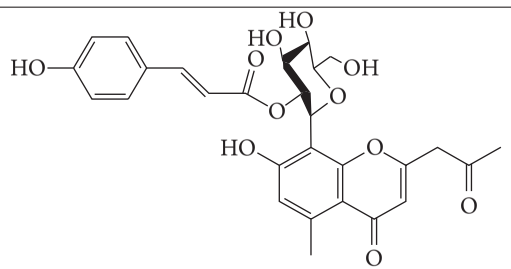
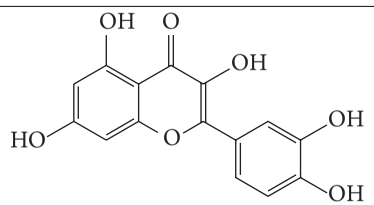
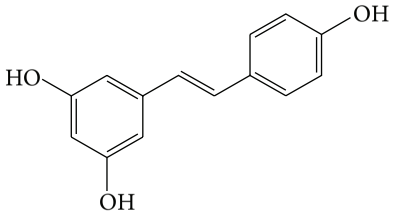
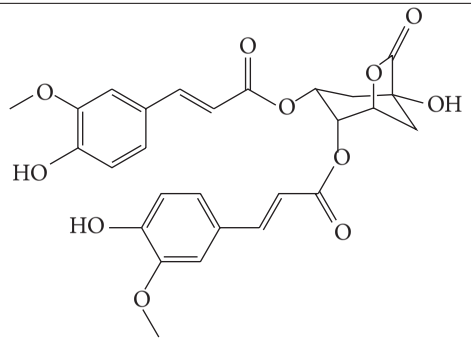
Herb	Compound name	Chemical structure	Antidiabetic mechanism(s)	References
A. linearis	Aspalathin		Regulate two or more pathways (insulin tolerance, β-cell function, and inhibition of α-glucosidase)	[113, 114]
	Rutin (quercetin-3-O-rutinoside)			[114–117]
A. vera	Aloeresin A		Regulate two or more pathways (suppression of α-glucosidase activity (gut glucose absorption) and insulin resistance)	[118]
Commonly found in plants, vegetables, and fruits	Quercetin		Regulate glucose absorption in guts.	[119, 120]

Table 1: Continued.

Herb	Compound name	Chemical structure	Antidiabetic mechanism(s)	References
Commonly found in plants and fruits	Resveratrol		Regulate two or more pathways (activates AMPK and downstream molecules, prevents cell death of pancreatic β cells, and activates SIRT1)	[121–125]
Coffee	Quinides (e.g. (1R,3R,4S,5R)-3,4-diferuloyl-1,5-quinide)*		Regulate two or more pathways	[126, 127]

N.A.: not applicable.
*Quinides are derived from chlorogenic acid during roasting.

and dyslipidemia [40] indicating that licorice and its active amorfrutins exert their antidiabetic function via the PPAR-γ pathway.

4.1.2. Dioscorea Polysaccharides and Dioscorea. The rhizome of *Dioscorea* is used as a traditional Chinese medicine for asthma, abscesses, chronic diarrhea, and ulcers [41]. Several studies on rodent models of diabetes have reported that *Dioscorea* extract improves glycemic control and insulin resistance [41–44]. Further, *Dioscorea* extract reduced blood glucose in high fat diet-induced rats [41]. The antidiabetic mechanism of *Dioscorea* extract involves reduction of insulin resistance by diminution of the phosphorylation of ERK and pS6K and increase of the phosphorylation of Akt and glucose transporter 4 (Glut4) [41]. Another study demonstrated that *Dioscorea* polysaccharides reduced insulin resistance mediated by inflammatory cytokines as evidenced by the phosphorylation of insulin receptor substrate (IRS) and Akt [42].

4.1.3. Anthocyanins and Blueberry. Blueberry (*Vaccinium* spp.) was demonstrated to lower systolic and diastolic blood pressure and lipid oxidation and improve insulin resistance, diabetes, diabetic complications, and digestion [45, 129–132]. Notably, blueberries contain powerful antioxidants that can neutralize free radicals that cause neurodegenerative disease, cardiovascular disease, and cancer [45]. Accordingly, phenolics and anthocyanins were proposed as active compounds for diabetes and insulin resistance [45, 46].

One clinical study showed that obese or T2D patients consuming 22.5 g blueberry, twice a day for 6 weeks, reduced insulin resistance to a greater extent than those consuming a placebo [47]. The data confirm the beneficial effect of blueberry on metabolic syndrome. However, the active compounds related to this claim need further investigation.

4.1.4. Astragalus Polysaccharides and Astragalus. The root of *Astragalus membranaceus* has long been used as a Chinese medicine and shows antioxidant, antidiabetic, antihypertensive, and immunomodulatory activities [133]. The extract of *A. membranaceus* was shown to treat diabetes and diabetic complications [134]. Moreover, treatment with *Astragalus* polysaccharides resulted in better glycemic control in diabetic rodents via an increase in insulin sensitivity [48–50]. The mode of action of *Astragalus* polysaccharides includes Akt activation and upregulation of Glut4 and inhibition of inflammation via the PTP1B/NFκB pathway [48, 50, 51].

4.1.5. Gastrodia elata. *G. elata* has been utilized as Chinese medicine for blood circulation and memory [52]. More recently, the extract of *G. elata* has been reported to improve insulin resistance [52]. Vanillin and 4-hydroxybenzaldehyde were proposed as the active compounds. Both compounds reduced insulin resistance through a decrease in fat accumulation in adipose tissues and an increase in fat oxidation and potentiation of leptin signaling in obese rats [52]. So far, no clinical study has been conducted in human diabetic patients.

4.1.6. Cinnamon. Both common cinnamon (*Cinnamomum verum* and *C. zeylanicum*) and cassia (*C. aromaticum*) have long been used as flavoring agents and in drinks and medicines worldwide [135]. Cinnamon has traditionally been used for rheumatism, wounds, diarrhea, headaches, and colds [136]. Recently, extensive studies have been performed on the action of cinnamon on diabetes and metabolic syndrome [135]. Cinnamon was shown to reduce blood glucose via reduction of insulin resistance and increase of hepatic glycogenesis [135, 137]. Cinnamon phenolics were proposed to be the active compounds in modulation of insulin signaling [53, 138, 139]. Moreover, cinnamaldehyde had antihyperglycemic and antihyperlipidemic effects on rodent models of diabetes [53]. This compound from cinnamon extract is thought of as a potential antidiabetic agent [139]. Unfortunately, the molecular target of cinnamon and cinnamaldehyde remains unclear.

4.1.7. Fenugreek. The seeds of fenugreek (*Trigonella foenum-graecum*) are used as a food supplement and also have a long history of medicinal use for labor induction, helping digestion and improving metabolism and health [34]. Animal studies have shown that extract of fenugreek seeds can lower blood glucose levels [140, 141]. Fenugreek is considered a promising agent for diabetes and its complications [34]. The glucose-lowering action of this plant involves reduction of insulin resistance [142]. Diosgenin, GII, galactomannan, trigoneosides, and 4-hydroxyisoleucine have been identified as the active antidiabetic compounds in fenugreek. However, little is known about the mechanisms of these compounds [55]. Among them, diosgenin was shown to reduce adipocyte differentiation and inflammation, implying its action in reduction of insulin resistance [54]. A clinical study indicated that fenugreek exerts hypoglycemic control via increasing insulin sensitivity [143].

4.1.8. Lychee. Lychee (*Litchi chinensis*) is an evergreen fruit tree. Its seeds are used as a Chinese herbal medicine for pain, gastrointestinal diseases, and others. Recently, lychee seed was reported to have antidiabetic activity in rats [56] and human patients [144]. Lychee seed extract exerts its action through reduction of insulin resistance [56]. In addition, oligonol from lychee fruit showed anti-oxidative activity and, thus, protected the liver and kidney in T2D animal models [57, 58].

4.2. Herbs and Compounds That Regulate β-Cell Function. In this section, plant chemicals and/or extracts are listed according to their impact on β cells. Their chemical structures and antihyperglycemic activities and actions on β-cell function ((re)generation and survival) in cells, animals, and T2D patients are discussed. The chemical and biological properties of the compounds discussed in this section are summarized in Table 1.

4.2.1. Carica papaya and Pandanus amaryllifolius. The ethanol extracts of *P. amaryllifolius* and *C. papaya* reduced hyperglycemia in streptozotocin- (STZ-) treated mice [59].

Histological staining data showed that these extracts significantly induced the regeneration of the β cells as evidenced by reduced blood glucose level [59]. So far, no active components have been identified. However, the flavonoids, alkaloids, saponin, and tannin in both plants were speculated to be bioactive phytochemicals [59].

4.2.2. Conophylline and Tabernaemontana divaricata. Conophylline, a plant alkaloid present in *T. divaricata* or *Ervatamia microphylla*, facilitates differentiation and generation of pancreatic β cells *in vitro* and *in vivo* [60–62]. This phytochemical was also shown to decrease the fibrosis of pancreatic islet cells [63]. Crude extract of *T. divaricata* was able to increase the level of blood insulin and reduce the level of blood glucose in STZ-treated mice [145]. These data imply a plausible role for conophylline and *T. divaricata* in β-cell function.

4.2.3. Kinsenoside. Kinsenoside, a major constituent of *Anoectochilus roxburghii*, exhibited hypoglycemic activity in STZ-treated mice [64]. This effect was partially attributed to β-cell repair and/or regeneration. However, the clinical potential of this compound in β-cell survival and regeneration awaits further investigation.

4.2.4. Nymphayol. Nymphayol, a plant sterol, was initially isolated and identified from *Nymphaea stellata*. One study showed that this compound promoted the partial generation of pancreatic islet cells [65]. Oral administration of Nymphayol significantly diminished the blood glucose level and increased the insulin content in diabetic rats. In addition, Nymphayol increased number of β cells enormously [65]. However, the impact of this compound on T2D patients is largely unknown.

4.2.5. Silymarin. Silymarin is a flavonoid mixture composed of silybin, silydianin, and silychristin, which are active components of the plant milk thistle (*Silybum marianum*) [146]. Aside from antioxidant, anti-inflammatory, and hepatoprotective activities, the modes of action through which silymarin and/or milk thistle exert antidiabetic activity are not well understood [66–74]. It has been reported that silymarin can rescue β-cell function in alloxan-treated rats [68].

4.2.6. Polyynes and Bidens pilosa. *B. pilosa* is used as an herbal medicine for a variety of diseases. Ubillas and colleagues showed that the aqueous ethanol extract of the aerial part of *B. pilosa* lowered blood glucose in db/db mice [75]. Based on a bioactivity-guided identification, 2 polyynes, 3-ß-D-glucopyranosyl-1-hydroxy-6(*E*)-tetradecene-8,10,12-triyne, and 2-ß-D-glucopyranosyloxy-1-hydroxy-5(*E*)-tridecene-7, 9,11-triyne were identified. Further, a mixture of both compounds significantly reduced blood glucose levels and food intake in db/db mice [75]. Another study confirmed that water extracts of *B. pilosa* at one and multiple doses significantly lowered fasting and postmeal hyperglycemia in db/db mice [76]. The anti-hyperglycemic effect of *B. pilosa* was inversely correlated with an increase in serum insulin levels, suggesting that BPWE acts to lower blood glucose via increased insulin production. Moreover, *B. pilosa* protected against islet atrophy in mouse pancreata. Despite the variation in the percentage of polyynes, *B. pilosa* varieties, *B. pilosa* L. var. *radiate* (BPR), *B. pilosa* L. var. *pilosa* (BPP), and *B. pilosa* L. var. *minor* (BPM) all displayed antidiabetic activity in db/db mice [76]. Another polyyne isolated from *B. pilosa*, 2-ß-D-glucopyranosyloxy-1-hydroxytrideca-5,7,9,11-tetrayne (cytopiloyne) showed better glycemic control than the previously-mentioned polyynes [53]. Mechanistic study demonstrated that similar to *B. pilosa*, cytopiloyne exerts antidiabetic function through regulation of β-cell function involving the increase insulin expression/secretion and islet protection [53]. Furthermore, cytopiloyne regulated β-cell function through a signaling cascade of calcium influx, diacylglycerol, and protein kinase Cα. Collectively, *B. pilosa* and cytopiloyne derivatives can treat T2D via acting on β cells.

Like all antidiabetic drugs, cytopiloyne failed to prevent and cure diabetes completely but reduced diabetic complications [53]. Together the data also imply that combination therapy that targets multiple pathways involved in metabolism could be a better remedy for T2D.

4.2.7. Gymnema sylvestre. *G. sylvestre* is an Indian medicinal herb that has been used to treat diabetes for centuries. The extract of *G. sylvestre* has been shown to reduce blood glucose. Its action involves insulin secretion and (re)generation of pancreatic β cells in rodents [77, 78]. *G. sylvestre* increased plasma insulin and C-peptide levels and decreased blood glucose concentrations in T2D patients [79]. Collectively, this plant exerts its antidiabetic effect via regulation of β-cell function.

4.3. Herbs and Compounds That Regulate GLP-1 Homeostasis. The chemical and biological properties of plants and phytochemicals regulating GLP-1 secretion and/or DPP-4 activity discussed in this section are summarized in Table 1.

4.3.1. Fructans. The American Diabetes Association (ADA) established a link between high intake of soluble dietary fiber and improved hyperglycemia and insulin secretion in T2D patients [147]. Inulins (Raftilose) are soluble dietary fibers made of short-chain fructans present in the roots of chicory, *Agave tequilana*, *Dasylirion* spp., and so on. One study showed that inulin-type fructans could prevent obesity, steatosis, and hyperglycemia. Moreover, fructans were demonstrated to stimulate incretin secretion in the colon of rats through their fermentation [80, 81]. In addition, 5-week feeding with inulin significantly lowered body weight gain, food intake, and blood glucose levels in C57BL/6J mice [82]. An elevation of GLP-1 levels was observed in the portal vein and proximal colon [82]. It remains unclear whether fructans can enhance incretin production in humans with T2D.

4.3.2. Monounsaturated Fatty Acid. Epidemiological investigations have established an association between dietary fat and T2D. A sedentary lifestyle with a diet overly high in fat

usually accompanies obesity and T2D [148, 149]. However, fat was found to stimulate incretin secretion [150]. Decrease in gastric emptying, level of postprandial blood glucose and insulin, and an increase in plasma GLP-1 level were caused by ingesting fat before a carbohydrate meal in T2D patients [86]. In addition, T2D patients took control meals and control meals supplemented with olive oil (74% monounsaturated fatty acid) or butter (72% saturated fatty acid). In contrast to the control diet, both fat-rich meals induced a 5- to 6-fold increase in plasma GLP-1 and 3- to 4-fold increase in GIP [85]. However, no significant differences in the level of blood glucose, insulin, or fatty acids were observed [85]. In normal and lean Zucker rats, olive oil enhanced GLP-1 secretion, leading to improved glycemic tolerance [83, 84]. Data from humans and rodents suggest that fat, particularly unsaturated fatty acid, can stimulate GLP-1 secretion.

4.4. Herbs and Compounds That Regulate Glucose Absorption in the Gut. The chemical and biological properties of plants and phytochemicals regulating α-glucosidase activity discussed in this section are summarized in Table 1.

4.4.1. Serotonin Derivatives and Safflower. Safflower (*Carthamus tinctorius*) seeds are used as a herbal medicine for menstrual pain, trauma, constipation, and diaphoresis in Korea and Asian countries [87]. Hydroalcoholic extract of safflower exhibited antidiabetic properties through enhancing insulin secretion in alloxan-induced diabetic rats [151]. Two serotonin derivatives isolated from safflower seed were shown to suppress α-glucosidase activity to a greater degree than the positive control acarbose [87].

4.4.2. Butyl-isobutyl-phthalate and Laminaria japonica. Rhizoid of Japanese kelp, *L. japonica*, has been used to treat diabetes. Butyl-isobutyl-phthalate, an active compound of *L. japonica*, exhibited inhibition of α-glucosidase activity *in vitro* and a hypoglycemic effect on diabetic mice induced by STZ [88].

4.5. Herbs and Compounds with Multiple Antidiabetic Actions. Some plants and plant compounds can target multiple metabolic pathways. The chemical and biological properties of the compounds discussed in this section are summarized in Table 1.

4.5.1. Berberine. Berberine, an isoquinoline alkaloid, was first isolated from *Berberis vulgaris*. This compound has multiple functions ranging from inflammation inhibition and cancer suppression to reduction of metabolic syndrome and other activities [93, 152–155]. With respect to T2D, this compound lowered hyperglycemia, increased insulin resistance, stimulated pancreatic β-cell regeneration, and decreased lipid peroxidation in a mouse model of T2D [89–92]. Thus, it may be useful for treatment of T2D and other types of diabetes. A meta-analysis study suggests that berberine *per se* does not show glycemic control in T2D patients. Combination treatment of berberine with other OAAs showed better glycemic control than either treatment alone. Of note, berberine had a mild antidyslipidemic effect on patients [94].

4.5.2. Bitter Melon. Bitter melon, the fruit of the plant *Momordica charantia* is used in Ayurvedic medicine [156]. The biochemistry and bioactivities associated with the antidiabetic effect of the extracts of bitter melon and *M. charantia* as a whole have been extensively studied. One *in vitro* study showed that bitter melon could increase insulin secretion from β cells. Moreover, immunostaining data indicated that the juice of the bitter melon increased β cells in the pancreas of STZ-treated rats. Modes of action of bitter melon and *M. charantia* include insulin secretion, inhibition of glucose reabsorption in guts, preservation of islet β cells and their functions, increase of peripheral glucose utilization, and suppression of gluconeogenic enzymes [38]. Of note, momorcharin and momordicin, isolated from *M. charantia* and its fruit, act to lower blood glucose likely because they possess insulin-like chemical structures [38].

4.5.3. Capsaicin and Chili Pepper. Chili peppers, the fruits of the *Capsicum* plants, are commonly used as food and medicine. Chili pepper extract exerts an insulinotropic action, implying its action on β cells [157]. Capsaicin, a pungent component of chili pepper, activates AMPK in 3T3-L1 preadipocytes [95]. The data suggest that the chili pepper and its active ingredients prevent T2D via regulation of insulin resistance and probably β cells. However, there is a discrepancy over the use of capsaicin to treat T2D. Capsaicin might cause T2D via impairment of insulin secretion [96]. Therefore, precaution should be taken in the use of capsaicin for T2D.

4.5.4. Ginseng. Ginseng (*Panax ginseng*) has been viewed as a panacea in oriental medicine. *P. ginseng* and North American ginseng (*P. quinquefolius*) were demonstrated to lower blood glucose in rodent models [158, 159]. Roots, berries, and/or leaves were found effective against T2D in humans and/or rodents [3, 160–164]. Some clinical studies have demonstrated that *P. ginseng* and North American ginseng improve glycemic control in T2D patients [165, 166]. However, another study reported that neither ginseng had an antidiabetic effect on diabetic patients [167]. This discrepancy may be the result of a variation in active ginsenosides in ginseng [168]. The glucose-lowering mechanisms of both ginsengs may involve a reduction in insulin resistance and β-cell function [97, 169–173]. Ginsenosides are the primary constituents present in ginseng roots that are claimed to benefit health. Extracts of ginseng root have been shown to protect against apoptosis of the pancreatic β-cell line, Min-6 cells [171]. One study proposed that ginseng alters mitochondrial function as well as apoptosis cascades to ensure cell viability in pancreatic islet cells [174]. Moreover, ginsenosides from ginseng extracts were reportedly responsible for this protection *in vitro*. One study reported that ginsenoside Rh2 is an active compound that improves insulin resistance in fructose-rich chow-fed rats [97]. Besides, ginsenoside Re was showed to possess antioxidant activity via upregulation

of glutathione and malondialdehyde in kidney and/or eye [98]. However, the *in vivo* protective role of the extracts and ginsenosides in β cells remains to be further verified.

4.5.5. Turmeric. Like many spices such as garlic and ginger, turmeric shows hypoglycemic and hypolipidemic effects on diabetic mice [175]. Turmeric also increased postprandial serum insulin levels to maintain blood glucose levels in healthy subjects [99]. Curcumin is a major constituent of the rhizomatous powder of turmeric (*Curcuma longa*) and is commonly used as food and medicine in southern Asia. Curcumin and turmeric rhizomes show a number of bioactivities such as antioxidant, anti-inflammatory, antidiabetic, and immunomodulatory [176]. Curcumin has been used to treat T2D [177, 178]. Weisberg and colleagues pointed out that curcumin reverses many of the inflammatory and metabolic derangements associated with obesity and improves glycemic control in mouse models of type 2 diabetes [178]. Chuengsamarn and colleagues showed that after 9 months of treatment, a curcumin-treated group showed a better β-cell function, with higher homeostatic measurement assessment (HOMA)-β and lower C-peptide. Also, the curcumin-treated group showed a lower level of HOMA insulin resistance (IR). This study demonstrated that curcumin can prevent T2D in humans [177]. Consistently, another clinical study exhibited that the ingestion of turmeric increased postprandial serum insulin levels in healthy subjects. These data suggest that curcumin, a bioactive compound of turmeric, ameliorates T2D via regulation of insulin resistance and β-cell function [99]. Further, turmerin, an anti-oxidant protein identified from turmeric, was also shown to inhibit α-glucosidase activity [100]. Overall, turmeric exerts antidiabetic actions likely via regulation of insulin resistance, β-cell function, and gut absorption.

4.5.6. Dicaffeoylquinic Acids, Matesaponins, and Mate Tea. Mate tea is made from the leaves of mate, *Ilex paraguariensis* (Aquifoliaceae), in South America [101]. Mate has been claimed to have neuroprotective, antioxidant, hepatoprotective, choleretic, diuretic, hypocholesterolemic, antirheumatic, antithrombotic, anti-inflammatory, antiobese, and cardioprotective activities [179–181]. Additionally, mate has been developed as an herbal supplement to control body weight [182]. Long-term consumption of mate tea significantly increases serum insulin and ameliorates hyperglycemia and insulin resistance in mice [101]. Mate also induces significant decreases in food intake and weight gain in high fat diet-fed ddY mice. 3,5-*O*-dicaffeoyl quinic acid and matesaponin 2, two major constituents of mate, significantly elevated serum GLP-1 levels in ddY mice. However, neither inhibited DPP-4 activity [101]. Collectively, these findings suggest that mate and probably its active compounds act as an antidiabetic medicine through augmentation of GLP-1 production.

4.5.7. Gingerol and Ginger. Ginger, *Zingiber officinale*, is commonly used as an ingredient in foods and medicine. Compelling data show that ginger extract has hypoglycemic, insulinotropic, and sensitizer effects on healthy humans and on experimental animals [103, 183–185]. More recently, Li and colleagues reported that ginger extract enhanced insulin release and reduced insulin resistance [103]. One clinical study reported that consumption of ginger powder, 3 g per day for 30 days, significantly reduced blood glucose and lipids in T2D patients [186]. Conversely, another study stated that consumption of ginger powder, 4 g daily for 3 months, did not alter blood sugar and lipids in patients with coronary artery disease [187]. This discrepancy may result from the variation in chemical composition of different ginger preparations. Gingerol and shogaol are the main active compounds in ginger extract. Gingerol was shown to attenuate sodium arsenite-induced T2D. This attenuation is related to islet-cell protection and increased insulin receptor signaling [102]. The role of shogaol in T2D treatment is not clear although this compound showed an elevation of glucose uptake in response to insulin in muscle and adipose cells [103].

4.5.8. Epigallocatechin 3-Gallate (EGCG) and Chinese Tea. Chinese tea has been used as a beverage and food supplement since antiquity in China. It is made of the leaves and leaf buds of the *Camellia sinensis* species. One of the claimed health benefits of this tea is reduction of T2D risk and amelioration of T2D. Chinese green tea and oolong tea can prevent and/or ameliorate type 2 diabetes in humans [188–190] and experimental mouse models [191, 192]. EGCG, a major flavonol in tea, was shown to have antidiabetic activities in rodents [104, 105]. EGCG appears to have multiple antidiabetic actions including islet protection, increasing insulin secretion, decreasing insulin tolerance, and decreasing gluconeogenesis and insulin-mimetic action [104–106]. The role of EGCG in islet protection was shown to protect against β-cell death mediated by islet amyloid polypeptide (IAP) *in vitro* [193]. EGCG was reported to activate AMPK in adipocytes [95].

4.5.9. Ishige okamurae. *I. okamurae*, an edible brown seaweed, lowers blood glucose in diabetic db/db mice [88]. Its mode of action involves reduction of insulin resistance and regulation of the hepatic glucose metabolic enzymes [88]. Diphlorethohydroxycarmalol, a phlorotannin of *I. okamurae*, inhibits the activity of α-glucosidase and α-amylase. This compound also decreases postprandial blood glucose level in streptozotocin-treated or normal mice [107].

4.5.10. Soybean. Soybeans are thought to be an important protein source for food. Soybean isoflavones have been reported to treat atherosclerosis, cancer, osteoporosis, and others [194]. In addition, soy protein and isoflavonoids in soybeans improve insulin resistance and enhancement of insulin release [195, 196]. Genistein is a key isoflavone present in soybean (*Glycine max*) and other edible plants and has been reported to have anticancer, antioxidant, anti-inflammatory, and antiosteoporosis activities. More recently, genistein has been reported to treat obesity and diabetes [197]. This compound preserved islet mass by increasing β-cell count, proliferation, and survival in the pancreas [108, 109].

The data demonstrated that genistein could prevent T2D via a direct protective action on β cells without alteration of periphery insulin sensitivity [108]. Moreover, its antidiabetic mechanism involves activation of protein kinase A (PKA) and extracellular-signal-regulated kinases (ERK)1/2. However, another review stated that genistein could activate AMPK and, in turn, led to a reduction in insulin sensitivity [95, 110, 111]. Genistein improved diabetic complications such as vascular dysfunction and wound healing [198, 199]. In a clinical trial, genistein and/or soybean extract reduced the risk of T2D in overweight women [200].

Of note, soybean has been demonstrated to promote the secretion of insulin and GLP-1 [201]. Glyceollins, the phytoalexins produced by soybeans in response to fungi, were demonstrated to reduce hyperglycemia. These compounds could improve glucose-stimulated insulin secretion and prevent apoptosis and dysfunction in β cells in the presence of palmitate [112]. Accordingly, glyceollins enhanced GLP-1 secretion in NCI-H716 cells, an intestinal enteroendocrine L cell line [112]. Further, the antidiabetic action of glyceollin-rich soybean extract was confirmed in diabetic mice [121].

In conclusion, soybean and/or its active components can treat T2D via multiple pathways mainly involving insulin resistance, β-cell function, and GLP-1 production.

4.5.11. Rooibos. Rooibos (*Aspalathus linearis*) is endemic to South Africa. It can be used as a herbal tea to treat diabetes in STZ-treated rats [114]. Aspalathin, a dihydrochalcone C-glucoside of rooibos, reduced hyperglycemia and ameliorated glucose intolerance through increased glucose uptake and insulin secretion in db/db mice [113]. Rutin, quercetin-3-*O*-rutinoside, is an inhibitor of α-glucosidase [115]. Rutin also decreased plasma glucose levels and increased insulin release in STZ-treated rats [116]. An aspalathin/rutin mixture at a ratio of 1 : 1 synergistically reduced blood glucose level in diabetic rats induced by STZ [114]. Additionally, rutin increased glucose uptake in hepatocytes *in vitro* and in mice *in vivo*, implying the function of rutin in insulin resistance [117]. Taken together, rooibos and probably its active compounds can treat T2D via targeting insulin tolerance, β-cell function, and inhibition of α-glucosidase.

4.5.12. Aloe vera. Extract of *A. vera* reduces hyperglycemia and hypercholesterolemia in diabetic patients [202, 203]. Similar antidiabetic effects were observed in alloxan- and STZ-treated animal models [204–206]. Aloeresin A, an active compound of *A. vera*, inhibited α-glucosidase activity [118]. *A. vera* and probably its active compounds exert their antidiabetic actions via inhibition of α-glucosidase and intestinal glucose absorption. In addition, extract of *A. vera* resulted in a reduction of hyperglycemia and insulin resistance [207]. As a whole, *A. vera* and its active components may treat diabetes via suppression of α-glucosidase activity (gut glucose absorption) and insulin resistance.

4.5.13. Quercetin. Quercetin is a flavonoid that is widely distributed in plants and their products. It is commonly used to treat inflammation, viral infections, cancers, and metabolic syndrome. Early studies indicated that quercetin can treat T2D in STZ- and alloxan-treated mouse models and db/db mice [119, 120, 208, 209]. More recently, this compound was shown to lower fasting and postprandial blood glucose levels in diabetic db/db mice without any alteration in serum insulin level [210]. Moreover, like acarbose, quercetin inhibited α-glucosidase activity [211]. On the other hand, quercetin also suppressed DPP-4 activity *in vitro* [208]. However, it remains unclear whether this compound can stimulate GLP-1 production *in vivo*. To sum up, the data suggest that quercetin, a flavonoid commonly found in plants, controls glycemic control via reduction of intestinal glucose absorption and, probably, GLP-1 secretion.

4.5.14. Resveratrol. Resveratrol is a stilbene compound, commonly found in plants and their products. It has a broad spectrum of bioactivities such as hepatoprotective, anticancer, anti-inflammatory, immunomodulatory, antidiabetic, and other activities [212]. Resveratrol has been demonstrated to treat diabetes [213, 214] and related complications [215–220] in different rodent models. When used as a T2D therapy evidence suggests that resveratrol exerts its action through multiple mechanisms. First, this compound can activate AMPK and the downstream molecules, leading to diminution of insulin resistance in db/db mice [121, 122]. It also prevented cell death of pancreatic β cells induced by IAP in culture [123] and in STZ-treated mice [221]. In addition, resveratrol enhanced glucose-mediated insulin secretion in β cells via the activation of SIRT1 [124], one of the cellular targets of resveratrol [222, 223]. A clinical study indicated that resveratrol can improve glycemic control in T2D patients [125]. Collectively, resveratrol ameliorates T2D and complications via the regulation of insulin resistance and β-cell functions.

4.5.15. Coffee. Coffee is one of the most commonly consumed drinks worldwide. Recently, several studies have demonstrated an association between coffee intake and improvement in glucose tolerance and insulin sensitivity and a lower risk of T2D [224]. However, the active compound(s) and responsible target(s) are poorly understood. Accumulating data imply that constituents other than caffeine are active in glycemic control and/or insulin sensitivity. A study on people who consumed caffeinated and decaffeinated coffee showed no difference in the risk of T2D and insulin sensitivity in those drinking either type of coffee after 8 weeks of consumption [225]. However, caffeine improved the function of adipocytes and the liver [225]. Coffee is one of the major sources of dietary antioxidants. Roasting at high temperature can convert chlorogenic acid into quinides, which are known to reduce blood glucose levels in animal models [126]. In addition, coffee consumption might also mediate levels of GLP-1 [127]. Taken together, adequate coffee consumption is beneficial for T2D and its complications.

4.5.16. Therapeutic Application. The paradigm of antidiabetic therapy has shifted from monotherapy to combination therapy. So far, no antidiabetic agents, used alone or in

combination, have been able to cure this disease in humans. A major challenge in the search for efficacious therapies is that the molecular basis of T2D etiology has not yet been fully deciphered. Insulin resistance, β-cell function, glucose (re)absorption in the gut and kidney, and incretin production are the primary targets of current drugs. Compelling data on T2D treatment suggest that multiple targeting of the previous metabolic pathways is a plausible, albeit not yet fully developed approach to reversing T2D. Pharmacological interference of these targets with antidiabetic agents has undesirable side effects. Due to the richness and complexity of the compounds in plants, herbal therapy has always been thought to act on multiple targets in the human body. Even one single compound can have multiple targets, as shown by the role of quercetin in inhibition of DPP-4, α-glucosidase, and other enzymes. Multiple targeting is a double-edged sword in diabetes therapies. The multiple targets associated with antidiabetic herbal medicine make clinical trials complicated, but such an approach is more likely to lead to an eventual cure for T2D. In this review, the antidiabetic potential of the selected glucose-lowering herbs and their different mechanisms of action were summarized and discussed. The up-to-date information presented can be considered a cornerstone for further basic research and investigation into clinical applications of medicinal plants as T2D therapies.

5. Conclusions and Perspective

T2D, a disease known to man for many millennia, causes serious morbidity and mortality in humans. Despite significant progress in T2D and the development of antidiabetic drugs, no cures have been found. Medicinal herbs, long used in alternative and complementary medicine systems, are an extremely rich source of T2D remedies. Currently, understanding of the mechanisms through which herbal therapies mediate T2D is evolving, and they are generally being viewed as modulating of multiple metabolic pathways. Based on safety and their multiple targeting actions, herbal therapies are potent therapeutic means in T2D. Here, we summarized the chemistry and biology of nearly 40 extracts and compounds of plant origin that have been demonstrated to prevent and treat T2D via the regulation of insulin resistance, β-cell function, incretin pathways, and glucose (re)absorption. In addition, the actions, mechanisms and therapeutic potential of plant compounds and/or extracts, and new insights into the advantage of herbal therapy, which simultaneously governs distinct metabolic pathways immune cells and β cells, were discussed for T2D. Systematic information about the structure, activity, and modes of action of these plants and compounds will pave the way for research and development of antidiabetic drugs.

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Research Article

Alternanthera sessilis Red Ethyl Acetate Fraction Exhibits Antidiabetic Potential on Obese Type 2 Diabetic Rats

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The antidiabetic potential of *Alternanthera sessilis* Red was investigated using the obese type 2 diabetic rats induced by high fat diet and streptozotocin. Three fractions (hexane, ethyl acetate, and water) were obtained from the crude ethanol extract of *Alternanthera sessilis* Red. *Alternanthera sessilis* Red ethyl acetate fraction (ASEAF) was found to possess the most potent antihyperglycemic effect through oral glucose tolerance test. The ASEAF was subsequently given to the diabetic rats for two weeks. It was found that two-week administration of ASEAF reduces the fasting blood glucose level, triglyceride level, and free fatty acid level of the rats. ASEAF-treated diabetic rats showed higher pancreatic insulin content and pancreatic total superoxide dismutase activity compared to the untreated diabetic rats. Also, the insulin sensitivity indexes suggested that ASEAF ameliorates the insulin resistant state of the diabetic rats. In conclusion, ASEAF could be developed into a potential antidiabetic agent for the management of type 2 diabetes.

1. Introduction

Type 2 diabetes mellitus (T2D) is a group of metabolic disorders that affect more than 90% of the diabetes population. Prevalence of diabetes mellitus in Malaysia is increasing alarmingly. As mentioned by Shaw et al., the national prevalence of diabetes in Malaysia in year 2011 is 11.7% [1]. This is contributed by several factors such as diabetes care facilities, ethnicity, socioeconomic status, urbanization, and most importantly the western lifestyles adopted [2]. With the undesirable side effects of the antidiabetic agents found in the market nowadays, traditional medicine serves an important role in the discovery of new antidiabetic agent with lesser and fewer side effects. One classic example is the alkaloid berberine. Berberine is an inhibitor of dipeptidyl peptidase IV [3]. Yin et al. [4] showed that berberine has similar antihyperglycemic potency to metformin (a common antidiabetic agent) and possessed antihypertriglyceridemic effect which is not seen in metformin. Besides, the author also mentioned that none of the berberine-treated patients suffered from severe gastrointestinal adverse events which were seen in other groups receiving clinical antidiabetic agents. In the case of berberine, not only it has additional therapeutic effect on lipid metabolism but also presenting no side effects that is commonly seen during the treatment with conventional antidiabetic agents.

The wild type of *A. sessilis* has green aerial parts. A decoction of *A. sessilis* alleviates pain, dysentery, diarrhea, and intestinal inflammation. Also, *A. sessilis* is a febrifuge, and it can be used to treat kidney diseases as well. It is often consumed as vegetable in India. Several therapeutic benefits of the wild (green) *A. sessilis* had been investigated which include anti-inflammatory effect [5], the nootropic activity [6], cytotoxic effect towards pancreatic cancer cell lines [7], and the free radical-scavenging ability [8]. The cultivar of this plant has red instead of green aerial parts. This cultivar is more commonly found in Malaysia and Singapore than the wild type. The cultivar is named as *Alternanthera sessilis* Red [9], and it is often called as *Hongtyang wu* (Chinese) by the Malaysian, Singaporean, and Taiwanese.

Traditionally, this cultivar is believed to be able to reduce the risk of cardiovascular disease. In our lab, we found that

the crude ethanol extract was able to reduce the formation of atheroma plaque in the blood vessel of the rabbits treated with high cholesterol diet. On the other hand, the crude ethanol extract showed blood glucose lowering effect in the preliminary study carried out in our lab. In the present study, we further explored the antidiabetic potential of the crude ethanol extract and identified the active fraction which is responsible for the antidiabetic effect as well as the physiological mechanism of antidiabetic action.

2. Methods

2.1. Preparation of Crude Ethanol Extract and Fractions from Alternanthera sessilis Red. The plant *Alternanthera sessilis* Red (ASR) were obtained from Bukit Tinggi, Selangor, Malaysia. It was identified by a botanist Dr. Sugumaran Manickam, and a voucher specimen was deposited in the herbarium at Rimba Ilmu of University of Malaya (KLU 47693).

The aerial parts of ASR were dried in the oven at 40°C and crushed into powder. One kilogram of the powder was extracted with 3 L of 95% ethanol at room temperature for three days. This extraction step was repeated three times. The mixture was then filtered with Whatman number 1 filter paper. The solvent was removed using RV10 rotary evaporator (IKA, Guangzhou), and the resulting residue was the crude ethanolic extract (112.5 g).

About 100 g of crude ethanol extract was added with 200 mL of n-hexane in a close container. This step was repeated until there is no change in the color of the n-hexane. The mixture was filtered, and the filtrate was concentrated by the rotary evaporator, yielding the hexane fraction (ASHXF). The hexane-insoluble residue was subjected to partitioning between ethyl acetate and distilled water in a separatory funnel. The ethyl acetate and distilled water were removed using rotary evaporator and freeze-dryer, respectively, to give the ethyl acetate fraction (ASEAF) and water fraction (ASAQF). The amount of ASHXF, ASEAF, and ASAQF is 42.1 g, 39.7 g, and 18.2 g, respectively.

2.2. Induction of Obese Type 2 Diabetes in Rats. Male Sprague-Dawley (SD) rats weighed between 200–230 g were obtained from the Laboratory Animal Centre, Faculty of Medicine, University of Malaya. The rats were housed in the animal room in Department of Physiology, Faculty of Medicine, University of Malaya, under standard environmental conditions, relative humidity, and dark/light cycle. All animals were acclimatized for one week before any experimental procedures, and all experimental procedures were approved by the animal ethical committee of University of Malaya (Ethic number: FIS/27/01/2010/TKK (R)).

The induction of diabetes was done according to Srinivasan et al. [10] with slight modification. Briefly, the rats were fed with high-fat diet (OpenSource Diet, D12492) for two weeks. On day 15, the overnight fasted (16 hours) rats were injected with 40 mg/kg of streptozotocin (STZ) intraperitoneally. After the injection of STZ, the rats were given 5% glucose solution for 24 hours to prevent hypoglycemia. Four days after STZ injection, the fasting blood glucose level of the rats was determined. Rats with blood glucose level more than 16.5 mmol/L were selected for subsequent experiment.

2.3. Determination of Active Fraction. The blood glucose lowering effect of the fractions was determined by oral glucose tolerance test (OGTT) [11]. Briefly, the diabetic rats were divided into 5 groups ($n = 5$) and were fasted overnight for 16 hours. On the next day, the diabetic rats in different groups were fed with 500 mg/kg fractions (hexane, ethyl acetate, and water), 30 mg/kg glibenclamide (positive control), and a dose volume of 2 mL/kg of 1% carboxymethylcellulose (CMC) prepared in distilled water (negative control), respectively. All fractions and glibenclamide were dissolved in 1% carboxymethylcellulose. After 30 minutes, the blood glucose level of the diabetic rats was measured using a glucometer (AccuChek Advantage). Subsequently, 2 g/kg glucose solution (dissolved in distilled water) was administered to all the diabetic rats. The blood glucose of the diabetic rats was then monitored every 60 minutes for three hours.

An OGTT curve was plotted, and the area under the curve was calculated using the trapezoid rule [12] as follows:

$$\text{AUC} = \frac{C1 + C2}{2} \times (t2 - t1), \quad (1)$$

where $C1$ and $C2$ are blood glucose concentrations at time points $t1$ and $t2$, respectively.

2.4. Effect of Two-Week Administration of ASEAF on the Diabetic Rats. Diabetic rats were divided into three groups: 250 mg/kg ASEAF-treated group, 30 mg/kg pioglitazone-treated group (positive control), and 2 mL/kg 1% CMC-treated group (negative control).

2.4.1. Sample Collection. Blood samples were collected from the tail vein. The blood glucose levels were determined using a glucometer. The pancreas and liver were harvested after the rats being sacrificed through cervical dislocation.

2.4.2. Biological Assays. Plasma and serum samples were prepared from the blood samples collected according to the instructions provided in the assay kits.

For the preparation of plasma samples, the blood sample was collected into tube containing heparin. Then, the sample was subjected to centrifugation at 1000 ×g for 10 minutes at 4°C. The top layer is the plasma sample.

For the preparation of serum samples, the blood sample collected was first allowed to clot for 30 minutes at room temperature. Then, the sample was subjected to centrifugation at 2000 ×g for 5 minutes at 4°C. The top layer is the serum sample.

The samples were labeled and stored at −80°C. On the day of experiment, the samples were thawed at room temperature. The organs harvested were washed with saline solution and stored at −80°C until used.

The plasma triglyceride level (Cayman Chemical, USA), plasma-free fatty acid level (EnzyChrom, USA), serum insulin level (Mercodia, Sweden), and superoxide dismutase

activity (Cayman Chemical, USA) were determined by using the commercial assay kits.

2.4.3. Liver Triglyceride Content. Adopted from Lian et al. [13], 0.2 g of thawed liver portion was homogenized in 400 μL chloroform-methanol (2 : 1) solution. The mixture was subjected to sonication for 15 minute at room temperature. Subsequently, the mixture was centrifuged at 5000 rpm for 10 minutes. The supernatant was washed with 0.2 volume of 0.9% NaCl saline and centrifuged again at 2000 rpm for 5 minutes. Two phases were obtained, and the lower phase was evaporated. The residual was dissolved in 0.5 mL of isopropanol which contained 10% Triton X-100 before subjected to the triglyceride assay using the assay kit. The liver triglyceride content was expressed as mg/g wet tissue.

2.4.4. Pancreatic Insulin Content and Pancreatic Total Superoxide Dismutase Activity. The extraction of pancreatic insulin was adopted from Portha et al. [14]. 0.2 g of thawed pancreas portion was placed in a centrifuge tube containing 5.0 mL of ice-cold acid-alcohol solution. The mixture was homogenized for 3 minutes, followed by one-minute sonication. The solution was left to stand at −20°C overnight and centrifuged at 3000 rpm at 4°C for 15 minutes in the next day. The supernatant was transferred into a new centrifuge tube and stored at −20°C, while the pellet was subjected to extraction again. Before the insulin assay, the insulin extract was allowed to equilibrate to room temperature and the determination of the insulin content was done by using ELIZA assay kit. The pancreatic insulin content was expressed as μg/mg wet tissue.

About 0.2 g of thawed pancreas portion was homogenized in 5.0 mL of cold 20 mM HEPES buffer, pH 7.2. The mixture was subjected to centrifugation at 3000 rpm for 5 minutes at 4°C. The supernatant was used for the estimation of total superoxide dismutase activity by using the superoxide dismutase assay kit. The superoxide dismutase activity was expressed as unit/mg wet tissue.

2.4.5. Insulin Sensitivity Indexes (HOMA and QUICKI). The homeostasis model assessment (HOMA) and quantitative insulin sensitivity check index (QUICKI) were calculated according to Cacho et al. [15] as follows:

$$\text{HOMA} = \frac{\text{fasting BGL} \times \text{fasting SIL}}{2430},$$
$$\text{QUICKI} = \frac{1}{\log_{10}(\text{fasting BGL}) + \log_{10}(\text{fasting SIL})}, \quad (2)$$

where the fasting BGL was in mg/dL, while the fasting SIL was in μU/mL.

2.5. Preliminary Phytochemical Screening. The ASEAF was subjected to a preliminary phytochemical screening. ASEAF was dissolved in dichloromethane and spotted on a thin-layer chromatography (TLC) plate. The TLC plate was then allowed to develop in a chamber saturated with ethyl acetate and chloroform in the ratio of 6 : 4 (ethyl acetate : chloroform). Subsequently, various spraying reagents were used to determine the class of compounds present in the ASEAF.

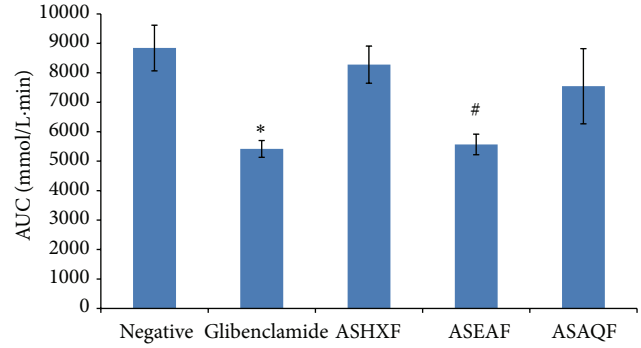

Figure 1: Effect of different fractions of the crude ethanolic extract of *Alternanthera sessilis* Red on OGTT. The diabetic rats were administered with 500 mg/kg of hexane fraction (ASHXF), ethyl acetate fraction (ASEAF), and water fraction (ASAQF). The data shown are the AUC of OGTT and are expressed as mean ± SEM, 5 rats ($n = 5$) per group. $^{*}P < 0.05$, $^{\#}P < 0.01$ versus negative control group.

2.6. Statistical Analysis. Values were represented as mean ± standard error from mean (SEM) with "n" represents sample size. Paired and unpaired Student's t-test was conducted using SPSS 17.0. A P value less than 0.05 ($P < 0.05$) was considered significant.

3. Results

3.1. Determination of Active Fraction. Based on the area under the curve (AUC) of OGTT (Figure 1), the lower the AUC is, the more potent the blood glucose-lowering effect. In this experiment, glibenclamide is a clinical anti-diabetic agent and was used as positive control in the OGTT. It is clear that 10 mg/kg of glibenclamide significantly suppressed the rise in the BGL ($P < 0.05$) when compared with the negative control group which received 1% CMC. Among the fractions, 500 mg/kg EAF produced a more significant blood glucose-lowering effect than glibenclamide ($P < 0.01$) when compared with the negative control group. HXF and AQF did not show significant antihyperglycemic effect in this experiment ($P > 0.05$). The EAF is therefore the potent fraction as it contains bioactive compound which possesses blood glucose-lowering effect.

3.2. Determination of Effect of Two-Week Administration of ASEAF on the Diabetic Rats. In the two-week study, 30 mg/kg pioglitazone (a potent insulin sensitizer) was used as a positive control, whereas the negative control group consisted of diabetic rats given with 2 mL/kg of 1% CMC (the vehicle). The fasting blood glucose level (BGL) of both ASEAF-treated group and pioglitazone-treated group was significantly lower than the negative control group ($P < 0.01$) and their respective day 0 values ($P < 0.05$) as shown in Figure 2. However, the serum insulin levels (SIL) of both

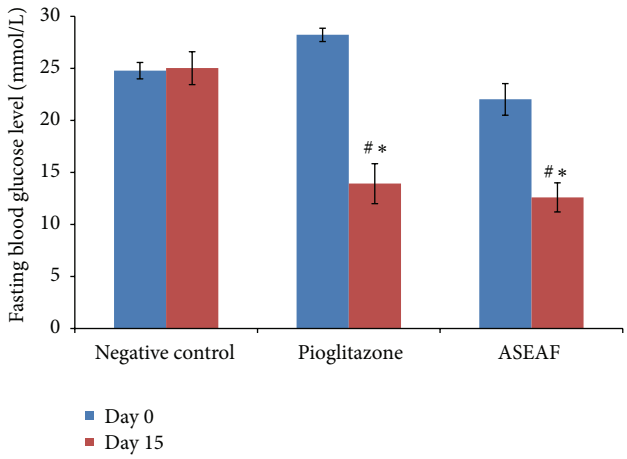

Figure 2: Effect of ASEAF on the fasting blood glucose level of the diabetic rats after two-week administration of 250 mg/kg ASEAF. Data shown are the fasting blood glucose level (mmol/L) and are expressed as mean ± SEM 6 rats ($n = 6$) per group. $^{*}P < 0.05$ versus negative control; $^{\#}P < 0.05$ versus day 0 value (before treatment).

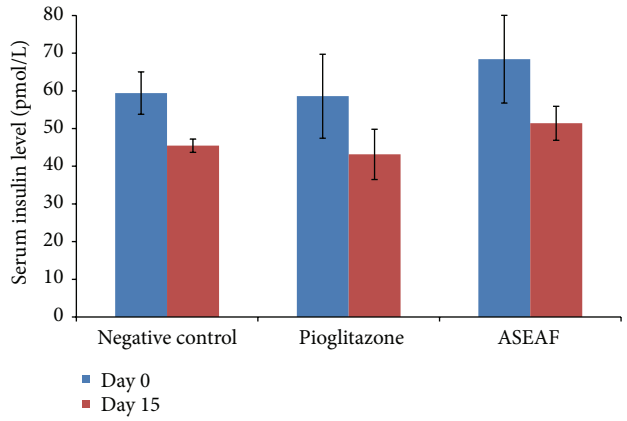

Figure 3: Effect of ASEAF on the fasting serum insulin level of the diabetic rats after two-week administration of 250 mg/kg ASEAF. Data shown are the fasting serum insulin level (pmol/L) and are expressed as mean ± SEM, 6 rats ($n = 6$) per group. No significant difference was found.

groups did not show any significant difference ($P > 0.05$) when compared with the negative control group and their respective day 0 values (Figure 3).

The salient point is that the decrease in the fasting BGL leads us to reckon that the possible mechanism of action of ASEAF was through the alleviation of insulin resistance. Hence, the HOMA and QUICKI were computed. As shown in Table 1, the HOMA indexes of the ASEAF-treated group and pioglitazone-treated group were significantly lower than the negative control group ($P < 0.001$) and their respective values before treatment ($P < 0.05$ and $P < 0.001$, resp.). On the other hand, the QUICKI values of both treatment groups were significantly higher than the negative control group ($P < 0.001$) and their respective values before treatment. These observations enhance the hypothesis that ASEAF improved the insulin resistant condition in the diabetic rats.

Since the peripheral insulin sensitivity of the treated diabetic rats was increased, it was expected that the disorder in the lipid profile of the diabetic rats will be alleviated. Indeed, the plasma triglyceride (TG) level and plasma free fatty acid (FFA) level of the pioglitazone-treated group and ASEAF-treated group were significantly lower than the negative control group ($P < 0.05$) as shown in Figures 4 and 5, respectively. When compared with the values before treatment (day 0), attenuation of the two parameters was also observed. After treatment with 30 mg/kg pioglitazone, the plasma TG level, and plasma FFA level of the treated rats decreased 52.21% and 35.57%, respectively. Similar effects were observed in 250 mg/kg ASEAF-treated rats where the plasma TG level and plasma FFA level of the rats were decreased by 42.04% and 34.38%, respectively.

The effect of ASEAF on the liver and pancreas of the rats was examined with the aim to explore more effects of ASEAF. As shown in Figure 6, ASEAF did not significantly alter the liver triglyceride content of the diabetic rats when compared to the negative control group ($P > 0.05$). On the contrary, pioglitazone significantly decreased the liver triglyceride content when compared with the negative control group ($P < 0.05$).

Considering that the SIL was not significantly altered ($P > 0.05$), it was presumed that the functionality of the pancreas is preserved. As can be seen in Figure 7, both ASEAF and pioglitazone increased the pancreatic insulin content of the diabetic rats ($P < 0.05$). The pancreatic total superoxide dismutase (SOD) activity of the rats was measured to gain insight on the integrity of the antioxidant system of the pancreas. Figure 8 shows that the pancreatic total SOD activity of both ASEAF-treated group and pioglitazone-treated group was significantly higher than the negative control group ($P < 0.05$).

3.3. Phytochemical Screening. Spraying reagents contain chemicals that bind to specific functional group of a compound and producing colors that allows the researcher to identify the presence of a specific class of compound. Preliminary phytochemical screening indicates that ASEAF contains phenolic compound, terpenoids, alkaloid, and secondary amines.

4. Discussion

The combination of high fat diet and low dose streptozotocin (STZ) has been commonly used to induce obese type 2 diabetes in the rats [10]. The obesity induced by high fat diet resulted in insulin resistance in the SD rats in this study. The low dose of STZ injected to the insulin resistant rats produced frank hyperglycemia which mimics human type 2 diabetes.

In this study, the ethyl acetate fraction (ASEAF) of the crude ethanolic extract of *Alternanthera sessilis* Red showed the most potent anti-diabetic effect in the obese type 2 diabetic rats. Judging from the decreased fasting BGL and the statistically unchanged SIL, it was hypothesized that ASEAF

Table 1: Effect of ASEAF on the insulin resistant state of the diabetic rats. The insulin sensitivity indexes (HOMA and QUICKI) were used to quantify the effect. The index values before and after 250 mg/kg ASEAF oral administration were shown.

	HOMA index		QUICKI	
	Before	After	Before	After
Negative	1.563 ± 0.169	1.197 ± 0.038	0.280 ± 0.003	0.289 ± 0.001
Pioglitazone	1.746 ± 0.348	0.646 ± 0.103[a,b]	0.279 ± 0.005	0.316 ± 0.006[a,d]
ASEAF	1.616 ± 0.302	0.689 ± 0.109[a,c]	0.282 ± 0.006	0.313 ± 0.005[a,b]

Data shown are the calculated index values and are presented as mean ± SEM, 6 rats ($n = 6$) per group; [a]$P < 0.001$ versus negative control group; [b]$P < 0.01$; [c]$P < 0.05$; [d]$P < 0.001$ versus before treatment.

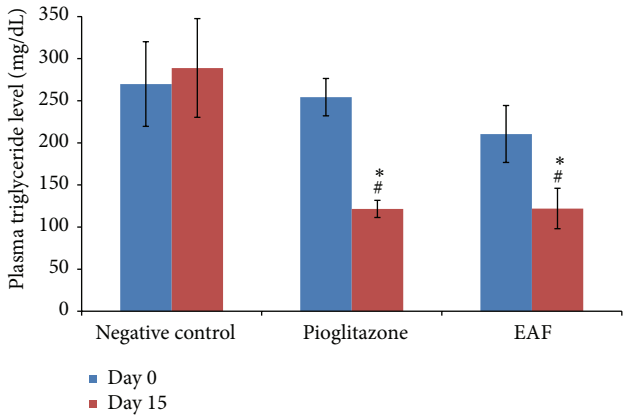

Figure 4: Effect of ASEAF on the fasting plasma triglyceride level of the diabetic rats after two-week administration of 250 mg/kg ASEAF. Data shown are fasting plasma triglyceride level (mg/dL) and are expressed as mean ± SEM, 6 rats ($n = 6$) per group. $^{*}P < 0.05$ versus negative control; $^{\#}P < 0.05$ versus day 0 value (before treatment).

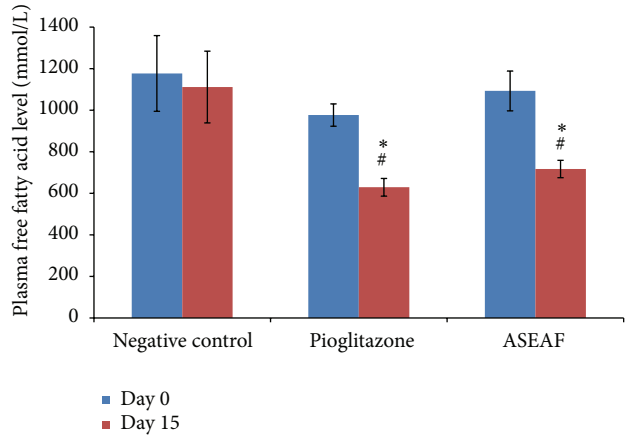

Figure 5: Effect of ASEAF on the fasting plasma-free fatty acid level of the diabetic rats after two-week administration of 250 mg/kg ASEAF. Data shown are fasting plasma free fatty acid level (mmol/L) and are expressed as mean ± SEM, 6 rats ($n = 6$) per group. $^{*}P < 0.05$ versus negative control; $^{\#}P < 0.05$ versus day 0 value (before treatment).

does not stimulate the pancreatic β cells to secrete more insulin. Rather, it improved the peripheral insulin sensitivity. This is because at a similar level of insulin in the blood of the diabetic rats in different groups, the fasting BGL of the treatment groups (ASEAF treated and pioglitazone treated) were lower, indicating that the decrease in BGL was brought about by the enhanced insulin action. Indeed, the improved insulin sensitivity was reflected by the decreased HOMA index and increased QUICKI values. HOMA [16] and QUICKI [17] are often used in replacement to the hyperinsulinemic-euglycemic clamp study due to the high positive correlation between these indexes to the results obtained from the hyperinsulinemic-euglycemic clamp study.

ASEAF reduced the plasma FFA level, halting the worsening of the diabetic condition in the rats. When ASEAF increases peripheral insulin sensitivity, lipolysis is suppressed, and FFA level is reduced. ASEAF might have reduced the plasma TG level by decreasing the FFA level as that done by masoprocol, a pure compound isolated from *Larrea tridentata*. Masoprocol inhibits hormone-sensitive lipase (HSL) via, possibly, the dephosphorylation of HSL by increasing phosphatase activity [18]. Pioglitazone improves peripheral insulin resistance by activating peroxisome proliferator-activated receptor-gamma (PPARγ). Hence, a similar trend of changes in the plasma TG level and plasma FFA level

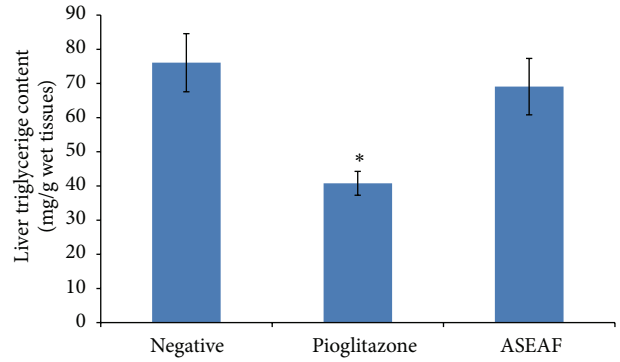

Figure 6: Effect of ASEAF on the liver triglyceride content of the diabetic rats after two-week administration of 250 mg/kg ASEAF. Data shown are liver triglyceride content (mg/g wet tissues) and are expressed as mean ± SEM, 6 rats ($n = 6$) per group. $^{*}P < 0.05$ versus negative control.

observed in the ASEAF-treated group was seen in the pioglitazone-treated group as well.

Hypertriglyceridemia was not observed in the ASEAF-treated group and the liver triglyceride content was of no significant changes when compared with the negative control

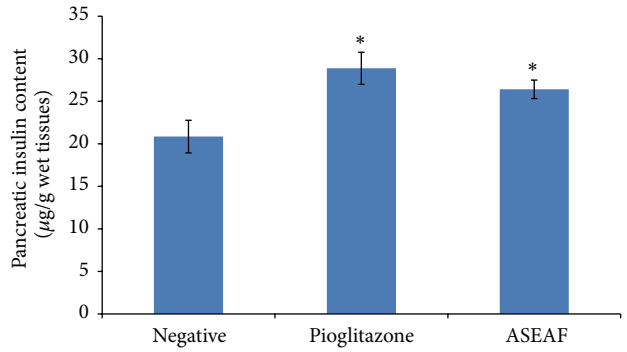

Figure 7: Effect of ASEAF on the pancreatic insulin content of the diabetic rats after two-week administration of 250 mg/kg ASEAF. Data shown are pancreatic insulin content (μg/g wet tissues) and are expressed as mean ± SEM, 6 rats ($n = 6$) per group. $^{*}P < 0.05$ versus negative control.

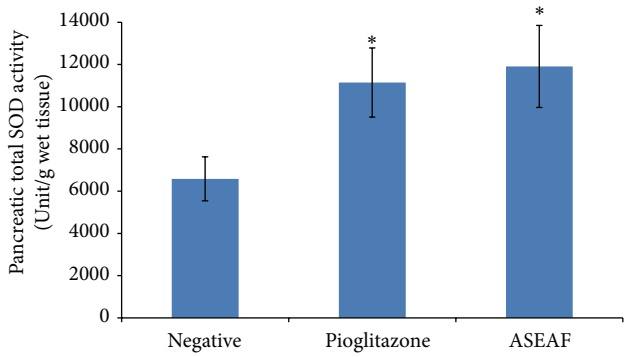

Figure 8: Effect of ASEAF on the pancreatic total superoxide dismutase activity of the diabetic rats after two-week administration of 250 mg/kg ASEAF. Data shown are pancreatic total superoxide dismutase activity (unit/g wet tissues) and are expressed as mean ± SEM, 6 rats ($n = 6$) per group. $^{*}P < 0.05$ versus negative control.

group. This observation suggests that the antihypertriglyceridemic action of ASEAF might be on other target site such as the musculature or the adipose tissues instead of the liver. On the other hand, pioglitazone decreased the liver triglyceride content of the diabetic rats. Murase et al. [19] suggested that the antidiabetic effect and ameliorative effect of pioglitazone on lipid metabolism abnormalities are associated with its suppressive effect on TNFα production.

The liver and muscles are two common sites of insulin resistance with muscle insulin resistance come after hepatic insulin resistance [20]. Plasma FFA level has been known to cause hepatic insulin resistance [21]. When insulin resistance is developed, a vicious cycle takes place, whereby lipid metabolism will be deranged, and more FFA will be produced. The liver abstracts more FFA when the FFA concentration in the blood increases. Subsequently, the hepatocytes synthesize more TG from the FFA and incorporate the TG into VLDL. Furthermore, lipolysis is not suppressed under insulin resistance, producing and releasing more FFA into the circulation. Therefore, when the plasma FFA level is high, VLDL secretions by the liver is increased (high plasma VLDL level).

Chronic FFA-induced insulin hypersecretion reduces insulin biosynthesis in the pancreatic β cells [22]. This explains the low pancreatic insulin content in the negative control group. Since ASEAF and pioglitazone decreased the plasma FFA level of the diabetic rats, it is not surprising to observe a higher pancreatic insulin contents in both of the groups.

The pancreatic total superoxide dismutase activity of both of the treatment groups was higher than the negative control group. Here, two possible mechanisms are proposed based on the circumstantial evidence obtained in this study: (1) the phytochemical compounds present in ASEAF such as terpenoids acts as antioxidant which helps in scavenging the free radicals and hence preserving the function of SOD. Terpenoids are known to possessed antioxidative capacity [23]. Quintans-Júnior et al. demonstrated that (+)-camphene, which is a type of terpenoids, possessed powerful antioxidant effect [24], or (2) ASEAF improves peripheral insulin sensitivity which reduces blood glucose concentration. When hyperglycemia is improved, the rate of protein glycation (SOD in this case) can be slowed down. Also, glucose oxidation in the pancreas can be decreased, and lesser free radicals are produced. Thence, the total SOD activity was preserved. Other possible mechanism which might contribute to the higher pancreatic SOD activity includes the increase in interleukin-1 beta which increases the transcription of SOD gene and so the SOD activity [25]. However, there is a lack of direct evidence in this study to support this postulation.

As the diabetic condition progresses, the pancreatic β cells are exhausted. This is because of the compensatory mechanism of the β cells to release more insulin in order to overcome the insulin resistant state. Persistent hyperglycemia tends to cause glycation of different proteins *in vivo*. Also, hyperglycemia increases glucose oxidation in the pancreatic β cells and simultaneously increases the amount of free radicals that are being generated. Superoxide dismutase (SOD) is an important antioxidant system in the pancreas, the Cu-Zn-SOD subtype in particular. Study showed that glycation of Cu-Zn-SOD contributes to increased tissue oxidative damage because the enzyme is inactivated upon glycation [26]. Thus, preserving the function of SOD would no doubt reduce the oxidative damage to the pancreatic β cells and maintain the normal function of the β cells in insulin biosynthesis and secretion.

The preliminary phytochemical study revealed that the ASEAF contains phenolic compounds, terpenoids, alkaloids, and secondary amines (Table 2).

5. Conclusions

We demonstrated that ASEAF possesses antihyperglycemic effect, antitriglyceridemic effect, and pancreatic protective effect in obese type 2 diabetic rats. The major suggested physiological mechanism of anti-diabetic actions of ASEAF is its ameliorative effect on insulin resistance in the diabetic rats, and the pancreatic protective effect is contributed by

Table 2: Preliminary results of the phytochemical screening on ASEAF. Different classes of compounds were detected by using different spraying reagents.

Reagents	Type of compound	Observations	Inferences
0.5% ninhydrin in acetone	Amino acid	Yellow spots	Absence of amino acid but presence of secondary amine
10% $SbCl_3$ in chloroform	Terpenoid	Green zones with different intensities	Absence of carotenoid; presence of terpenoids
Vanillin in H_2SO_4: CH_3COOH:CH_3OH	Terpenoid	Purple zone	Presence of terpenoids
Dragendorff's reagent	Alkaloid	Orange zone	Presence of alkaloids
5% $FeCl_3$ (aq)	Phenolic compound	Some green zones	Presence of phenolic

the antioxidative potential of ASEAF via the superoxide dismutase. Further studies are on the way to pile up more data and evidence in order to elucidate the molecular mechanism of action using both *in vitro* and *in vivo* methods. Also, isolation and identification of the bioactive compound(s) that responsible for the antidiabetic effect are in progress. Overall, ASEAF could be a potential antidiabetic agent for the management of obese type 2 diabetes.

Conflict of Interests

The authors declare that there is no conflict of interests.

Acknowledgment

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Research Article

Antidiabetic Effect and Mode of Action of Cytopiloyne

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Cytopiloyne was identified as a novel polyacetylenic compound. However, its antidiabetic properties are poorly understood. The aim of the present study was to investigate the anti-diabetic effect and mode of action of cytopiloyne on type 2 diabetes (T2D). We first evaluated the therapeutic effect of cytopiloyne on T2D in db/db mice. We found that one dose of cytopiloyne reduced postprandial glucose levels while increasing blood insulin levels. Accordingly, long-term treatment with cytopiloyne reduced postprandial blood glucose levels, increased blood insulin, improved glucose tolerance, suppressed the level of glycosylated hemoglobin A_{1c} (HbA_{1c}), and protected pancreatic islets in db/db mice. Next, we studied the anti-diabetic mechanism of action of cytopiloyne. We showed that cytopiloyne failed to decrease blood glucose in streptozocin- (STZ-)treated mice whose β cells were already destroyed. Additionally, cytopiloyne dose dependently increased insulin secretion and expression in β cells. The increase of insulin secretion/expression of cytopiloyne was regulated by protein kinase Cα (PKCα) and its activators, calcium, and diacylglycerol (DAG). Overall, our data suggest that cytopiloyne treats T2D via regulation of insulin production involving the calcium/DAG/PKCα cascade in β cells. These data thus identify the molecular mechanism of action of cytopiloyne and prove its therapeutic potential in T2D.

1. Introduction

Insulin is indispensable for glucose homeostasis in mammals. Insulin biosynthesis at transcriptional and translational levels, and its secretion in β cells, is well regulated by blood glucose [1]. Calcium ions, potassium ions, phospholipase C, DAG, phosphatidylinositol triphosphate (IP3), PKC, and protein kinase A (PKA) are involved in insulin secretion and, likely, insulin biosynthesis in pancreatic β cells [2]. On binding to insulin, an insulin receptor initiates a signaling cascade and eventually causes glucose uptake in peripheral tissues. Any defect in insulin synthesis/secretion or action, or both, may result in hyperglycemia, a major pathological feature of type 2 diabetes (T2D) [3]. Such hyperglycemia is detrimental to β cells and insulin target tissues, and this glucotoxicity is clinically relevant as a cause of diabetes-related complications such as nephropathy, retinal blindness, neuropathy, peripheral gangrene, and cardiovascular disease [4]. Therefore, maintenance of glycemic homeostasis is the most common therapeutic aim for patients with T2D.

Diabetes is a life-threatening metabolic disease, afflicting around 5% of the world population. Over 90% of the diabetic population is diagnosed with T2D mellitus [5, 6]. Current antihyperglycemic drugs are insulin secretagogues, insulin sensitizers, inhibitors of sugar cleavage, and glucagon-like peptide-1 (GLP-1), all of which control homeostasis of blood sugar by different mechanisms [7]. Common drawbacks of such drugs include significant side effects, decreased efficacy over time, low cost-effectiveness, and only partial anti-diabetic effect of each individual drug [8]. Of note,

secretagogues with the ability to prevent adverse effects (e.g., weight gain and hypoglycemia), to stimulate insulin biosynthesis, or to protect β cells from death are rare [7, 9]. GLP-1, an injectable peptide drug, may be the only one reported to fit these criteria [10]. In view of patients' welfare, there is still an obvious need for development of antidiabetics that protect against hypoglycemia, enhance insulin synthesis, or improve β-cell protection.

Plants are an extraordinary resource for anti-diabetic remedies [11, 12]. One outstanding example is metformin, a derivative of guanidine that was first isolated from French lilac and is a commonly prescribed insulin sensitizer for treatment of T2D [13]. Further, extracts prepared from the plant *Bidens pilosa*, a member of the Asteraceae family, were shown to have anti-diabetic properties in alloxan-treated mice [14] and have been used to treat patients with diabetes in America, Africa, and Asia [11, 15]. Two polyacetylenes isolated from *B. pilosa* have demonstrated anti-diabetic properties by two different laboratories [16, 17]. More recently, another polyacetylene, cytopiloyne, was identified in *B. pilosa* and shown to be highly potent in the prevention of type 1 diabetes via T-cell regulation [18]. *B. pilosa* and its three polyacetylenes showed glucose-lowering activities in diabetic mice [16, 19, 20]. However, the long-term therapy and mechanism of these three polyacetylenes for T2D are not known.

The db/db mice whose leptin receptor gene is mutated spontaneously develop diabetes because of insulin resistance [21]. STZ-treated mice represent a chemical-inducible model that exhibits insufficient insulin production [22]. Both models reflect main causes of T2D [23]. In this study, we studied the anti-diabetic potential and mechanism of cytopiloyne in db/db mice and STZ-treated C57BL mice and in pancreatic β cells.

2. Materials and Methods

2.1. Ethics Statement. All animals were maintained and handled according to the institutional guidelines and the protocol was approved by the Academia Sinica Animal Care and Utilization Committee (protocol number: OMiIBAYW2010043).

2.2. Chemicals, Cells, and Animals. Dimethyl sulfoxide (DMSO), STZ, nimodipine, EGTA, metformin, glimepiride, brefeldin A, hematoxylin, eosin, phorbol 12-myristate 13-acetate (PMA), glimepiride, 1-stearoyl-2-arachidonoyl-*sn*-glycerol, cholesterol, and diaminobenzidine tetrahydrochloride were purchased from Sigma-Aldrich (MO, USA). Anti-actin, anti-HA, and anti-insulin antibodies were purchased from Santa Cruz Biotechnology (CA, USA). Insulin (Novo Nordisk, NJ, USA) and anti-PKCα (Abcam, MA, USA) and antiphospho-PKCα (Millipore, MA, USA) antibodies were purchased. Cytopiloyne was prepared to 98% purity from *B. pilosa* as previously described [17, 18]. Cytopiloyne dissolved in DMSO was stored in a light protected vial at −20°C. After one year storage, more than 90% of the isolated cytopiloyne was stable, as was confirmed by structural determination by NMR spectroscopy. RIN-m5F cells (CRL-11605), a rat β-cell line, were obtained from the American Type Culture Collection. Primary pancreatic islets were isolated from fasted Wistar rats obtained from the National Laboratory Animal Center (NLAC) in Taipei, Taiwan. C57BL and db/db mice [24] were obtained from the NLAC. All animals were maintained in the institutional animal facility and handled according to the guidelines of the Academia Sinica Institutional Animal Care and Utilization Committee.

2.3. Drug Administration in db/db Mice. For single-dose administration, diabetic db/db males aged 6 to 8 weeks, with free access to food, were grouped and tube fed with either 0.2 mL vehicle (1 μL DMSO per 1 mL of PBS), cytopiloyne (0.1, 0.5, and 2.5 mg/kg body weight (BW)), or glimepiride (2.5 mg/kg BW). After 0.5 h, the levels of postprandial blood sugar and insulin from the mice were monitored for an additional 4 h. For continuous administration, diabetic db/db males were grouped and tube-fed with vehicle, cytopiloyne (0.5 or 2.5 mg/kg/day), or glimepiride (2.5 mg/kg/day) for the indicated time, while levels of blood sugar, insulin, and glycosylated HbA_{1c} and glucose tolerance in these mice were determined. Unless otherwise indicated, the mice were fasted for 16 h and then postprandial blood glucose and insulin in these mice were measured. A portion of mice were sacrificed for immunohistochemical staining. The rest were maintained to determine survival rates.

2.4. Drug Administration in STZ-Treated Mice. To deplete pancreatic β cells in mice, 6-week-old C57BL females were intraperitoneally injected with STZ at 200 mg/kg. STZ-treated females with postprandial blood sugar over 500 mg/dL were grouped. Each group was either tube-fed with vehicle (1 μL DMSO per 1 mL of PBS), cytopiloyne (0.1, 0.5, and 2.5 mg/kg), or glimepiride (2.5 mg/kg) or injected with insulin at 2.5 IU/kg BW. Blood glucose levels were monitored for 4 h. To distinguish sensitizer activity from releaser activity of cytopiloyne, STZ-treated C57BL mice were tube-fed with vehicle, glimepiride (2.5 mg/kg), metformin (60 mg/kg), and cytopiloyne (0.5 and 2.5 mg/kg) 1 h before insulin injection (2.5 IU/kg). Blood glucose levels in the mice were monitored from 0 to 4 h after insulin injection.

2.5. Measurement of Glucose, Insulin, and HbA_{1c}. Glucose levels in mouse blood samples were measured using an Elite glucometer (Bayer, PA, USA). Insulin levels in blood samples or islet cell supernatants were determined by ELISA assays (Mercodia, Uppsala, Sweden). The levels of glycosylated HbA_{1c} in blood samples were measured using a DCA 2000 analyzer (Bayer, PA, USA).

2.6. Insulin Secretion. Pancreatic islets (10 islets/mL) from fasted male Wistar rats were incubated with Krebs-Ringer bicarbonate (KRB) buffer [25] containing vehicle (1 μL DMSO per mL KRB buffer), glimepiride, or cytopiloyne in the absence or presence of glucose for 30 min. The KRB buffer was then collected for ELISA assays.

2.7. Intraperitoneal Glucose Tolerance Test (IPGTT). Male db/db mice were administered either vehicle, cytopiloyne at 0.5 and 2.5 mg/kg/day, or glimepiride at 2.5 mg/kg/day for

the indicated time. The mice were fasted for 16 h before the glucose tolerance test. On days 0 and 42, each group received an oral dose (0.2 mL) of vehicle (1 μL DMSO per 1 mL of PBS), glimepiride, or cytopiloyne (time 0) and one intraperitoneal injection with glucose (0.5 g/kg) 0.5 h later. The levels of blood sugar were monitored from −0.5 to 3 h after glucose administration.

2.8. Immunohistochemistry. Pancreata from db/db males with continuous drug administration were snap frozen in *OCT* compound and stained with hematoxylin and eosin or anti-insulin antibody, followed by diaminobenzidine tetrahydrochloride development as previously published [18]. Optimal cutting temperature compound (OCT) is an inert cryosection medium. Multiple parallel sections of each pancreas were analyzed by light microscopy.

2.9. Intracellular Staining for Insulin. Rat pancreatic islets were incubated with vehicle alone (1 μL DMSO per 1 mL of PBS), cytopiloyne, or glimepiride for 24 h. The islet cells were dissociated into single-cell suspension, stained with anti-insulin antibody and analyzed by fluorescence-activated cell sorting (FACS).

2.10. Real-Time RT-PCR Analysis. Rat pancreatic islets were incubated with vehicle (1 μL DMSO per 1 mL of PBS), cytopiloyne, or glimepiride for 24 h. Total RNA isolated from these islets was extracted and converted to cDNA. Real-time RT-PCR was performed with the above cDNA using insulin primers (5′-TGCGGGTCCTCCACTTCAC-3′ and 5′-GCCCTGCTCGTCCTCTGG-3′) or L13 primers (5′-AGA TAC CAC ACC AAG GTC CG-3′ and 5′-GGA GCA GAA GGC TTC CTG-3′).

2.11. Transfection and Luciferase Assays. The phINS-Luc and pRL-TK plasmids contain the human insulin promoter (2347 bp) from the phINS-DCR3 vector [26] linked to the firefly luciferase gene and the thymidine kinase promoter linked to the *Renilla* luciferase reporter gene, respectively. RIN-m5F cells were transfected with pHACE-PKCα DN plasmid (a gift from Dr J.-W. Soh), pcDNA3 (a control plasmid), phINS-Luc, and/or pRL-TK by lipofectamine or electroporation. After a 24 h recovery, the cells were treated with vehicle (1 μL DMSO per 1 mL of medium), glucose, GF109203X (a PKC inhibitor), glimepiride, or cytopiloyne for an additional 24 h. Subsequently, dual luciferase assays were performed as described [18].

2.12. Detection of Intracellular Calcium. RIN-m5F cells were preloaded with Fura 2-AM (5 μM) in modified Krebs-Henseleit buffer for 30 min at 25°C for 1 h. After washing, the cells were stimulated with 16.7 mM glucose or cytopiloyne (7, 14, and 28 μM). Intracellular calcium was measured using a fluorescence spectrophotometer (CAF 110, Jasco, Tokyo, Japan) at the excitation wavelengths of 340 and 380 nm and emission wavelength of 500 nm. The ratio of fluorescence intensity at 340 nm to that at 380 nm represents the level of intracellular calcium.

2.13. Extraction and Measurement of DAG. RIN-m5F cells were treated with glucose, cytopiloyne, PMA, and glimepiride for 5 min. Total lipids were extracted with ethyl acetate as previously described [27]. The cells were separated on a silica thin layer plate with the first developer of ethyl acetate : acetic acid : trimethylpentane (9 : 2 : 5) and the second developer of hexane : diethylether : methanol : acetic acid (90 : 20 : 3 : 2). The spot of DAG and cholesterol in each sample was visualized with 15% sulfuric acid and identified by 1-stearoyl-2-arachidonoyl-*sn*-glycerol and cholesterol.

2.14. Western Blot. RIN-m5F cells were starved in KRB buffer for 30 min. The cells were then treated with vehicle, PMA, 16.7 mM glucose, and cytopiloyne in the absence or presence of EGTA and nimodipine for the indicated time. After extensive washing, the cells were pelleted. The total lysate, cytosolic fraction, and membrane fraction were prepared, followed by SDS-polyacrylamide gel electrophoresis. The membrane was blotted with anti-PKCα, anti-phospho-PKCα, and anti-actin antibodies. The expression level of HA-tagged PKCα-DN in RIN-m5F cells was confirmed using Western blot with anti-HA antibody. The effect of GF109203X on PKCα inactivation in β cells was confirmed by Western blot (see the species list in the Supplementary Material of Figure 1 available online at http://dx.doi.org/10.1155/2013/685642).

2.15. Statistical Analysis. Data from three independent experiments or more are presented as mean ± SEM. ANOVA was used for statistical analysis of differences between groups, and P (*) less than 0.05 was considered to be statistically significant.

3. Results

3.1. Beneficial Effect of Cytopiloyne on Glucose Lowering, Glucose Tolerance Test, Glycosylation of HbA_{1c}, and Islet Preservation. We and others have previously identified three polyacetylenes present in *B. pilosa* that exhibit antihyperglycemic activities in different diabetic models [16–20]. However, their long-term benefit and mode of action remained unclear. In the study, we investigated the therapeutic effect and mechanism of cytopiloyne, a polyacetylenic glucoside (Figure 1(A)), on T2D. Glimepiride, an anti-diabetic sulfonylurea drug, acts to enhance insulin secretion in pancreatic β cells and, in turn, reduces blood glucose. We first evaluated single-dose effects of cytopiloyne on diabetic db/db mice. We found that like glimepiride (2.5 mg/kg), cytopiloyne at doses of 0.1, 0.5, and 2.5 mg/kg significantly reduced postprandial blood glucose levels in a dose-dependent manner in diabetic db/db mice (Table 1). We also compared blood insulin levels in the same mice. Both glimepiride and cytopiloyne significantly elevated the blood insulin levels in db/db mice compared to vehicle alone (Table 2). These data showed that a single dose of cytopiloyne had anti-hyperglycemic and insulin-releasing effects on db/db mice. Next, we investigated long-term therapeutic effects of cytopiloyne in diabetic db/db mice. We found that 0.5 mg/kg cytopiloyne had similar blood sugar-lowering effects on fed db/db mice as glimepiride at 2.5 mg/kg (Figure 1(B)). Additionally, cytopiloyne was

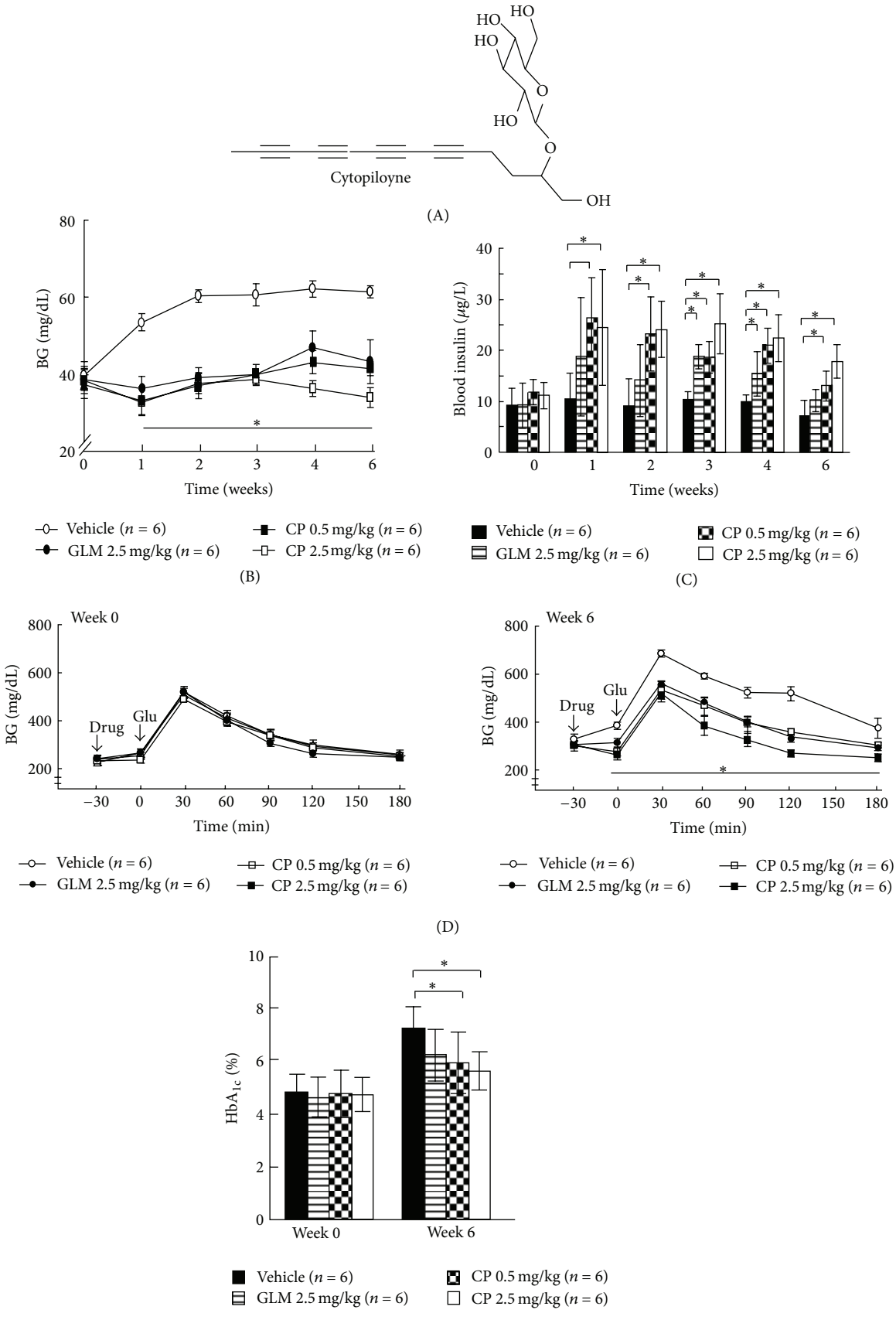

Figure 1: Continued.

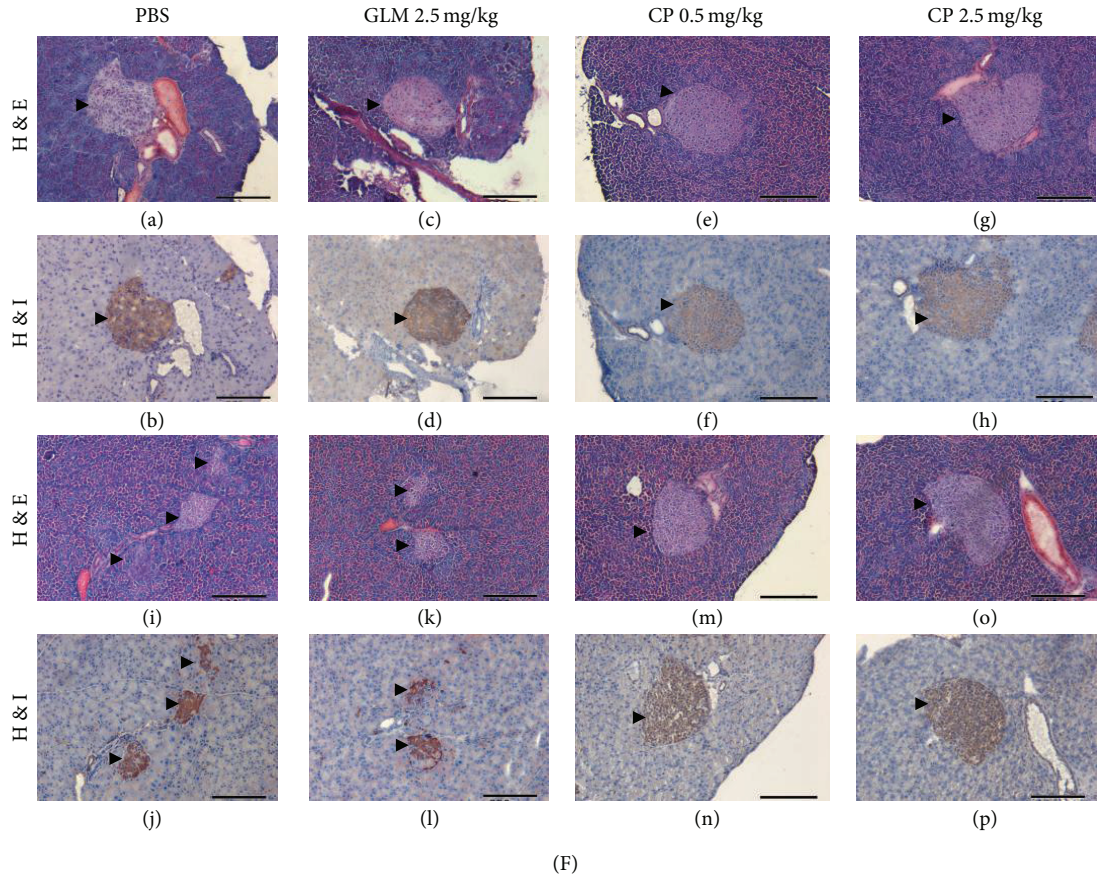

Figure 1: Anti-diabetic effects of cytopiloyne in db/db mice during long-term treatment. (A) Chemical structure of cytopiloyne. (B) Four groups of 6 to 8-week-old diabetic db/db mice were tube-fed with vehicle, cytopiloyne (CP, 0.5 and 2.5 mg/kg/day), or glimepiride (GLM, 2.5 mg/kg/day) from 0 to 6 weeks. Postprandial blood glucose (BG) levels in these mice were measured. (C) Blood insulin levels from the above mice (B). (D) IPGTT was performed in the above db/db mice (B) on weeks 0 and 6 after-treatment, and blood glucose levels were monitored for 3.5 h. (E) The percentage of glycosylated HbA_{1c} in whole blood from the above mice (B) was determined 0 and 6 weeks after-treatment. (F) Pancreata of 8- and 16-week-old db/db males, which had received the same treatment as described in (B) for 2 (images a–h) and 10 (images i–p) weeks, were stained with hematoxylin and eosin (H&E, images (a), (c), (e), (g), (i), (k), (m), and (o)) or hematoxylin and an antibody against insulin (H&I, images (b), (d), (f), (h), (j), (l), (n), and (p)). Arrowheads indicate pancreatic islets. Scale bars, 200 μm. Results are expressed as mean ± SEM from 3 independent experiments, and P (*) < 0.05 was considered to be statistically significant. The number of mice (n) is indicated in parentheses.

slightly more efficacious at 2.5 mg/kg than glimepiride at 2.5 mg/kg. Consistently, cytopiloyne increased blood insulin levels to a greater extent than glimepiride and this increase was dose dependent (Figure 1(C)). We also evaluated the effect of cytopiloyne on glucose tolerance. IPGTT assays showed no difference in glucose tolerance in treated and control mice at week 0 (upper panel, Figure 1(D)). By contrast, cytopiloyne treatment for 6 weeks improved glucose tolerance in db/db mice to a greater extent than glimepiride at the same dose (2.5 mg/kg) (lower panel, Figure 1(D)). Glycosylated HbA_{1c} is known to be an excellent indicator of long-term glycemic control. Therefore, we examined the percentage of glycosylated HbA_{1c} in db/db mice following different treatments. In the blood from 6- to 8-week-old db/db mice, 4.8% HbA_{1c} was glycosylated. However, by 12 to 14 weeks of age, this value had risen to 7.3% in untreated db/db mice. By contrast, 6.3%, 6%, and 5.6% of HbA_{1c} were glycosylated in the blood of age-matched mice following treatment with 2.5 mg/kg glimepiride or with 0.5 mg/kg or 2.5 mg/kg cytopiloyne, respectively (Figure 1(E)). These data suggest that cytopiloyne, which reduced glycosylated HbA_{1c} by 1.3% and 1.7% at concentrations of 0.5 mg/kg or 2.5 mg/kg, respectively, achieves relatively tighter glycemic control than glimepiride, which only decreased glycosylated HbA_{1c} by 1%, in db/db mice. Diabetic db/db mice usually develop severe atrophy of pancreatic islets. We assessed the protective effect of cytopiloyne on islet destruction in db/db mice aged 8 and 16 weeks, which corresponded to early and chronic stages of diabetes, respectively [28]. There was no significant difference in pancreatic islets of treated and untreated db/db mice at 6 to 8 weeks of age. Twelve- to 14-week-old db/db mice, which had received a long-term treatment with vehicle control and glimepiride, had sporadic islets. In sharp contrast, the age-matched db/db mice with

Table 1: Blood glucose levels following a single oral dose of cytopiloyne in fed db/db mice. Diabetic db/db mice aged 6 to 8 weeks, with free access to food, were grouped and tube-fed with vehicle, glimepiride (GLM) at 2.5 mg/kg/day, and cytopiloyne (CP) at 0.1, 0.5, and 2.5 mg/kg/day. A half-hour after tube feeding was set as time 0. Blood samples were collected from the mice at the indicated time intervals (0, 1, 2, and 4 h). The blood glucose levels were determined using a glucometer. The number of mice (n) tested is indicated in parentheses in the first column.

Treatment	Blood glucose level (mg/dL)			
	0	1	2	4 (h)
Vehicle ($n = 8$)	365 ± 9	309 ± 4	281 ± 9	240 ± 11
GLM 2.5 mg/kg ($n = 5$)	378 ± 9	237 ± 16*	178 ± 20*	187 ± 6*
CP 0.1 mg/kg ($n = 7$)	373 ± 6	267 ± 12	204 ± 17*	158 ± 18*
CP 0.5 mg/kg ($n = 7$)	369 ± 27	214 ± 15*	163 ± 8*	132 ± 11*
CP 2.5 mg/kg ($n = 7$)	365 ± 9	207 ± 11*	147 ± 12*	129 ± 11*

* $P < 0.05$ as determined by ANOVA.

cytopiloyne treatment showed much greater preservation of islet structure (Figure 1(F)). Accordingly, cytopiloyne treatment resulted in a better survival rate as compared to treatment with glimepiride or vehicle in db/db mice (see the species list in the Supplementary Material of Table 1 available online at http://dx.doi.org/10.1155/2013/685642). We also confirmed the preventive effect of cytopiloyne on T2D in db/db mice aged 4 weeks that has been previously reported [29, 30]. Cytopiloyne failed to stop the development of T2D in db/db mice, but it significantly reduced hyperglycemia in these mice compared to the control cohort (see the species list in the Supplementary Material of Figure 2 available online at http://dx.doi.org/10.1155/2013/685642). It should be noted that the blood glucose levels of db/db mice in preventive experiments (see the species list in the Supplementary Material of Figure 2(a) available online at http://dx.doi.org/10.1155/2013/685642) and therapeutic experiments (Figure 1(B)) were dissimilar because the ages of the mice examined were different. Collectively, cytopiloyne treatment for diabetes was better than glimepiride in terms of both dosage and therapeutic effects.

3.2. Cytopiloyne Acts as an Insulin Secretagogue rather than a Sensitizer. The sugar-reducing and insulin-increasing effects of cytopiloyne raised the possibility that cytopiloyne controls blood sugar in db/db mice primarily through stimulating insulin production from β cells. Rat primary pancreatic islets are commonly used to test insulin secretion/synthesis because rats have more abundant pancreatic islets than mice and the islets of both species respond to glucose similarly [1]. To examine the role of cytopiloyne in insulin secretion, we treated rat islets with cytopiloyne in KRB buffer containing 16.7 mM glucose. We found that cytopiloyne effectively enhanced insulin secretion in high glucose medium (Figure 2(a)) as well as glucose-free and low-glucose media (see the species list in the Supplementary Material of Figure 3 available online at http://dx.doi.org/10.1155/2013/685642). To confirm that cytopiloyne reduced hyperglycemia by stimulating insulin production from pancreatic β cells *in vivo*, we tested its ability to reduce hyperglycemia and to augment insulin levels in STZ-treated C57BL mice whose β cells were already depleted. As expected, cytopiloyne lost its ability to regulate both responses in these mice (Figure 2(b)). In sharp contrast, insulin treatment still diminished blood glucose levels in β-cell-depleted mice (Figure 2(b)). To exclude the possibility that cytopiloyne is an insulin sensitizer, we administrated STZ-treated C57BL/6 mice with an oral dose of vehicle, glimepiride, metformin, or cytopiloyne 60 min before an insulin injection. Both cytopiloyne and glimepiride had little, if any, lowering effect on blood sugar in these mice. However, metformin, an anti-diabetic biguanide drug, acts to sensitize insulin signaling and, in turn, significantly reduced blood glucose levels compared to vehicle alone in these mice (Figure 2(c)). Overall, our results support an insulin-releasing role of cytopiloyne in β cells.

3.3. Cytopiloyne Elevates the Level of Insulin mRNA and Protein in Pancreatic Islets. Glucose is known to modulate transcription, translation, and secretion of insulin in pancreatic β cells [1]. However, current secretagogues act to increase insulin secretion but not synthesis. We have shown that cytopiloyne increases insulin secretion from rat islets (Figure 2(a)). Therefore, we also evaluated the effect of cytopiloyne on insulin expression. We first used an insulin promoter-driven reporter construct to test the effect of different treatments on insulin transcription. Glimepiride had no significant effect on insulin transcription in RIN-m5F β cells, a rat β-cell line, compared to the low-glucose control (3.3 mM). By contrast, high glucose (16.7 mM) upregulated insulin transcription eleven times; 28 μM cytopiloyne augmented insulin transcription five times, and this increase was dose dependent (Figure 3(a)). Next, we examined the expression levels of insulin mRNA relative to those of L13, a house-keeping control gene, in rat islet cells pretreated with low glucose, high glucose, 10 μM glimepiride, or cytopiloyne at 7, 14, and 28 μM for 24 h. Glimepiride slightly decreased insulin transcription. By contrast, a high concentration of glucose up-regulated insulin transcription five times, while 28 μM cytopiloyne resulted in doubled insulin transcription (Figure 3(b)). Further, we examined the effect of cytopiloyne on insulin content inside pancreatic islet cells. FACS is a sensitive method to detect levels of an intracellular protein at the level of an individual cell. Therefore, we used FACS to monitor the content of intracellular insulin. Glucose treatment (16.7 mM) increased the intracellular insulin levels in these cells from 2.3% to 5.1% (Figure 3(c)). Consistent with the effect of cytopiloyne on insulin transcription, 28 μM cytopiloyne increased the intracellular insulin levels 5-fold compared to control treatment in these cells, and this effect on insulin content was dose dependent (Figure 3(c)). The overall data suggest that cytopiloyne stimulates insulin expression in pancreatic β cells.

3.4. Cytopiloyne Increases Calcium Influx, DAG Generation, and PKCα Activation. Secondary messengers such as calcium and DAG are involved in a variety of signaling pathways in β cells [31–35]. We wanted to understand the mechanism of cytopiloyne in the insulin expression and the release in

Table 2: Blood insulin levels following a single oral dose of cytopiloyne in fed db/db mice. Diabetic db/db mice aged 6 to 8 weeks received the same treatment as those in Table 1. Blood samples at the indicated time interval (0, 0.5, 1, 2, and 4 h) were collected from the mice and the insulin levels in each blood sample were determined using ELISA kits. The number of mice (n) is indicated in parentheses in the first column.

Treatment	Blood insulin level (μg/L)				
	0	0.5	1	2	4 (h)
Vehicle ($n = 8$)	13.8 ± 1.9	11.5 ± 1.9	10.3 ± 2.4	8.3 ± 0.7	8.6 ± 1.9
GLM 2.5 mg/kg ($n = 6$)	13.9 ± 1.2	19.3 ± 4.2	26.3 ± 3.3*	15.9 ± 1.7*	11.8 ± 2.1*
CP 0.1 mg/kg ($n = 5$)	17.2 ± 4.7	21.0 ± 1.3*	15.5 ± 0.6*	14.1 ± 0.5	9.7 ± 1.3
CP 0.5 mg/kg ($n = 5$)	15.7 ± 1.9	22.6 ± 1.9*	16.0 ± 2.4*	12.8 ± 0.7*	12.2 ± 1.9
CP 2.5 mg/kg ($n = 5$)	12.5 ± 1.2	25.5 ± 1.2*	18.9 ± 3.3*	17.6 ± 1.7*	8.9 ± 2.1

* $P < 0.05$ as determined by ANOVA.

β cells. Our data showed that RIN-m5F cells responded to glucose and cytopiloyne in a similar way to primary rat islet cells (Figures 3(a) and 3(b)). Therefore, we used RIN-m5F cells to test the effect of cytopiloyne on calcium mobilization. We found that 16.7 mM glucose significantly increased intracellular calcium in β cells (Figure 4(a)). Similarly, cytopiloyne increased the level of intracellular calcium in a dose-dependent manner (Figure 4(a)). Next, we determined the effect of cytopiloyne on the production of lipids, DAG, and cholesterol, in RIN-m5F cells. PMA, glimepiride, and 16.7 mM glucose significantly increased the level of DAG in comparison with the vehicle control (Figure 4(b)). Of note, cytopiloyne dose dependently increased the level of DAG but not cholesterol (Figure 4(b)). Because PKCα has been previously implicated in insulin secretion of β cells [31, 33], we next assessed the effect of cytopiloyne on PKCα activation by examining its translocation and phosphorylation. Like PMA and 16.7 mM glucose, cytopiloyne dose dependently increased the membrane portion of PKCα (Figure 4(c)). Besides, like PMA, cytopiloyne increased the phosphorylation of PKCα (Figure 4(d)). This increase was abolished by nimodipine, a calcium channel blocker, and EGTA, a calcium chelator (Figure 4(d)). The data suggest that cytopiloyne activates PKCα through an increase of its activators, calcium and DAG.

3.5. Cytopiloyne Increases Insulin Secretion and Transcription via PKCα. Next, we tested whether PKCα regulated cytopiloyne-mediated insulin secretion in β cells. We found that cytopiloyne stimulated insulin secretion in β cells, similar to what was observed in positive controls, 16.7 mM glucose and PMA (Figure 5(a)). By contrast, GF109203X, a PKC inhibitor, inhibited cytopiloyne- and PMA-induced insulin secretion (Figure 5(a)). Accordingly, overexpression of a dominant-negative mutant of PKCα decreased cytopiloyne- and PMA-mediated insulin secretion (Figure 5(b)). On the contrary, overexpression of this mutant only slightly, if at all, inhibited glucose-mediated insulin secretion (Figure 5(b)). These data showed that cytopiloyne increased insulin secretion in β cells in a PKCα-dependent manner. We also tested the involvement of PKCα in insulin transcription in β cells. We found that like 16.7 mM glucose and PMA, cytopiloyne stimulated insulin transcription in β cells (Figure 5(c)). GF109203X completely abolished cytopiloyne-, glucose-, and PMA-mediated insulin transcription (Figure 5(c)). Similarly, overexpression of a dominant-negative PKCα significantly inhibited insulin transcription (Figure 5(d)). These data revealed that cytopiloyne increased insulin transcription in β cells via PKCα. Furthermore, we tested whether calcium mobilization affected cytopiloyne-mediated insulin secretion. We found that calcium intervention inhibited the insulin secretion by cytopiloyne in RIN-m5F cells (Figure 5(e)) and rat β pancreatic islets (see the species list in the Supplementary Material of Figure 4 available online at http://dx.doi.org/10.1155/2013/685642).

In summary, our mechanistic data suggest that cytopiloyne enhances insulin secretion and expression in β cells via the regulation of PKCα by calcium and DAG. The increase of insulin production in β cells and islet protection is associated with the therapy of cytopiloyne for T2D (Figure 6).

4. Discussion and Conclusions

Plants provide a promising source of anti-diabetic medicines. Cytopiloyne, a plant polyacetylene, represents a new class of anti-diabetic chemotherapeutics. This study not only demonstrates the anti-diabetic efficacy of cytopiloyne, but also reveals the mechanism of the anti-diabetic actions of cytopiloyne in cell and mouse models.

Cytopiloyne has several unique benefits over current secretagogues in the market, including enhancement of insulin expression and maintenance of islet architecture. First, nutrients such as glucose can stimulate insulin biosynthesis at transcriptional and translational levels and the secretion of insulin in β cells [1, 36]. However, current secretagogues for diabetes can stimulate insulin secretion but not insulin biosynthesis. Unexpectedly, cytopiloyne can increase the level of insulin mRNA and protein, and it may be functionally superior to sulfonylureas (Figure 3). Second, cytopiloyne improved islet protection and resulted in a higher survival rate of db/db mice than glimepiride or vehicle control (Figure 1(F) and see the species list in the Supplementary Material of Table 1 available online at http://dx.doi.org/10.1155/2013/685642). Similarly, cytopiloyne was reported to maintain pancreatic islet architecture in NOD mice, a type 1 diabetes model [18]. Whether cytopiloyne employs the same mechanism in prevention of β cell death in both types of diabetes needs to be further investigated.

Cytopiloyne has other advantages over glimepiride. First, cytopiloyne is a relatively potent anti-diabetic compound

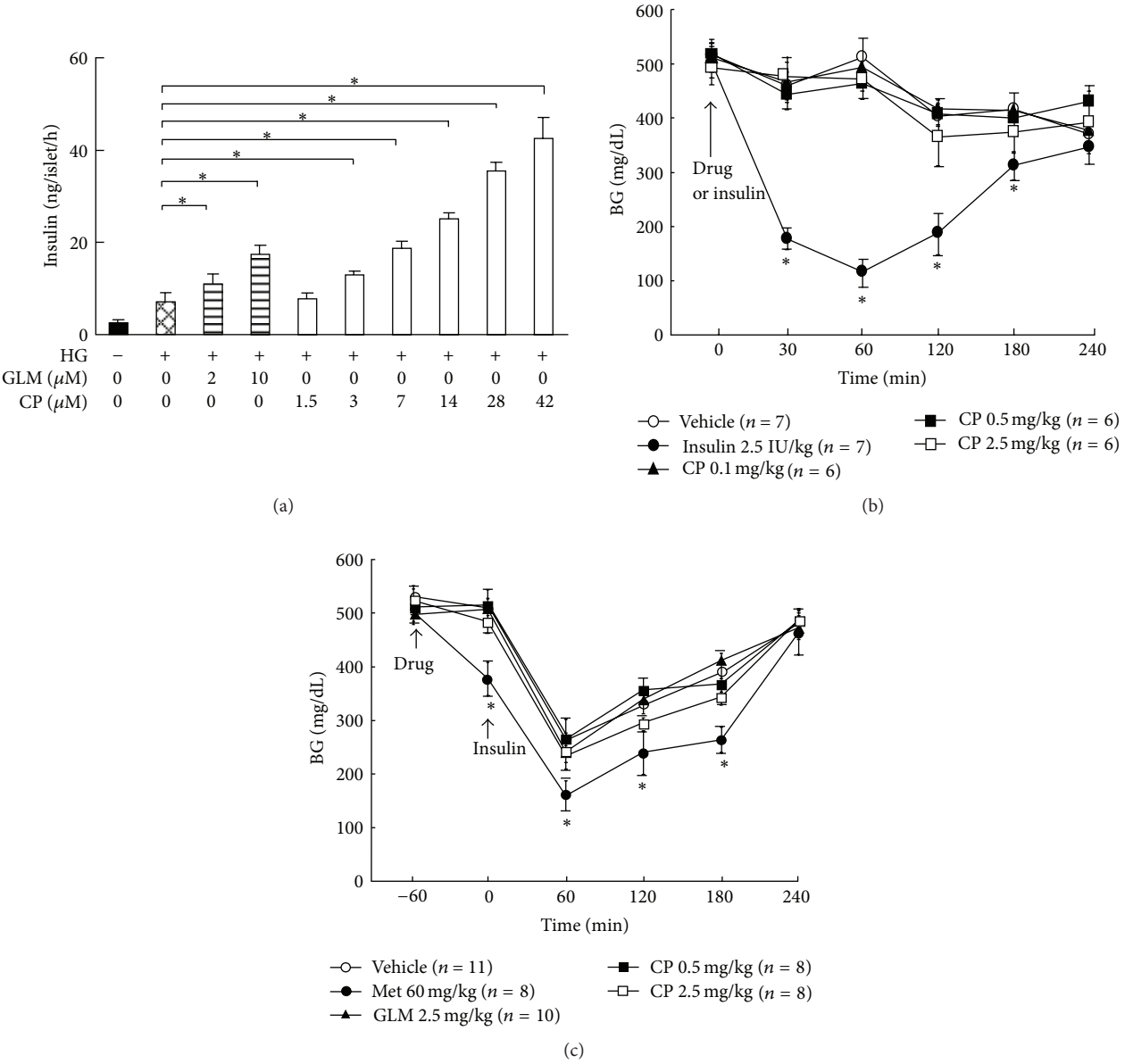

Figure 2: Cytopiloyne-mediated insulin secretion depends on pancreatic β cells. (a) Rat pancreatic islets were incubated with KRB buffer containing vehicle, glimepiride (GLM, 0 to 10 μM), or cytopiloyne (CP, 1.5 to 42 μM) in the absence or presence of 16.7 mM glucose (HG). The insulin levels were determined using an insulin ELISA kit. The data are presented as mean ± SEM of 3 independent experiments. (b) Fed C57BL mice, which had already received an injection of STZ, were administered an oral dose of vehicle, cytopiloyne (CP, 0.1, 0.5, and 2.5 mg/kg), and an intraperitoneal injection of insulin (Ins, 2.5 IU/kg). Postprandial blood sugar levels in the STZ-treated mice were determined using a glucometer. (c) Fed C57BL mice, which had already received STZ, were orally administered a single dose of vehicle, cytopiloyne (CP, 0.5 and 2.5 mg/kg), glimepiride (GLM, 2.5 mg/kg), or metformin (Met, 60 mg/kg), followed by an intraperitoneal injection with insulin (Ins). Postprandial blood sugar levels in the STZ-treated mice were determined using a glucometer. Results are expressed as mean ± SEM from 3 independent experiments, and $P < 0.05$ was considered to be statistically significant (*). The number of mice (n) is indicated in parentheses.

as compared to glimepiride. Cytopiloyne induced similar anti-diabetic effects as glimepiride at one-fifth of the dose and modestly better anti-diabetic effects at the same dose (Figure 1 and Tables 1 and 2). In comparison with low-potency insulin secretagogues, cytopiloyne may have other benefits such as increased efficacy or decreased toxicity. Second, cytopiloyne may have a different mechanism of action as that of glimepiride, based on the large differences in their chemical structures. In fact, cytopiloyne, but not glimepiride, is able to promote insulin transcription (Figure 3) and confer islet protection (Figure 1(F)). Cytopiloyne is structurally different from currently known secretagogues and thus may represent a new class of anti-diabetic agents. Thus, studying the anti-diabetic mechanism of cytopiloyne may elucidate

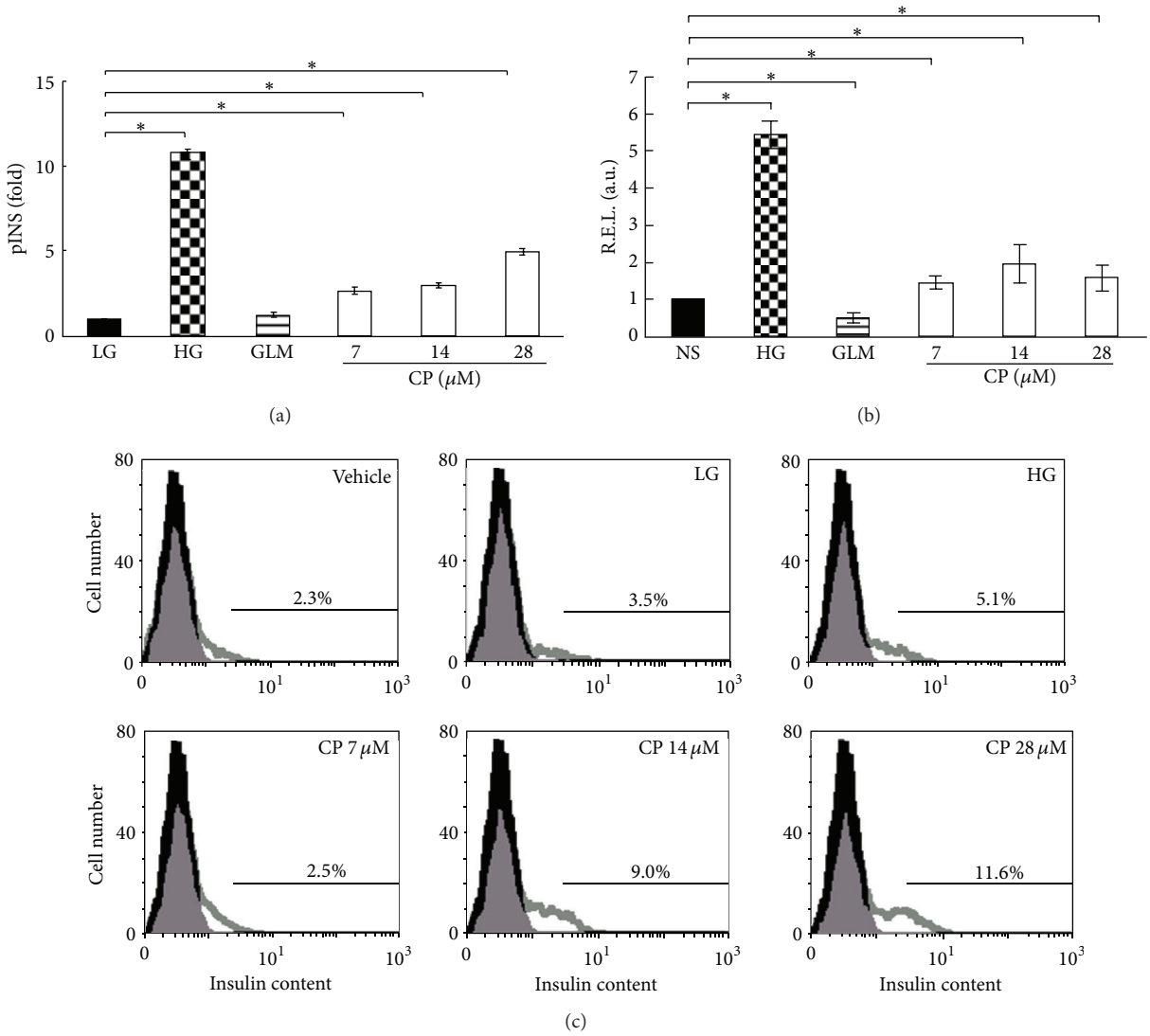

Figure 3: Increase in insulin mRNA and protein content by cytopiloyne in pancreatic islets. (a) RIN-m5F β cells transfected with phINS-Luc and pRL-TK plasmids were incubated with medium containing 3.3 mM glucose in the presence of vehicle (LG), glimepiride (GLM, 10 μM), and cytopiloyne (7, 14, or 28 μM) or 16.7 mM glucose (HG). Insulin promoter activity expressed as fold change relative to vehicle-treated control was measured using dual luciferase assays. (b) The relative expression level (R.E.L.) of insulin relative to L13 in rat primary pancreatic islets, which were already treated with 3.3 mM glucose in the presence of vehicle (LG), glimepiride (GLM, 10 μM), or cytopiloyne (7, 14, or 28 μM) or 16.7 mM glucose (HG) for 24 h, was determined by real-time RT-PCR. (c) Rat pancreatic islets received the same treatments as the islets in (b) in the presence of brefeldin A for 24 h. After anti-insulin antibody staining, these cells underwent FACS analysis. The percentage of insulin-positive β cells is shown. Results are expressed as mean ± SEM from 3 independent experiments, and $P < 0.05$ was considered to be statistically significant (*).

novel pathways that participate in insulin synthesis/secretion and islet preservation and the development of anti-diabetic agents.

No drug is perfect, and cytopiloyne presents challenges like other therapies. Current secretagogues occasionally reduce blood sugar to a detrimental degree, a condition known as hypoglycemia. The development of blood glucose-dependent secretagogues would prevent this dangerous side effect. At a low dose (e.g., 3 μM), cytopiloyne stimulates insulin secretion in pancreatic islets in a glucose-dependent manner (see the species list in the Supplementary Material of Figure 3 available online at http://dx.doi.org/10.1155/2013/685642). However, a high dose of cytopiloyne can still stimulate insulin secretion to some extent even in the absence of glucose (Figure 2(a)). The data show that cytopiloyne-mediated insulin secretion is partially glucose dependent. Thus, at a high dose, cytopiloyne may pose a similar potential risk for hypoglycemia as sulfonylureas, particularly in patients with low blood glucose levels. However, this problem may be alleviated by decreased dosage or use in combination with a sensitizer such as metformin, which has no hypoglycemic effect. Additionally, it should be

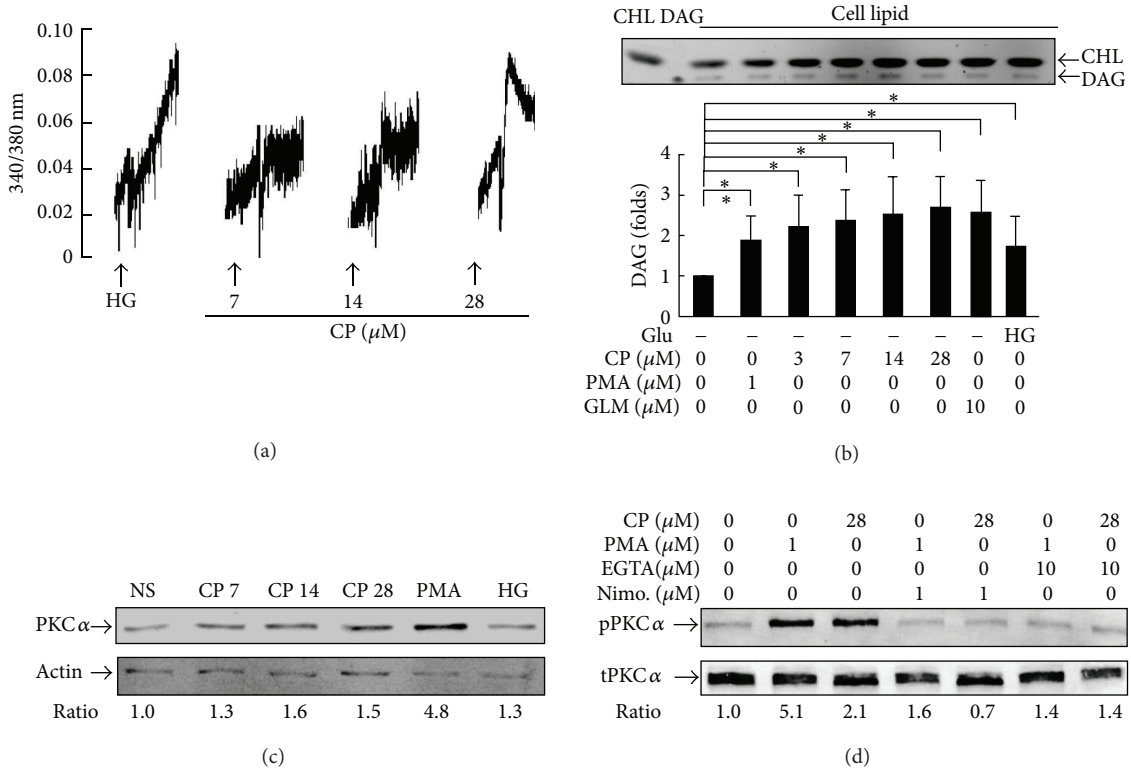

Figure 4: Effects of cytopiloyne on calcium mobilization, DAG generation, and PKCα activation. (a) After Fura 2-AM loading, RIN-m5F cells were stimulated with 16.7 mM glucose (HG) and cytopiloyne (CP) at 7, 14, and 28 μM. The level of intracellular calcium, as shown by the 340/380 nm ratio, was detected using a fluorescence spectrophotometer. (b) RIN-m5F cells were stimulated with glucose, cytopiloyne (CP), PMA, and glimepiride (GLM). Total cell lipids and their commercial standards, DAG and cholesterol (CHL), were resolved on a silica thin layer plate. The quantity of DAG and cholesterol in each sample is replotted into histograms. (c) RIN-m5F cells were incubated with vehicle (NS), cytopiloyne (CP, 7, 14, and 28 μM), PMA (1 μM), and 16.7 mM glucose (HG). Membrane proteins of each sample were subjected to Western blot with anti-PKCα and anti-actin antibodies. (d) RIN-m5F cells were incubated with vehicle, cytopiloyne (28 μM), and PMA (1 μM) in the absence or presence of EGTA (10 μM) and nimodipine (Nimo, 1 μM). Total proteins were subjected to Western blot with anti-PKCα (t-PKCα) and anti-phospho-PKCα (p-PKCα) antibodies.

noted that insulin secretagogues have a low clinical incidence of hypoglycemia, because patients with T2D usually have higher insulin resistance than healthy subjects.

Cytopiloyne contains a glucose moiety and therefore it is conceivable that it acts at glucose receptors to mediate insulin expression and secretion. However, our data argue against this possibility. At 28 μM, the concentration of glucose in cytopiloyne is about 600 times lower than the glucose in our experiments (16.7 mM). However, upregulation of insulin transcription by cytopiloyne at the same concentration is 40% (not 0.17%) of the up-regulation by 16.7 mM glucose (Figures 3(a) and 3(b)). Notably, 28 μM cytopiloyne was less effective in inducing insulin mRNA production but more effective in increasing insulin protein levels than 16.7 mM glucose in islets (Figure 3). This discrepancy may be due to differences in the regulation of insulin at the mRNA and protein levels by cytopiloyne. Indeed, glucose and GLP-1 strongly stimulate insulin translation but only modestly stimulate insulin transcription in β cells [1, 37].

DAG and calcium are common secondary messengers in β cells [31–35]. Both messengers activate PKCα. PKCα has been found to be involved in insulin secretion mediated by PMA and glucose [31–35] although the authors of one study excluded the participation of PKCα in glucose-mediated insulin secretion [31–35]. Consistent with the literature, our study shows that PMA activates PKCα in β cells to a greater extent than glucose (Figure 4(c)). In this study, cytopiloyne dose dependently activated PKCα (Figures 4(c) and 4(d)). PKCα activation of cytopiloyne was dependent on calcium (Figure 4(d)) and probably also DAG. Furthermore, interference with the PKC inhibitor, GF109203X, and the dominant-negative mutant of PKCα inhibited cytopiloyne-mediated insulin secretion/expression in β cells (Figures 5(a)–5(d)). The insulin secretion/expression of cytopiloyne is calcium dependent (Figure 5(e)). Therefore, our data suggest that cytopiloyne increases insulin production via PKCα activation that involves secondary messengers (Figure 6).

In conclusion, we showed that cytopiloyne suppressed the progression of T2D in db/db mice as evidenced by the decrease in the levels of blood glucose and HbA_{1c}, glucose tolerance, and islet atrophy in db/db mice. Insulin release/expression and protection of β cells contributed

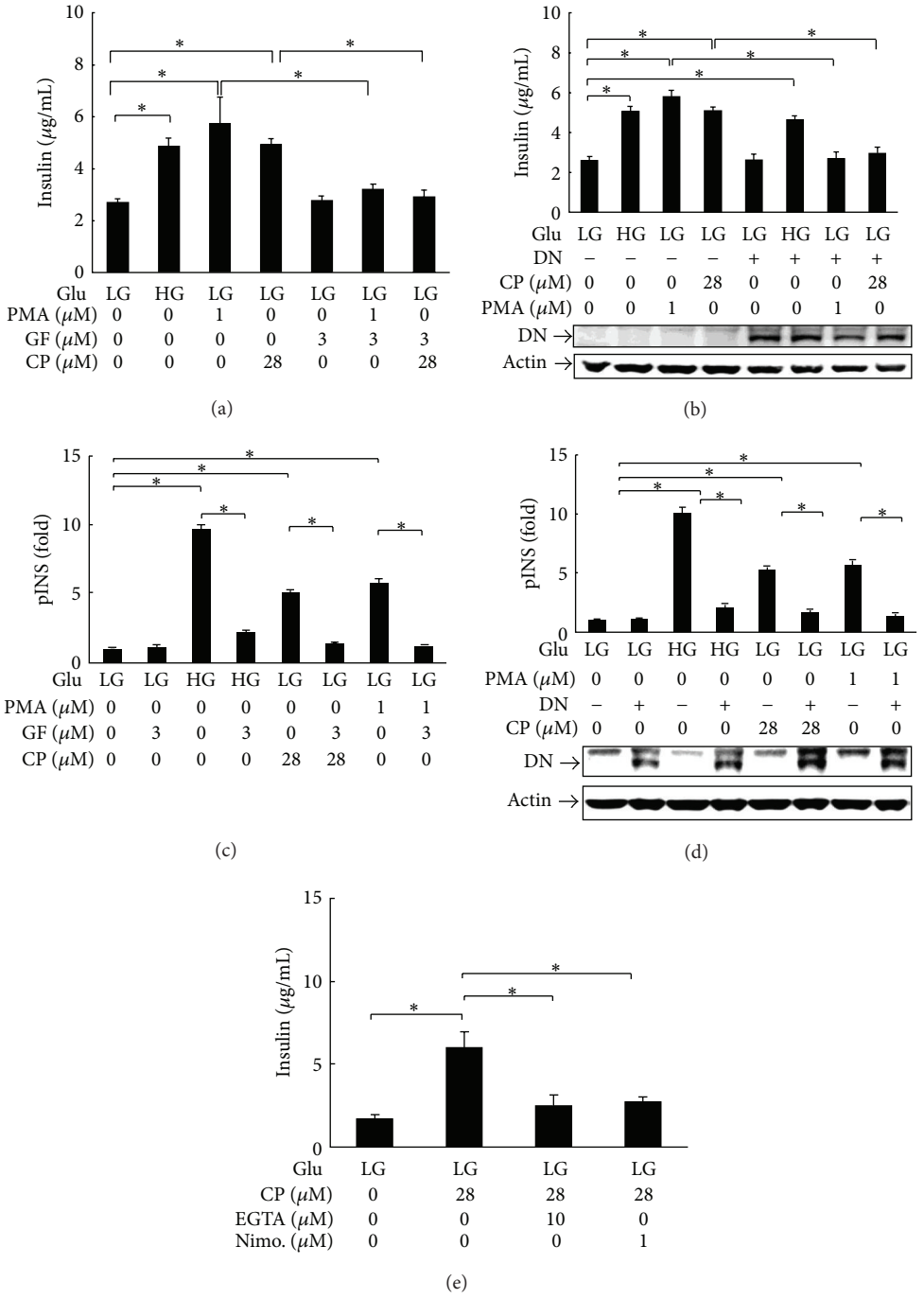

Figure 5: Cytopiloyne-mediated insulin secretion and expression are abolished by a dominant-negative mutant and a PKCα inhibitor. (a) RIN-m5F cells were grown in medium with 16.7 mM glucose (HG) or 3.3 mM glucose (LG) in the presence of PMA (1 μM), GF109203X (GF, 3 μM), and cytopiloyne (CP, 28 μM). The insulin level in the supernatants was determined using an ELISA kit. (b) RIN-m5F cells were transfected with 5 μg of pHACE-PKCα DN (+) or pcDNA3 (−) plasmid and grown in medium supplemented with 16.7 mM (HG) or 3.3 mM glucose (LG) in the presence of PMA and cytopiloyne. The insulin level was determined as described in (a). The expression level of dominant-negative HA-tagged PKCα (DN) and an internal control, actin, in the transfected cells was determined by Western blot using anti-HA and anti-actin antibodies. (c) RIN-m5F cells were transfected with phINS-Luc and pRL-TK plasmids. The cells were grown in medium with 16.7 mM (HG) or 3.3 mM glucose (LG) in the absence and presence of PMA, GF109203X, and cytopiloyne. The activity of the insulin promoter (pINS) in fold was measured using dual luciferase assays. (d) RIN-m5F cells were transfected with phINS-Luc and pRL-TK plus 5 μg of pHACE-PKCα DN (+) or pcDNA3 (−) plasmids. The cells were grown in medium with 16.7 mM (HG) or 3.3 mM glucose (LG) in the absence or presence of PMA and cytopiloyne. Insulin promoter activity expressed as fold change relative to vehicle-treated control was measured using dual luciferase assays. The expression level of dominant-negative HA-tagged PKCα (DN) and actin in the transfected cells was determined using Western blot and anti-HA and anti-actin antibodies. (e) RIN-m5F cells were grown in medium with 3.3 mM glucose (LG) and/or cytopiloyne (CP, 28 μM) in the presence of EGTA (10 μM) or nimodipine (Nimo, 1 μM). The insulin level in the supernatants was determined.

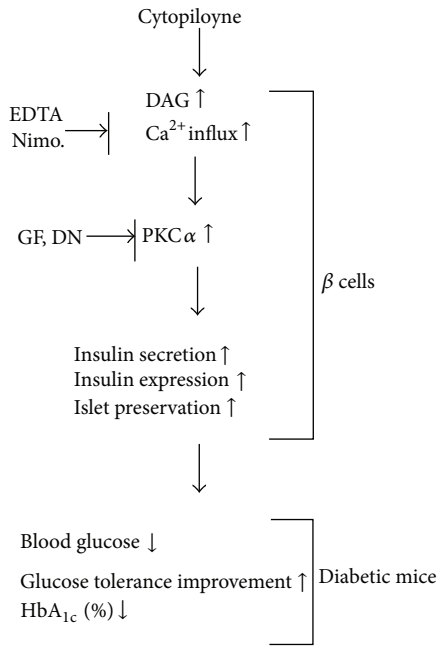

Figure 6: Schematic diagram of the likely mechanism by which cytopiloyne treats T2D in diabetic mouse models. Cytopiloyne shows anti-diabetic effects in diabetic mice, as evidenced by a reduction in the levels of blood sugar and glycosylated HbA_{1c}, improvement of glucose tolerance, and its regulation of β-cell functions (e.g., insulin secretion, insulin expression, and pancreatic islet protection). The regulation of insulin secretion/expression in β cells by cytopiloyne involves PKCα and its activators, calcium, and DAG.

to this suppression. We also showed that cytopiloyne up-regulated insulin release/expression that involved PKCα activation and its activators, calcium and likely DAG. This study reveals the anti-diabetic mode of action of cytopiloyne and suggests its use as a new anti-diabetic agent.

Abbreviations

BW: Body weight
DAG: Diacylglycerol
DMSO: Dimethyl sulfoxide
FACS: Fluorescence-activated cell sorting
GLP-1: Glucagon-like peptide-1
HbA_{1c}: Hemoglobin A_{1c}
IPGTT: Intraperitoneal glucose tolerance test
IP3: Phosphatidylinositol triphosphate
KRB: Krebs-Ringer bicarbonate
PBS: Phosphate-buffered saline
PKA: Protein kinase A
PKC: Protein kinase C
PMA: Phorbol 12-myristate 13-acetate
STZ: Streptozocin
T2D: Type 2 diabetes.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

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Research Article

Fructus Mume Formula in the Treatment of Type 2 Diabetes Mellitus: A Randomized Controlled Pilot Trial

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Background. "*Fructus Mume* or Dark Plum" (pilule form) has been used for many years in Traditional Chinese Medicine (TCM) and may be a valid treatment for type 2 diabetes mellitus (T2DM). *Aim*. One aspect toward efficacy validation is the evaluation of the blood glucose-lowering effect of *Fructus Mume* (*FM*) with T2DM patients in a randomized controlled trial (RCT). *Methods*. This pilot study uses a RCT procedure to assess efficacy of *FM* and Metformin. The trial was for 12 weeks, with 80 T2DM subjects. Both groups were standardized in their diet and exercise routine. Comparisons of several variables were analyzed. *Results*. No significant differences were found between groups in the fasting and postprandial glucose levels although both had significant decreases. The values of glycosylated hemoglobin were significantly reduced in both groups. For patients whose body mass index (BMI) was <23, neither *FM* nor Metformin had an effect on BMI; for those with a BMI between 23 and 25 or the BMI was >25, both *FM* and Metformin significantly reduce the BMI. *Conclusions*. In this pilot study, it was demonstrated that *Fructus Mume* formula may reduce the levels of blood glucose in patients with type 2 diabetes.

1. Introduction

It has been said that some traditional chinese medicinal (TCM) formulae could provide clinical benefits for patients with type 2 diabetes mellitus [1]. The reported benefits include optimal glycemic control, amelioration of clinical manifestations, and preventing or improving macrovascular or microvascular complications.

A TCM formula called "*Fructus Mume*" (Chinese: "Wumei Wan," Dark Plum fruit pilule) is used to lower blood glucose levels. It was first recorded in the *Treatise on Cold Damage Diseases* (Chinese: "Shanghanlun") which was written by the Sage of TCM-Zhang ZhongJing (circa 200–205 CE). Two animal experimental studies declared that it has blood glucose-lowering effect by facilitating the recovery of islet β-cells, increasing the concentration of hepatic glycogen, accelerating the glycogen synthesis, stimulating β-cells to excrete insulin, improving the glucose utility of peripheral tissues, and so forth [2].

The present researchers assert that the *FM* formula (composed of ten herbs; Table 1) has potential in the treatment of diabetes. First, it contains some special herbs which may be directly effective for diabetes. Among these herbs, *coptidis* is a herb of bitter flavor and cold property and *Dark Plum* is of sour flavor. *Four-qi* and *Five flavors* represent the main effects of Chinese medicinals and are one of the basic concepts

Table 1: Traditional chinese medicinal herbs used in the *Fructus Mume* formula.

PinYin name	Latin name	English name	Original dose	Modification
Wumei	*Fructus Mume*	Dark plum fruit	300 pieces	30 g
Xixin	*Herba Asari*	Manchurian wildginger	6 tael	Removed
Ganjiang	*Rhizoma Zingiberis*	Dried ginger	10 tael	15 g
Huanglian	*Rhizoma Coptidis*	Golden thread	16 tael	30 g
Danggui	*Radix Angelicae Sinensis*	Chinese angelica	4 tael	10 g
Fuzi	*Rhizoma Typhonii Gigantei*	Giant typhonium rhizome	6 tael	20 g
Huajiao	*Fructus Zanthoxyli*	Prickly ash peel	4 tael	5 g
Guizhi	*Ramulus Cinnamomi*	Cassia twig	6 tael	10 g
Renshen	*Radix Ginseng*	Ginseng	6 tael	10 g
Huangbai	*Cortex Phellodendri*	Amur corktree bark	6 tael	20 g

in TCM theory. It is therefore necessary in our research to explain the hypoglycemic effect of *FM* besides *Yin-Yang* theory. There is no direct correlation of the use of TCM concept vocabulary such as *flavors* (sour, bitter, and sweet) with Western scientific concepts, so we are limited in our explanation of these concepts. In TCM theory, bitter flavor is in direct opposition to sweet flavor, and sour flavor can neutralize sweet flavor [3]. So the combination of bitter and sour flavors is an excellent approach to counteract sweet flavor. Secondly, it incorporates many basic TCM principles into a formula by using herbs of various flavor and properties [4]. Both hot and cold properties are also in the *FM* formula. This reflects the basic concept of *Yin-Yang* in TCM theory, and we interpret this formula as a typical prescription where herbs of cold or hot property are used together to adjust the balance of *Yin-Yang*. Generally speaking, it is a formula highly revered by ancient and modern TCM practitioners.

The individual substance alone in the herbs used in *FM* may have beneficial effects on diabetes. For example, initial research with berberine and ginsenosides is promising.

Berberine. Coptidis is one of the most popular herbs for diabetes [5], as it contains *berberine*. Numerous studies have demonstrated that *berberine* could exert beneficial effects on the treatment of diabetes [6]. The potential mechanisms include improving insulin sensitivity, inhibiting gluconeogenesis, stimulating glucose uptake through the AMP-AMPK-p38 MAPK pathway, or correcting lipid disorders [7–11]. Some articles have examined the effects and safety of *berberine* among patients with type 2 diabetes and suggested that it is effective and safe [12–14].

Ginsenosides. A recent article [15] has declared that malonyl ginsenosides (one of the natural ginsenosides of ginseng, another herb used in *FM*) could alleviate hyperglycemia, hyperlipemia, and insulin resistance of type 2 diabetes. Cho and coworkers [16] reported that ginsenoside Re could lower blood glucose and lipid levels and exerts protective actions against the occurrence of oxidative stress in the eye and kidney of diabetic rats.

But all of these researches have not provided evidence to demonstrate the blood glucose-lowering effect of *FM* on well-designed randomized controlled trials.

Although many benefits have been reported by the TCM community, there is one question that remains unanswered; are TCM methods able to reduce the levels of blood glucose? The current debate over the role of TCM in management of diabetes mellitus revolves around its ability to serve as an independent monotherapy versus being delegated to the role of adjunct to hypoglycemia agents. The present pilot study is a randomized controlled trial designed to evaluate the blood glucose-lowering effect and safety of the *Fructus Mume* formula in patients with type 2 diabetes mellitus. One of the ingredients of the *FM* formula, *Herba Asari,* (Manchurian Wild Ginger) contains aristolochic acid and is therefore nephrotoxic [17]; we therefore removed this herb from the formula. As *Rhizoma Typhonii Gigantei* remained in our test drug and plays a major role as an extreme hot herb in *FM* [18], removing *Herba Asari* will not upset the balance of hot/cold, yin/yang. The ingredient herbs of the modified *FM* are shown in Table 1.

The present study was registered on the Chinese Clinical Trial Registry: ChiCTR-TRC-12002320. The results are presented according to the Consolidated Standards for Reporting Trials of Traditional Chinese Medicine (CONSORT for TCM) checklist [19].

2. Subjects and Methods

2.1. Subjects. The study was conducted at four centers in Sichuan Province, China. The ethics committee of the Teaching Hospital of Chengdu University of Traditional Chinese Medicine approved the protocol, and all patients provided the written informed consent. The diagnosis of type 2 diabetes was based on clinical history and the finding of blood glucose concentrations according to the China Guideline for Diabetes Prevention and Treatment. To be included in the study, all patients had to have 7.0 mmol/L (126 mg/dL) ≤ fasting plasma glucose (FPG) ≤ 13.3 mmol/L (240 mg/dL) or 11.1 mmol/L (200 mg/dL) ≤ 2 h postprandial plasma glucose (2hPG) ≤ 22.9 mmol/L (412 mg/dL). Other inclusion criteria included an age of 18 to 70 years and normal renal function, and the value of transaminase was lower than one and a half times the upper limit. Patients were excluded if they had

Table 2: Demographic and baseline characteristics of the FAS population of the two groups of patients.

Characteristic	*FM* group	Metformin group	*P* value
Sex			
male	25	14	0.025
female	16	25	
Age (years)	54.37 ± 8.89	54.77 ± 11.83	0.864
Diabetes duration (months)	16.41 ± 21.15	14.34 ± 27.20	0.703
Body weight (Kg)	64.76 ± 11.85	60.79 ± 7.39	0.085
BMI (Kg/m^2)	24.31 ± 4.34	24.04 ± 2.85	0.757
SBP (mmHg)	118.59 ± 11.02	121.31 ± 13.12	0.335
DBP (mmHg)	79.85 ± 7.42	78.57 ± 8.02	0.48
TC (mmol/L)	4.90 ± 1.01	4.59 ± 1.33	0.286
TG (mmol/L)	2.53 ± 3.48	2.37 ± 2.48	0.832
HDL (μmol/L)	1.68 ± 0.54	1.57 ± 1.20	0.630
LDL (μmol/L)	3.03 ± 0.94	3.06 ± 0.91	0.898
HbA1c (%)	7.66 ± 1.11	8.23 ± 1.95	0.352
FPG (mmol/L)	7.871 ± 1.461	7.494 ± 1.544	0.266
2hPG (mmol/L)	15.182 ± 2.715	14.006 ± 3.061	0.073
Insulin concentration (mU/L)	8.65 ± 5.60	6.89 ± 3.90	0.416

any of the following: pregnancy or lactation, a history of alcohol abuse, a history of cerebrovascular accident, malignant hypertension and acute coronary syndrome within the previous six months, allergic constitution, or an allergic history to TCM. Patients were also excluded if they had taken medications which are known to affect glucose metabolism such as thiazide diuretic; nicotinic acid within the past three months; or had comorbidity such as chronic heart failure; chronic renal failure; and hematopoietic system disease, mental disorders; had diseases which are known to affect glucose metabolism such as thyroid disease and adrenal gland disease; had participated in other clinical trials within the past three months.

The baseline characteristics of the two groups of patients are shown in Table 2. In this pilot study, there are more female subjects in the Metformin group. Other baseline characteristics did not vary greatly.

2.2. Interventions. A total of 85 subjects were randomized to receive either *FM* or Metformin. Metformin is commonly used for type 2 diabetes, and clinical trials demonstrate it is safe and efficacious [20] in reducing plasma glucose concentrations in patients with T2DM. All patients received diet and exercise therapy. The randomization sequence was generated with an SAS software package by the Good Clinical Practice (GCP) Center of the Teaching Hospital of Chengdu University of TCM. The sequence was concealed and disseminated using opaque envelopes. All patients were instructed with a diet therapy (developed by a national Chinese nutrition society) where the calculated daily calorie expenditure was given, and recommended daily dietary nutrients were provided.

To insure control over the quality of the decoction, this paper was standardized between research centers. All Chinese medicinals were processed according to the "Pharmacopoeia of the People's Republic of China" (2010 edition) and purchased from Sichuan Neautus Traditional Chinese Medicine, Inc., Ltd. Before decoction, the herbs of *FM* were infused for half an hour. The *Giant Typhonium Rhizome* was decocted firstly for one hour and then the other herbs of *FM* were added, decocted for another half an hour under 0.1 MPa, 120°C. Herbs were decocted with an automatic boiling and packaging machine, using three packages of decoction (200 mL/package). Patients were orally administered three packages daily. Metformin was taken at the dose of 500 mg twice daily.

2.3. Analytic Methods. Plasma glucose was measured every four weeks with a Hitachi analyzer by the hexokinase (HK) method, and glycosylated hemoglobin was measured by high-performance liquid chromatography at randomization and week 12. Blood chemical tests were performed with a 7170A automatic analyzer (Johnson & Johnson Medical (China) Ltd.) and urinalysis was performed with an AVE-763B automatic analyzer at randomization and week 12. The levels of insulin were determined by the electrochemiluminescence (ECL) method with a Cobase 601 analyzer at randomization and week 12.

2.4. Statistical Analyses. All analyses were performed on a modified intention-to-treat population. The full-analysis-set (FAS) population was the primary population for assessing efficacy. The FAS included patients who took at least one dose of the study drug and had at least one value on treatment. Missing data were imputed with the use of the last-observation-carried-forward method, whereby missing values were replaced by the last nonmissing value. A worst case scenario (WCS) was conducted for the proportion of patients with FPG < 7.0 mmol/L; namely, the patients who dropped out were assumed to have achieved FPG < 7.0 mmol/L in the Metformin group, and those in the *FM*

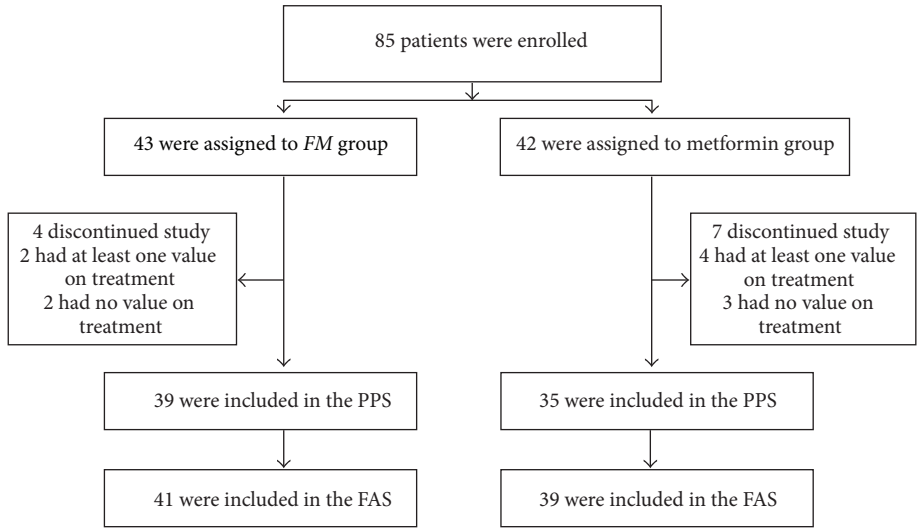

Figure 1

Table 3: Changes in FPG (means ± SD, mmol/L).

	Week 0	Week 4	Week 8	Week 12
PPS				
FM group	7.934 ± 1.470	7.164 ± 1.176	6.609 ± 1.117	6.339 ± 1.308
Metformin group	7.524 ± 1.577	6.831 ± 1.138	6.304 ± 1.009	5.966 ± 1.000
FAS				
FM group	7.871 ± 1.461	7.129 ± 1.161	6.601 ± 1.094	6.345 ± 1.279
Metformin group	7.494 ± 1.544	6.823 ± 1.129	6.349 ± 1.014	6.056 ± 1.029

group were assumed to have not. The Pearson Chi-Square test was used to determine the difference in the proportion of patients with FPG < 7.0 mmol/L or 2 h PG < 10.0 mmol/L between the two groups. Repeated measures and multivariate analysis of variance of the general linear model were applied to determine the changes in blood glucose levels. The changes in body mass index were summarized and subgroup analyses were made by *t*-test according to the Chinese body size, namely, BMI < 23 indicating normal, BMI 23 to 25 indicating overweight, and BMI > 25 indicating obesity.

3. Results

A total of 85 patients were recruited in the present study. Four patients in the *FM* group and seven patients in the Metformin group withdrew before week 12. Of these, two patients in the *FM* group and four patients in the Metformin group took at least one dose of study drug and had at least one value on treatment; therefore, the FAS was comprised of 80 subjects (41 in the *FM* group and 39 in the Metformin group) and the PPS was comprised of 74 subjects (39 in the *FM* group and 35 in the Metformin group). Enrollment, randomization and followup in the present study are depicted in Figure 1.

3.1. Glycemic Control. At week 12 the proportion of patients who had achieved fasting plasma glucose <7.0 mmol/L was similar in both groups (WCS: *FM* group versus Metformin group = 73% (30/41) versus 87% (34/39)). The proportion of patients who had achieved postprandial plasma glucose <10.0 mmol/L was also similar in both groups (WCS: *FM* group versus Metformin group = 68% (28/41) versus 69% (27/39)). The results of analyses for per-protocol set were in line with those for WCS (PPS analyses: for FPG, *FM* group versus Metformin group = 77% versus 86%; for 2hPG, *FM* group versus Metformin group = 72% versus 65%).

3.2. Fasting Plasma Glucose and Postprandial Plasma Glucose. By week 12 the fasting plasma glucose concentration had decreased by 1.53 mmol/L to 6.35 mmol/L in the *FM* group and decreased by 1.44 mmol/L to 6.06 mmol/L in the Metformin group (for the comparison of *FM* with Metformin, $P > 0.05$, Table 3 & Figure 2). At week 12, the postprandial plasma glucose levels decreased by 5.45 mmol/L to 9.73 mmol/L in the *FM* group and decreased by 4.29 mmol/L to 9.72 mmol/L in the Metformin group (for the comparison of *FM* with Metformin, $P > 0.05$, Table 4 and Figure 3). The results of the perprotocol set analyses (Tables 3 and 4) were in line with those of FAS analyses.

3.3. Glycosylated Hemoglobin (HbA1c). The levels of HbA1c decreased from 7.66 percent to 6.78 percent in the *FM* group and decreased from 8.23 percent to 6.76 percent in the Metformin group ($P > 0.05$).

Table 4: Changes in 2hPG (means ± SD, mmol/L).

	Week 0	Week 4	Week 8	Week 12
PPS				
FM group	15.274 ± 2.748	11.045 ± 2.56	9.901 ± 1.844	9.646 ± 2.691
Metformin group	14.076 ± 3.178	11.11 ± 2.236	9.958 ± 1.790	9.633 ± 1.890
FAS				
FM group	15.182 ± 2.715	11.063 ± 2.544	9.974 ± 1.891	9.732 ± 2.696
Metformin group	14.006 ± 3.061	11.085 ± 2.148	10.000 ± 1.702	9.716 ± 1.803

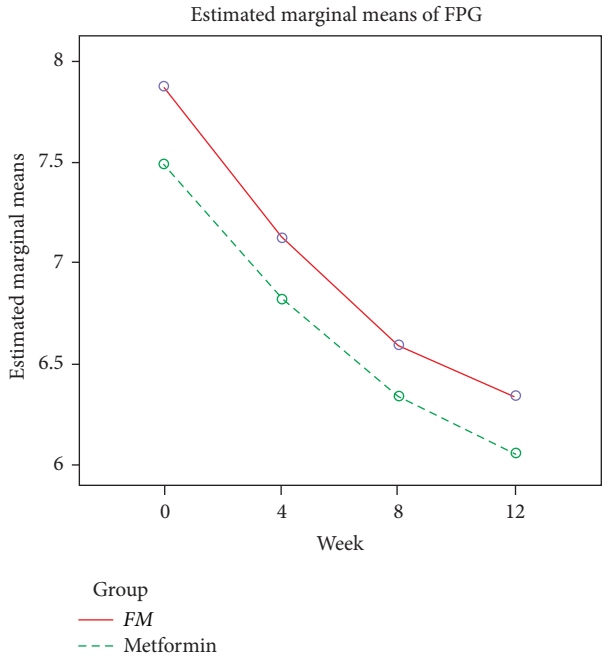

Figure 2

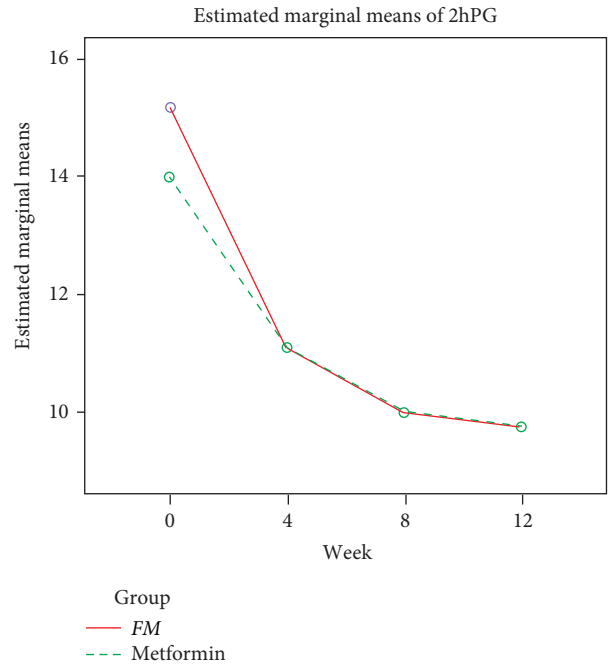

Figure 3

3.4. Insulin Concentrations. The fasting insulin concentrations were similar in the *FM* and Metformin groups ($P > 0.05$) and they did not significantly change during the 12-week period (data not shown).

3.5. Lipid Profiles. The lipid profiles (TC, TG, LDL, and HDL) were similar in the *FM* and Metformin groups ($P > 0.05$), and they did not significantly change during the 12-week period (data not shown).

3.6. Body Mass Index. For subjects whose BMI was between 23 and 25 and >25, *FM* could significantly decrease BMI, with no significant differences compared with Metformin. For subjects whose BMI <23, neither Metformin nor *FM* could decrease BMI. The changes in BMI were shown in Table 5.

3.7. Adverse Events. One patient in the *FM* group withdrew because of adverse events. The patient had palpitation and diarrhea, and they disappeared after the discontinuation of *FM*. The adverse events were classified as moderate in severity and probably related to *FM*.

4. Discussion

Surprisingly, *Fructus Mume* formula is as effective as the most eminent hypoglycemia agent—Metformin in reducing blood glucose levels. In the WCS, there were 73 percent patients with FPG < 7.0 mmol/L. The fasting plasma glucose reduced by 1.53 mmol/L and the postprandial glucose reduced by 5.45 mmol/L in the twelve-week period. What is more, the HbA1c decreased by 0.88 percent points. The results of FAS were in line with those of PPS in significance for glycemic outcomes, which suggested that our results were robust. However, we must interpret the results cautiously because of the following reasons.

First, blood glucose levels can be affected by many factors. The reduction in blood glucose levels cannot be attributed only to *FM*, as subjects underwent dietary and exercise therapy, and this might affect blood glucose significantly.

Table 5: Changes in BMI (means ± SD, Kg/m^2).

	Week 0			Week 12		
	BMI < 23	23 < BMI < 25	BM I > 25	BMI < 23	23 < BMI < 25	BMI > 25
FM group	20.07 ± 1.23	24.07 ± 0.61	28.43 ± 3.64	20 ± 1.15	23.49 ± 0.75*	27.35 ± 3.57*
Metformin group	20.77 ± 1.18	24.08 ± 0.53	26.64 ± 1.47	20.49 ± 0.95	23.76 ± 0.65*	25.68 ± 1.82*

*Indicates that there is significant difference between the values at week 0 and week 12 ($P < 0.05$).

However, since both groups received dietary and exercise therapy, the results still have clinical implications.

Second, as the trial lasted only a short 12-week period, we were not able to arrive at any conclusions concerning the long-term effect and safety of *FM*. Regarding the safety of this formula, since there was one subject who dropped out because of palpitation and diarrhea, it raises the question of the long-term toxicity of *FM* which contains both *Rhizoma Typhonii Gigantei*, as well as *Herba Asari* (removed in this study).

Thirdly, we went to great lengths to make the present trial well designed. However, it was impossible to carry out a double blind trial due to the obvious difference between TCM and Metformin.

The TCM community has developed many approaches for the treatment of diabetes, both theoretically and clinically. Frankly speaking, these, may have enriched the management portfolio of diabetes, but may have impaired the standardization of TCM. Is there a common TCM way to reduce the level of blood glucose? Taking into account the *Yin-Yang* theory, we think that managing diabetes with herbs of hot or cold properties together might be a good choice. In *Yin-Yang* theory, sweetness is a flavor of the *Yang* property (an expansive process) and bitterness and sourness are flavors of the *Yin* property (contracting). One TCM explanation of diabetes is that sweet flavor develops to an extreme point (excessive *Yang*), triggering the onset. The relationship of sugar consumption is obvious here. Therefore, we use herbs of bitter flavor—such as *Rhizoma Coptidis* and herbs of sour flavor—such as *Fructus Mume* in the treatment of T2DM (bitter flavor can counteract sweet flavor because it is in direct opposition to sweetness, and sour flavor can neutralize sweet flavor). As herbs of cold property could impair the healthy Qi of the body, herbs of hot property should be added to counteract the side-effects. As *Fructus Mume* formulais such a famous classical formula, we chose it for assessing the effects of TCM in reducing blood glucose levels. In our opinion, *Fructus Mume* formula reduces the blood glucose levels by adjusting the balance of *Yin* and *Yang*. However, the question to be answered is "By removal of some of the classic formula ingredients how does it effect the balance of Yin and Yang? [sic]" Of course, it is not enough to explain the hypoglycemic effects of *FM* just by Yin-Yang theory. It is notable that although many articles declared that TCM may have insulinotropic effects, our results demonstrated that neither *FM* nor Metformin could affect the insulin concentration. Since published literature reported that *Radix Ginseng, Rhizoma Coptidis, Cortex Phellodendri,* three herbs used in *FM,* that have antioxidant effects [16, 21–23], we took the results into account and therefore hypothesized that *FM* may exert its hypoglycemic effect on type 2 diabetes primarily by improving sensitivity of peripheral tissues and hence decreasing insulin resistance and by the reducing oxidative stress.

We also should note that the baseline levels of blood glucose were not high (mean values, 7.87 mmol/L), which suggested the severity of disease of observed subjects. This indicates that the diabetes severity level, to which TCM can be applied as a monotherapy that is still limited. Regarding the differences in Western and Asian body types, the dose of Metformin (i.e., 500 mg bid) might not be strong enough to produce optimal glycemic control for Caucasians, but this dose may be appropriate as the starting therapy for Chinese patients.

Although numerous articles declared that TCM could provide clinical benefits for the management of diabetes, most published documents were of poor methodology and no firm conclusions could be drawn. It is really very easy to yield clinical trials evaluating the effects of TCM on diabetes mellitus in some less-than-rigorous medical journals. Lack of scientific rigor is a current major problem with Chinese editors in the assimilation of scientific methodology. Some trials declared that TCM could reduce the levels of blood glucose, and some declared that TCM could provide clinical benefits for diabetic patients with microvascular or macrovascular complications—but without well-designed controlled research, it is not a solid demonstration of the evidence.

In contrast, almost all of the systematic reviews or meta-analyses which summarized the results of published articles concluded that observational trials are generally of poor methodology and well-designed randomized controlled trials (RCT) are warranted to confirm the effects of TCM on diabetes mellitus. For example, *Liuwei Dihuang Wan* is a widely used Traditional Chinese Medicinal formula for the management of diabetes. One systematic paper examined the efficacy and safety of *Liuwei Dihuang Wan* and included five RCTs. Although the five observational RCTs were of poor methodology, this review still reached the conclusion that *Liuwei Dihuang Wan* is effective and safe for type 2 diabetes [24] (which really baffles readers). Obviously, to date, most clinical evidence from the TCM community is not persuasive. Our study, albeit not conclusive, introduced higher methodology in TCM clinical trials. At any rate, the present pilot study deserves a seat in the field of TCM for diabetes because it introduces the strict methodology and it is, without doubt, brave enough to evaluate the effect of TCM, when used as a monotherapy, on "hard outcomes."

Compared with the established hypoglycemic effects of western medicine, the blood glucose-lowering effect of TCM

remains controversial. However, it is a topic many experts would like to clarify. He et al. [25] evaluated the antidiabetic efficacy and mechanisms of 34 TCMs. Their results showed that 13 out of the 34 herbs showed a statistically significant plasma glucose-lowering action compared with the diabetic control group.

To our current knowledge, most TCM formulae aimed at diabetes focus on alleviating diabetic symptoms other than decreasing blood glucose. Although few TCM formulae have recognized blood glucose-lowering effects, it is not difficult to yield articles in medical journals where the authors declared their test TCM had blood glucose-lowering effects. A brief look at the evidence demonstrates that TCM may have hypoglycemic potential. In a Chinese article, Zhong et al. [26] concluded "the TCM *Yitangyin* (YTY) granule is an effective hypoglycemic agent." Zhao and coworkers [27] reported that a TCM formula, referred to as *JCU*, has sustained glucose-lowering effects in male Zucker diabetic fatty rats. Li et al. [28] assessed the effect of *qiangyi jiangtang* capsules (*QJC*) on diabetes mellitus model Wistar rats, and their results demonstrated "*QJC* could remarkably lower the levels of blood glucose, HbA1c." Since published literature with respect to the hypoglycemic effects of TCM is increasing, the hypoglycemic effect of TCM remains an explorative issue and the debate about it needs to be clarified.

5. Conclusions and Looking Forward

In summary, *Fructus Mume* formula may reduce the levels of blood glucose in patients with type 2 diabetes to some extent. The present study also discloses the potential of TCM strategy with the use of herbs of cold or hot property together to adjust the balance of *Yin* and *Yang* in the treatment of type 2 diabetes. Future research on this topic should address (1) *FM* ingredients which are toxic, (2) a closer look at individual patient characteristics that may be prone to the side effects, and (3) if TCM methods are able to significantly reduce the levels of blood glucose across levels of BMI.

Conflict of Interests

The authors declared that there is no conflict of interests.

Acknowledgments

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Research Article

Improvement of Liquid Fructose-Induced Adipose Tissue Insulin Resistance by Ginger Treatment in Rats Is Associated with Suppression of Adipose Macrophage-Related Proinflammatory Cytokines

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Adipose tissue insulin resistance (Adipo-IR) results in excessive release of free fatty acids from adipose tissue, which plays a key role in the development of "lipotoxicity." Therefore, amelioration of Adipo-IR may benefit the treatment of other metabolic abnormalities. Here we found that treatment with the alcoholic extract of ginger (50 mg/kg/day, by oral gavage) for five weeks attenuated liquid fructose-induced hyperinsulinemia and an increase in the homeostasis model assessment of insulin resistance (HOMA-IR) index in rats. More importantly, ginger reversed the increases in the Adipo-IR index and plasma nonesterified fatty acid concentrations during the oral glucose tolerance test assessment. Adipose gene/protein expression profiles revealed that ginger treatment suppressed CD68 and F4/80, two important macrophage accumulation markers. Consistently, the macrophage-associated cytokines tissue necrosis factor alpha and interleukin-6 were also downregulated. In contrast, insulin receptor substrate (IRS)-1, but not IRS-2, was upregulated. Moreover, monocyte chemotactic protein (MCP)-1 and its receptor chemokine (C-C motif) receptor-2 were also suppressed. Thus these results suggest that amelioration of fructose-induced Adipo-IR by ginger treatment in rats is associated with suppression of adipose macrophage-related proinflammatory cytokines.

1. Introduction

Insulin resistance is the thread that runs through many chronic afflictions of modern times: obesity, cardiovascular disease, and, most conspicuously, type 2 diabetes [1]. While hepatic and muscle insulin resistance plays important roles, an additional component, adipose tissue insulin resistance (Adipo-IR), is also a significant factor to systemic insulin resistance, especially to the development of obesity-related insulin resistance [2, 3]. Adipose tissue is increasingly recognized as a secretory organ that plays many important roles in homeostasis, of which energy expenditure and insulin sensitivity are included. Adipose tissue is understood to exert its effects through both paracrine and endocrine mechanisms. Adipose tissue is emerging as a key mediator of cardiometabolic disorders in the general population and of liver disease in nonalcoholic fatty liver disease, likely through the modulation of lipotoxic free fatty acid metabolism and of pro- and anti-inflammatory cytokine secretion [3, 4]. Recent evidence suggests that the severity of Adipo-IR is closely correlated with metabolic derangements and hepatic histological damage in patients with nonalcoholic steatohepatitis [4–7]. Treatment with the insulin-sensitizing agent pioglitazone that improves adipose tissue functions [8] decreased Adipo-IR, which was correlated with the decreases in hepatic

fat accumulation and necroinflammation in patients with nonalcoholic steatohepatitis [5, 7, 9]. These findings suggest that amelioration of Adipo-IR may play an important role in the treatment of nonalcoholic fatty liver disease.

Strong evidence suggests that consumption of diets high in fructose results in fatty liver, hyperlipidemia, and insulin resistance [10–12]. After absorption, fructose is almost completely metabolized in the liver, where fructose increases de novo lipogenesis [12]. The intrahepatic effects of fructose overconsumption have been extensively addressed [12]. It is also well known that fructose overconsumption is associated with adiposity [10–12]. Recent findings in clinic suggest that, in adolescents, higher fructose consumption is associated with multiple markers of cardiometabolic risk, but it appears that these abnormalities are mediated by visceral obesity [13]. Unfortunately, we still know much less about the adverse effects of fructose overconsumption on adipose tissue functions.

Ginger (*Zingiber officinale* Roscoe, Zingiberaceae) is one of the most commonly used spices and medicinal plants around the world. It has been found that ginger treatment ameliorates fatty liver and hyperlipidemia in rats fed cholesterol-enriched diet [14] and high-fat diet [15]. Recently, we have also demonstrated that ginger treatment ameliorates fructose-induced metabolic abnormalities, such as fatty liver and hypertriglyceridemia in rats [16]. Further, the hepatic pathways have been suggested in the lipid-lowering effects [14–16]. In the present study, we investigated the effects of fructose overconsumption on adipose tissue insulin functions and the impact of ginger treatment in rats.

2. Materials and Methods

2.1. The Alcoholic Extract of Ginger. The alcoholic extract of ginger was prepared and identified as described previously [16]. Briefly, 5 kg sliced ginger rhizomes including the skin were immersed in 5 L 95% ethanol with intermittent shaking for 24 h, then refluxed for 3 h by heating. The filtrate was evaporated under reduced pressure below 45°C. The residue (yield: 9.6%) was designated as an alcoholic extract. The extract was quantified by HPLC method to contain two representative components: [6]-gingerol and [6]-shogaol in concentrations of 4.4% and 1.1%, respectively.

2.2. Animals: Diet and Experimental Protocol. All animal procedures were in accordance with the "Principles of laboratory animal care" (http://grants1.nih.gov/grants/olaw/references/phspol.htm) and were approved by the Animal Ethics Committee, Chongqing Medical University, China.

Male Sprague-Dawley rats weighing 210–230 g and the standard diet were supplied by the laboratory animal center, Chongqing Medical University, China. Rats were housed in a temperature-controlled facility (21 ± 1°C, 55 ± 5% relative humidity) with a 12 h light/dark cycle. Animals were allowed free access to water and the standard diet for at least 1 week prior to starting the experiments.

Given that sugar-sweetened nonalcoholic beverages, such as soft drinks, appear as the major source of fructose for the population aged 6–50 years [12], liquid fructose was used in the present study. In initial experiments, we noted that compared to vehicle, ginger treatment significantly increased fructose intake when the rats had free access to 10% fructose in drinking water. In order to exclude the influence of the difference in intake of fructose (the primary pathogenic factor in the development of the adverse metabolic effects in this model), we adjusted the fructose consumption in ginger-treated rats to that of fructose controls. 24 rats were divided into 4 groups ($n = 6$ per group, 2 rats/cage): (1) water control, free access to water; (2) fructose control, free access to 10% fructose solution (w/v, preparation every day); (3) fructose ginger 20 mg/kg; and (4) fructose ginger 50 mg/kg, in which the fructose consumption was adjusted (by regulating the concentration of fructose solution) daily to that in the fructose-control group on the previous day. There was no difference in body weight between the groups before treatments commenced. Animals in ginger-treated groups were administered ginger extract 20 and 50 mg/kg (oral gavage, once per day) for 5 weeks, respectively. The rats in water- and fructose-control groups received vehicle (5% Gum Arabic) alone. All rats had free access to the standard chow. The consumed chow and fructose solution were measured daily, and the intake of fructose was calculated. At the end of week 4, oral glucose tolerance test (OGTT) was performed. After rats were deprived of chow but still had free access to water or fructose solution for 14 h on day 35, animals were weighed and killed. Epididymal white adipose tissue (eWAT) was collected and weighed. Segments of eWAT were snap-frozen in liquid nitrogen and stored at −80°C for subsequent determination of gene expression.

2.3. OGTT. After being fasted for 14 h with free access to water, all rats received a glucose solution (2 g/kg in 10 mL) by the oral route. Blood samples were collected prior to and 20, 60, and 120 min after administration of glucose solution for determination of plasma concentrations of glucose (kit from Kexin Institute of Biotechnology, Shanghai, China), insulin (kit from Morinaga Biochemical Industries, Tokyo, Japan), and nonesterified fatty acid (NEFA) (NEFA-C kit, Wako, Osaka, Japan) using enzymatic methods or by ELISA. Hepatic insulin sensitivity was expressed as the homeostasis model assessment of insulin resistance (HOMA-IR) index {[fasted insulin (μIU/mL) × fasted glucose (mM)]/22.5} [6, 17]. Adipo-IR index was calculated as the following formula: [Adipo-IR index = fasted insulin (mmol/L) × fasted NEFA (pmol/L)] [4–6, 9].

2.4. Histological Examination. A portion of eWAT was fixed with 10% formalin and embedded in paraffin. 10-micron sections were cut and stained with hematoxylin and eosin for examination of adipose tissue histology (IX-81, Olympus Corporation, Tokyo, Japan). The adipocyte cross-sectional area was measured using an ImageJ 1.43 analyzing system.

2.5. Real-Time PCR. Total RNA was isolated from eWATs of individual rats using TRIzol (Takara, Dalian, China). cDNA was synthesized using M-MLV RTase cDNA Synthesis Kit (Takara, Dalian, China) according to the manufacturer's

instructions. Real-time PCR was performed with the CFX 96 Real Time PCR Detection System (Bio-rad Laboratories Inc, Hercules, CA, USA) using the SYBR Premix Ex Taq II (Takara, Dalian, China). The sequences of primers are shown in Table 1. Gene expression in individual samples was determined in duplicate and was normalized against the reference β-actin. Expression in water-control rats was arbitrarily assigned a value of 1.

2.6. Data Analysis. All results are expressed as means ± SEM. Data obtained from experiments with more than two groups of animals were analyzed by ANOVA using StatView and followed by Student-Newman-Keuls testing to locate the differences between groups. Data obtained from experiments with two groups of animals were analyzed by the Student's t-test. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Effects of Fructose Consumption in Rats. Recently, we have demonstrated that fructose-overconsumption-induced fatty liver and hypertriglyceridemia in rats are improved by ginger treatment [16]. In the present study, we focused on adipose function-related variables.

Five-week intake of 10% fructose solution decreased chow intake (water control: 1993.1 ± 100.0 versus fructose control: 1082.8 ± 75.6 g/2 rats/5 weeks, $P < 0.05$). Compared to water drinking, fructose feeding did not significantly affect body weights (Figure 1(a)) and body weight gain (Figure 1(b)). eWAT weight (Figure 1(c)), the ratio of eWAT weight to body weight (Figure 1(d)), and adipocyte size (Figures 1(f), 2(a) and 2(b)) had a trend to increase, whereas adipocyte number (Figure 1(e)) had a trend to decrease.

Although fructose feeding did not alter basal plasma glucose concentration (Figure 3(a)), it significantly increased plasma insulin concentration under fasted condition (Figure 3(b)). The increase in insulin concentration resulted in an increase in the HOMA-IR index (Figure 3(c)).

During the OGTT assessment, changes in plasma glucose (Figures 4(a) and 4(b)) and insulin (Figures 4(c) and 4(d)) concentrations were not significantly different from those of water-control group.

Importantly, basal plasma NEFA concentrations (Figure 5(a)) and Adipo-IR index (Figure 5(b)) in fructose controls were higher compared to those in water controls. Furthermore, plasma NEFA concentrations (Figure 5(c)) and the NEFA AUC (Figure 5(d)) during the OGTT assessment were also increased.

3.2. Effects of Ginger Treatment in Fructose-Fed Rats. Fructose intake was uniform in fructose-control and fructose-ginger groups (fructose control: 845.4 ± 33.1; fructose ginger 20 mg/kg: 827.2 ± 13.6; fructose ginger 50 mg/kg: 831.8 ± 18.1 g/2 rats/5 weeks, $P > 0.05$). Ginger treatments did not significantly affect chow intake (fructose control: 1082.8 ± 75.6; fructose ginger 20 mg/kg: 1156.2 ± 29.1; fructose ginger 50 mg/kg: 1155.8 ± 52.0 g/2 rats/5 weeks, $P > 0.05$), body weights (Figure 1(a)), and body weight gain (Figure 1(b)). eWAT weight (Figure 1(c)), the ratio of eWAT weight to body weight (Figure 1(d)), and adipocyte size (Figures 1(f), 2(c), and 2(d)) tended to decrease, and adipocyte number (Figure 1(e)) tended to increase after ginger treatment. Basal plasma glucose and insulin concentrations, as well as the HOMA-IR index were decreased after treatment with 50 mg/kg ginger extract (Figures 3(a)–3(c)). During the OGTT assessment, the changes in plasma glucose concentrations (Figures 4(a) and 4(b)) were similar, but insulin concentrations (Figures 4(c) and 4(d)) were significantly lower in fructose ginger 50 mg/kg group than fructose-control group.

Ginger treatments did not significantly decrease basal plasma NEFA concentrations (Figure 5(a)), but fructose-induced increases in the Adipo-IR index (Figure 5(b)) and plasma NEFA concentrations (Figures 5(c) and 5(d)) during the OGTT assessment were reversed after treatment with ginger 50 mg/kg.

3.3. Adipose Gene/Protein Expression Profiles in Rats. By real-time PCR, fructose-control rats showed significant increase in adipose β-actin, the house-keeping gene, compared to water-control rats. However, there was no difference in β-actin expression between fructose-control and fructose-ginger groups (data not shown). Thus, comparisons in gene expression are restricted to fructose-control and fructose-ginger groups.

Ginger treatment substantially suppressed adipose expression of CD68 (Figure 6(a)), F4/80 (Figure 6(b)), tumor necrosis factor (TNF)-α (Figure 6(c)), and interleukin (IL)-6 (Figure 6(d)). In contrast, expression of insulin receptor substrate (IRS)-1 (Figure 6(e)), but not IRS-2 (Figure 6(f)), was upregulated. Moreover, monocyte chemotactic protein (MCP)-1 (Figure 6 (g)) and chemokine (C-C motif) receptor-2 (CCR2) (Figure 6(h)) were also suppressed.

In addition, ginger treatment had no significant effect on carbohydrate-response-element-binding protein (ChREBP) (Figure 7(a)), sterol-regulatory-element-binding protein (SREBP)-1c (Figure 7(b)), fatty acid synthase (FAS) (Figure 7(c)), acetyl-CoA carboxylase (ACC)-1 (Figure 7(d)), stearoyl-CoA desaturase (SCD)-1 (Figure 7(e)), peroxisome-proliferator-activated receptor (PPAR)-γ (Figure 7(f)), adiponectin (Figure 7(g)), and CD36 (Figure 7(h)).

4. Discussion

4.1. Ginger Treatment Improves Liquid-Fructose-Overconsumption-Induced Adipo-IR in Rats. Insulin action in adipose tissue involves stimulation of glucose uptake and inhibition of lipolysis. However, adipose tissue only accounts for about 10% of insulin-stimulated glucose disposal [18]. In contrast, adipose tissue is the primary source of free fatty acids (~ 70%) for hepatic triglyceride synthesis [19]. In the setting of insulin resistance, insulin is unable to properly suppress lipolysis, resulting in an increase in free fatty acid release into the plasma [20]. Excess release of free fatty acids plays a key role in the development of lipotoxicity including liver injuries [6, 21–23]. Increased delivery of free fatty acids from adipose tissue leads to increases in fat accumulation, gluconeogenesis, and insulin resistance in liver [24, 25]. Therefore,

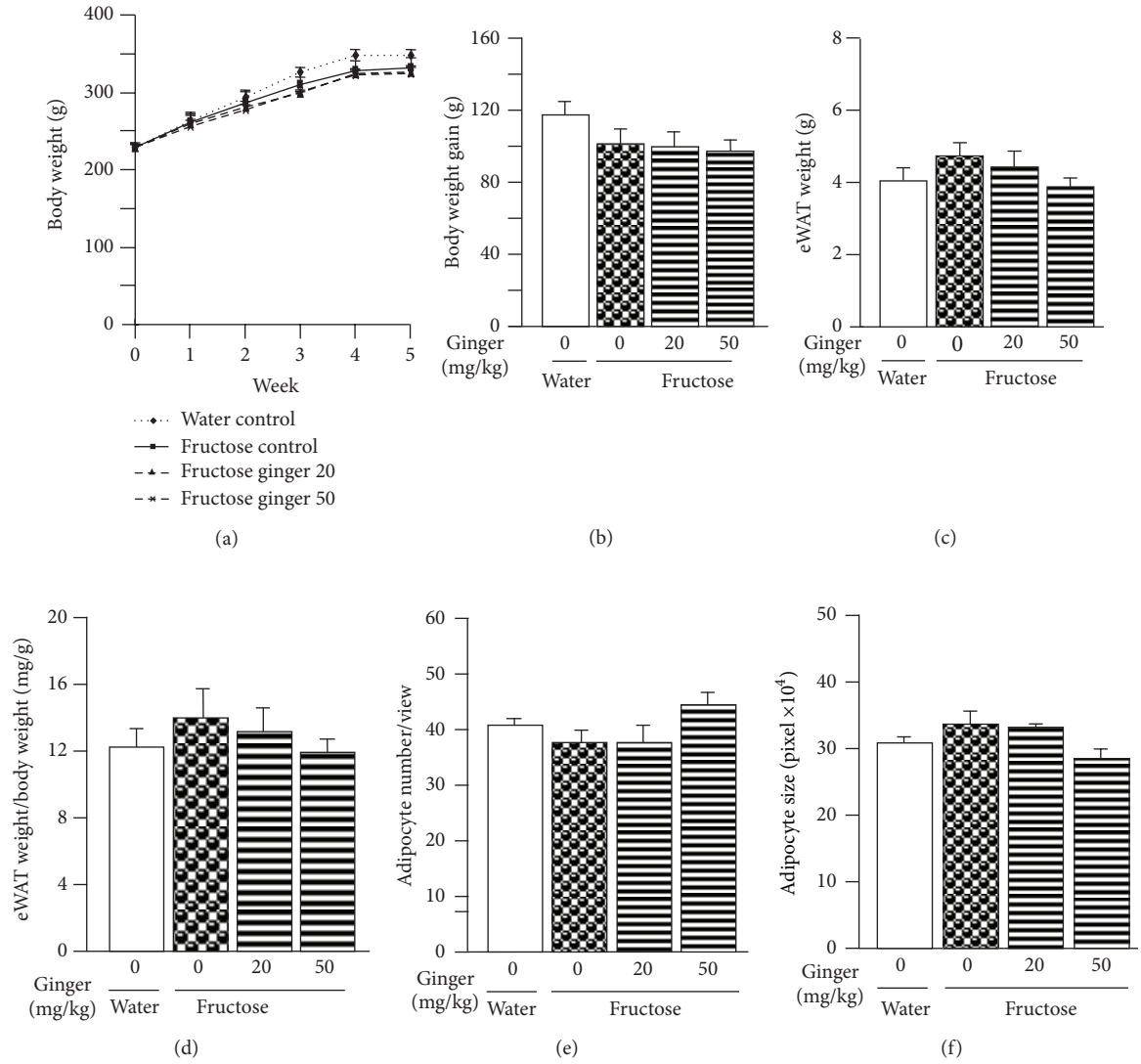

Figure 1: Body weight (a), body weight gain (b), epididymal white adipose tissue (eWAT) weight (c), ratio of eWAT weight to body weight (d), adipocyte number (e) and adipocyte size (f) in water-control and fructose-pair-fed rats. The fructose control rats had free access to 10% fructose in their drinking of water over 5 weeks, while the consumption of fructose in the ginger-(20 or 50 mg/kg) treated (by gavage daily) rats was adjusted to that of the fructose-control rats. Data are means ± SEM ($n = 6$ each group).

plasma fatty acid changes during the OGTT assessment and the Adipo-IR index are used to evaluate insulin action in adipose tissues and analyze the contribution to the development of hepatic injuries including fatty liver [4–6, 9]. We have recently demonstrated that ginger treatment improves fructose-induced fatty liver in rats [16]. In the present study, long-term liquid fructose overconsumption increased the index of Adipo-IR and plasma NEFA and insulin concentrations at baseline and during the OGTT assessment in rats, indicating that fructose overconsumption induces Adipo-IR. The increases in Adipo-IR index and plasma concentrations of NEFA and insulin during the OGTT assessment were attenuated after treatment with ginger extract. These effects were accompanied by decrease in the HOMA-IR index, which reflects hepatic insulin sensitivity [5, 6]. Thus, these results suggest that ginger treatment improves fructose-induced Adipo-IR.

4.2. Suppression of Adipose Macrophage-Associated Proinflammatory Cytokines Contributes to Ginger-Treatment-Elicited Amelioration of Adipo-IR in Fructose-Fed Rats. It is well known that inflammation in white adipose tissues (especially visceral fat) is associated with insulin resistance [1, 26, 27]. The macrophage accumulation in adipose tissue under an inflammatory state is a hallmark of obesity-induced insulin resistance, and the macrophages are responsible for almost all adipose tissue expression of TNF-α and IL-6, the markers of adipose macrophage polarization and inflammation [28, 29]. TNF-α is an important mediator of insulin resistance in obesity and diabetes through its ability to decrease the tyrosine kinase activity of the insulin receptor. TNF-α directly decreases insulin sensitivity and increases lipolysis in adipocytes [30, 31]. It has been demonstrated that treatment of cultured murine adipocytes with TNF-α induced serine phosphorylation of IRS-1 and converted IRS-1 into an

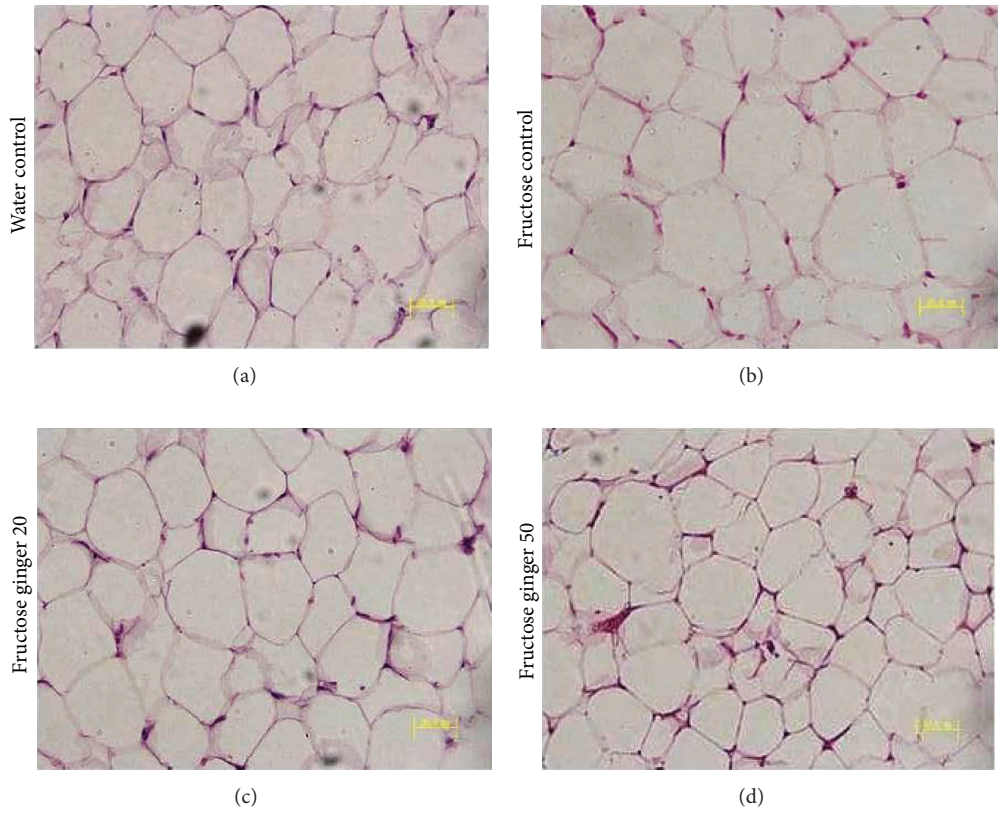

Figure 2: Representative images showing histology of eWAT (hematoxylin and eosin staining) in water-control or fructose-pair-fed rats (a)–(d). The fructose-control rats had free access to 10% fructose in their drinking water over 5 weeks, while the consumption of fructose in the ginger-(20 or 50 mg/kg) treated (by gavage daily) rats was adjusted to that of the fructose-control rats.

inhibitor of the insulin receptor tyrosine kinase activity in vitro [32]. IL-6 plays a crucial role in metabolic processes and has adverse effects on insulin action in liver and adipose tissue [33]. IL-6 stimulates lipolysis to increase plasma NEFA concentrations in rats [34] and in human adipose tissue [35]. Insulin binding induces receptor tyrosine autophosphorylation, which is followed by the recruitment of scaffolding proteins known as IRS-1 and IRS-2 [18]. It has been suggested that IRS-1 works on the metabolism by regulating insulin signals in muscle and adipose tissues, whereas IRS-2 is a major player of hepatic insulin action [36]. IRS-1-deficient mice showed a phenotype of peripheral insulin resistance (mainly in muscle and white adipose tissue) [37, 38]. IL-6-mediated insulin resistance involves activation of proinflammatory kinases that converge at the IRS-1 level [39]. In the present study, 5-week fructose feeding tended to increase adiposity in rats, which was diminished by treatment with ginger extract. Importantly, ginger treatment suppressed adipose expression of CD68 and F4/80 (two important macrophage markers [29, 31]), indicating that ginger treatment decreases macrophage content in adipose tissue. Consistently, expression of the macrophage-associated proinflammatory cytokines, TNF-α and IL-6, was also downregulated. Further, the level of mRNA encoding IRS-1, but not IRS-2, was upregulated. Thus, these findings suggest that ginger treatment improves fructose-induced Adipo-IR via suppression of adipose macrophage accumulation-associated proinflammatory cytokines.

Macrophages may populate in adipose tissue during obesity through recruitment of chemokine-mediated chemotaxis. MCP-1 is an adipokine with insulin-resistance-inducing capacity that is related to increased adipose tissue mass in obesity and insulin resistance [40]. MCP-1 causes infiltration of macrophages to release proinflammatory proteins, such as TNF-α and IL-6 [30, 41, 42]. Studies have shown that overexpression of MCP-1 in adipose tissues causes macrophage recruitment and insulin resistance [30, 42]. In contrast, MCP-1 or its receptor CCR-2 knockout mice have fewer macrophages and less inflammation in adipose tissue and are protected from high-fat-diet-induced insulin resistance [41, 42]. Therefore, MCP-1 plays a pivotal role in the development of insulin resistance and is an important therapeutic target for improvement of insulin resistance [30, 31, 40–42]. In the present study, ginger treatment significantly suppressed adipose expression of MCP-1 and CCR-2 in fructose-fed rats, which was consistent with the downregulation of the adipose expression of macrophage-accumulation-mediated proinflammatory cytokines. Thus, it is likely that modulation of the adipose MCP-1-mediated

Table 1: Primer sequences for real time PCR assays.

Gene	Forward primers	Reverse primers
β-actin	ACGGTCAGGTCATCACTATCG	GGCATAGAGGTCTTTACGGATG
ACC-1	AACATCCCGCACCTTCTTCTAC	CTTCCACAAACCAGCGTCTC
Adiponectin	CGTTCTCTTCACCTACGACCAGT	ATTGTTGTCCCCTTCCCCATAC
CCR-2	GAAGACCCAAAGACCAAGATGC	TCTGACAACAAAGCAGGAGGTG
CD36	AACCCAGAGGAAGTGGCAAAG	GACAGTGAAGGCTCAAAGATGG
CD68	ACTGGGGCTCTTGGAAACTACAC	CCTTGGTTTTGTTCGGGTTCA
ChREBP	TTGTTGGTGAGAAGTTCCGAAGG	CCCAGTAGAAGGGGTAAATGTTGAG
F4/80	ATCGCTGCTGGCTGAATACG	GCAACCTCGTATCCTTGAGCTTAG
FAS	ACCTCATCACTAGAAGCCACCAG	GTGGTACTTGGCCTTGGGTTTA
IL-6	GTTGCCTTCTTGGGACTGATGT	GGTCTGTTGTGGGTGGTATCCT
IRS-1	CTTCTGTTACACCTCAAGGGGC	GGTTATGGTTGGGACTTAGGTTCA
IRS-2	GACCAGTCCCACATCAGGCTT	CTGCACGGATGACCTTAGCG
MCP-1	CGGTTTCTCCCTTCTACTTCCTG	GCTCTGCCTCAGCCTTTTATTG
PPAR-γ	GCCCTTTGGTGACTTTATGGAG	GCAGCAGGTTGTCTTGGATGT
SCD-1	CAGTTCCTACACGACCACCACTA	GGACGGATGTCTTCTTCCAGAT
SREBP-1c	CTGTCGTCTACCATAAGCTGCAC	ATAGCATCTCCTGCACACTCAGC
TNF-α	ATGGGCTCCCTCTCATCAGTTC	CTCCTCCGCTTGGTGGTTTG

Sequences: 5′ to 3′.

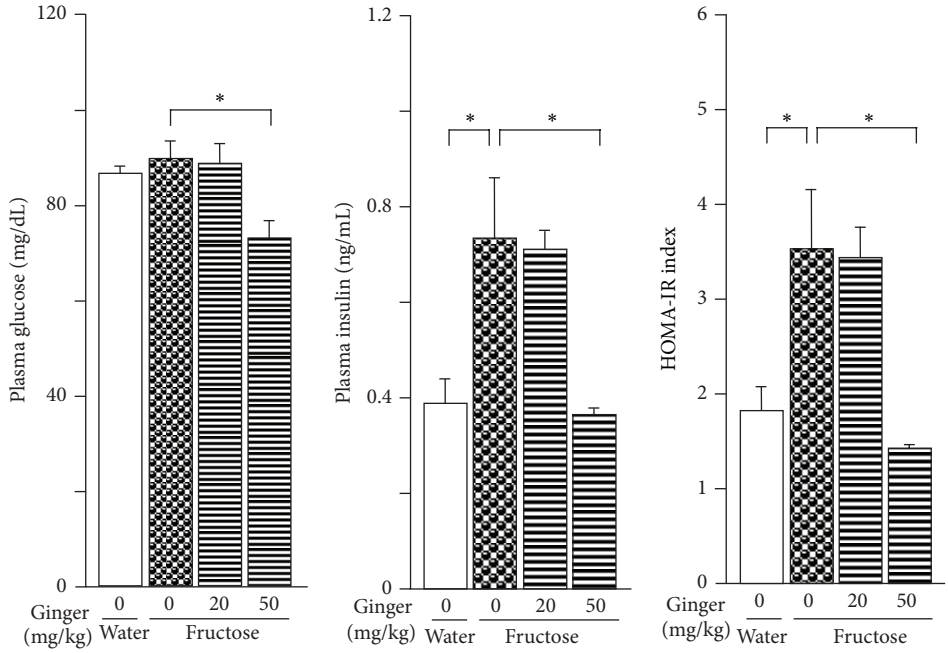

Figure 3: Plasma glucose (a) and insulin (b) concentrations at the baseline (fasted) and HOMA-IR index (c) in water-control and fructose-pair-fed rats. The fructose-control rats had free access to 10% fructose in their drinking water, while the consumption of fructose in the ginger-(20 or 50 mg/kg) treated (by gavage daily) rats was adjusted to that of the fructose-control rats. The variables were determined at the end of week 4. Data are means ± SEM ($n = 6$ each group). $^*P < 0.05$.

pathway is involved in ginger-treatment-elicited suppression of the proinflammatory cytokines.

The constituents of ginger are numerous. Gingerols and shogaols (the latter is a dehydrated form of gingerols) are major components derived from ginger. [6]-gingerols and [6]-shogaol have been implicated in most of the pharmacological activities of ginger [43]. It has been reported that ginger components gingerols, [6]-shogaol and 1-dehydro-[10]-gingerdione, inhibit lipopolysaccharide-stimulated release and gene expression of proinflammatory cytokines including

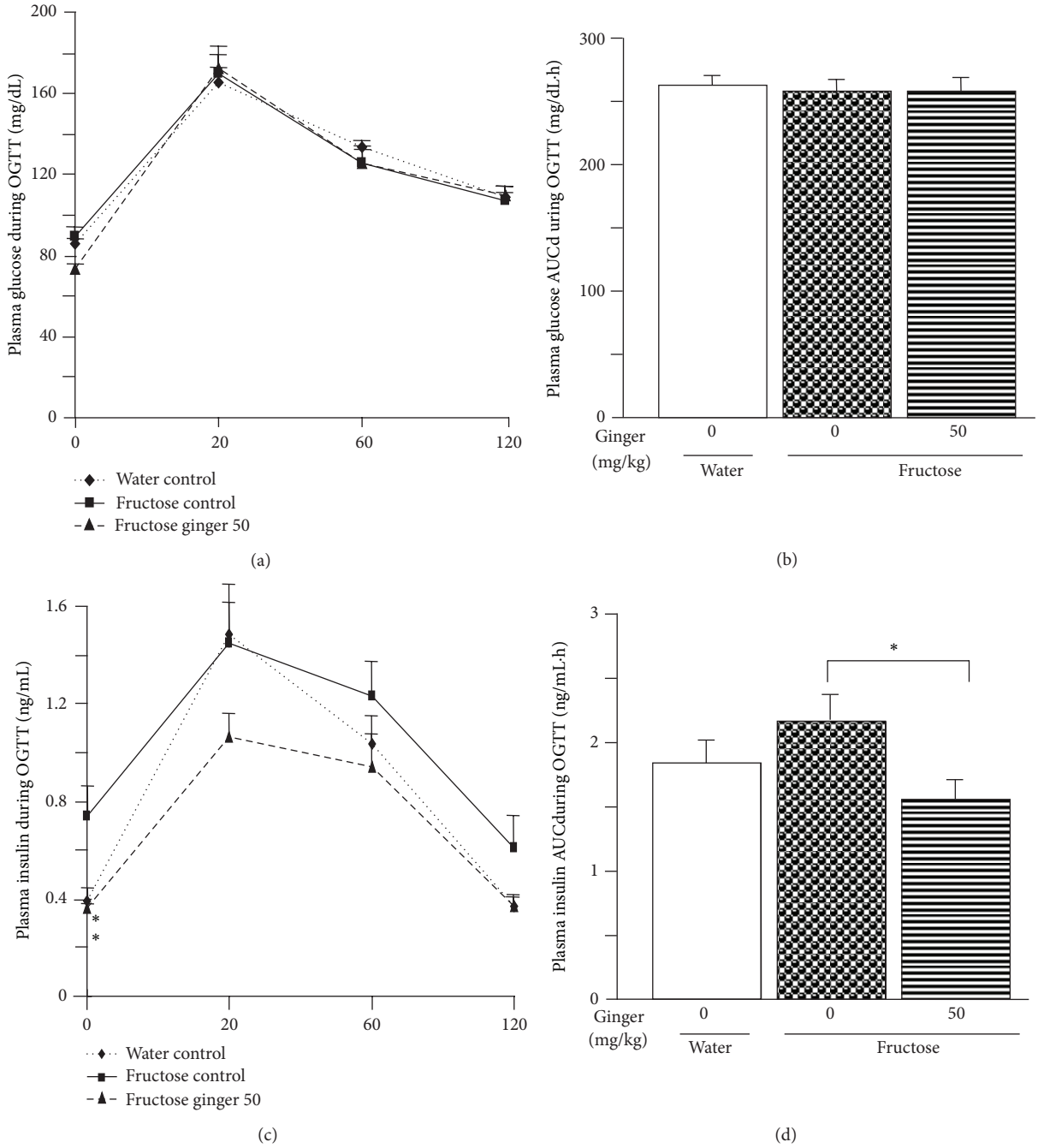

Figure 4: Plasma concentrations of glucose (a) and insulin (c) and their area under curve (AUC)s ((b) and (d)) during oral glucose tolerance test (OGTT, glucose: 2 g/kg) assessment in water-control and fructose-pair-fed rats. The fructose-control rats had free access to 10% fructose in their drinking water, while the consumption of fructose in the ginger-(20 and/or 50 mg/kg) treated (by gavage daily) rats was adjusted to that of the fructose-control rats. OGTT was performed at the end of week 4. Data are means ± SEM ($n = 6$ each group). $^{*}P < 0.05$.

MCP-1 and IL-6 in RAW 264.7 macrophages or cultured primary rat astrocytes [44–47]. On the other hand, recent studies indicate that the recruited and activated neutrophils in adipose tissue produce chemokines and cytokines in response to excess energy intake [48, 49]. These neutrophils can facilitate macrophage infiltration, thereby contributing to the chronic low-grade-inflammation-associated insulin resistance [48, 49]. From here, thus, further investigation is needed to understand more about ginger detail by detail, such as the components responsible for the improvement of fructose-induced Adipo-IR, the specific function on macrophages, the manner in suppressing TNF-α and IL-6, and the role in modulating the functions of adipose neutrophils.

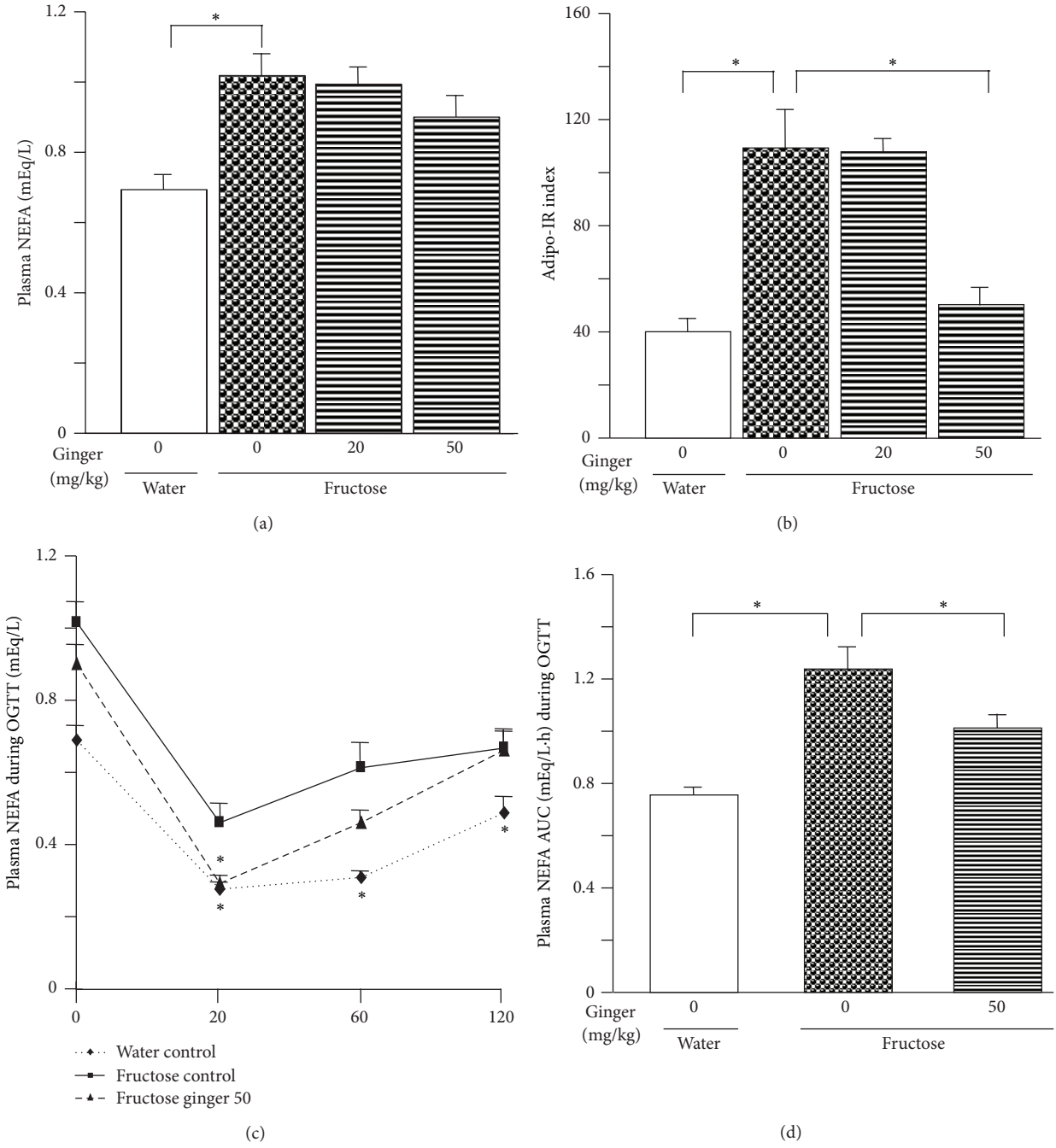

Figure 5: Plasma NEFA concentrations at the baseline (fasted) (a) and during oral glucose tolerance test (OGTT, glucose: 2 g/kg) assessment (c), the AUC of NEFA concentrations during OGTT (d), and the adipose tissue insulin resistance (Adipo-IR) index (b) in water-control and fructose-pair-fed rats. The fructose-control rats had free access to 10% fructose in their drinking of water, while the consumption of fructose in the ginger-(20 and/or 50 mg/kg) treated (by gavage daily) rats was adjusted to that of the fructose-control rats. OGTT was performed at the end of week 4. Data are means ± SEM ($n = 6$ each group). $^*P < 0.05$.

4.3. Ginger Treatment Does Not Alter Adipose PPAR-γ-, ChREBP-, and SREBP1c-Mediated Gene Expression in Fructose-Fed Rats. PPAR-γ is a member of the ligand-activated nuclear receptor superfamily, expressed at high levels in adipose tissue; PPAR-γ-activating ligands improve adipose tissue function by altering fat topography and adipocyte phenotype and by upregulating genes encoding molecules that promote a combination of lipid storage and lipogenesis, such as CD36, SREBP-1, and SCD-1; PPAR-γ agonists promote the production of adiponectin in adipose tissue [8, 18]. Treatment with PPAR-γ agonist troglitazone upregulates the gene expression of PPAR-γ, ChREBP, and SREBP-1c in 3T3-L1 adipocytes [50]. It is known that ChREBP may play an important role in mediating de novo lipogenesis [12, 25].

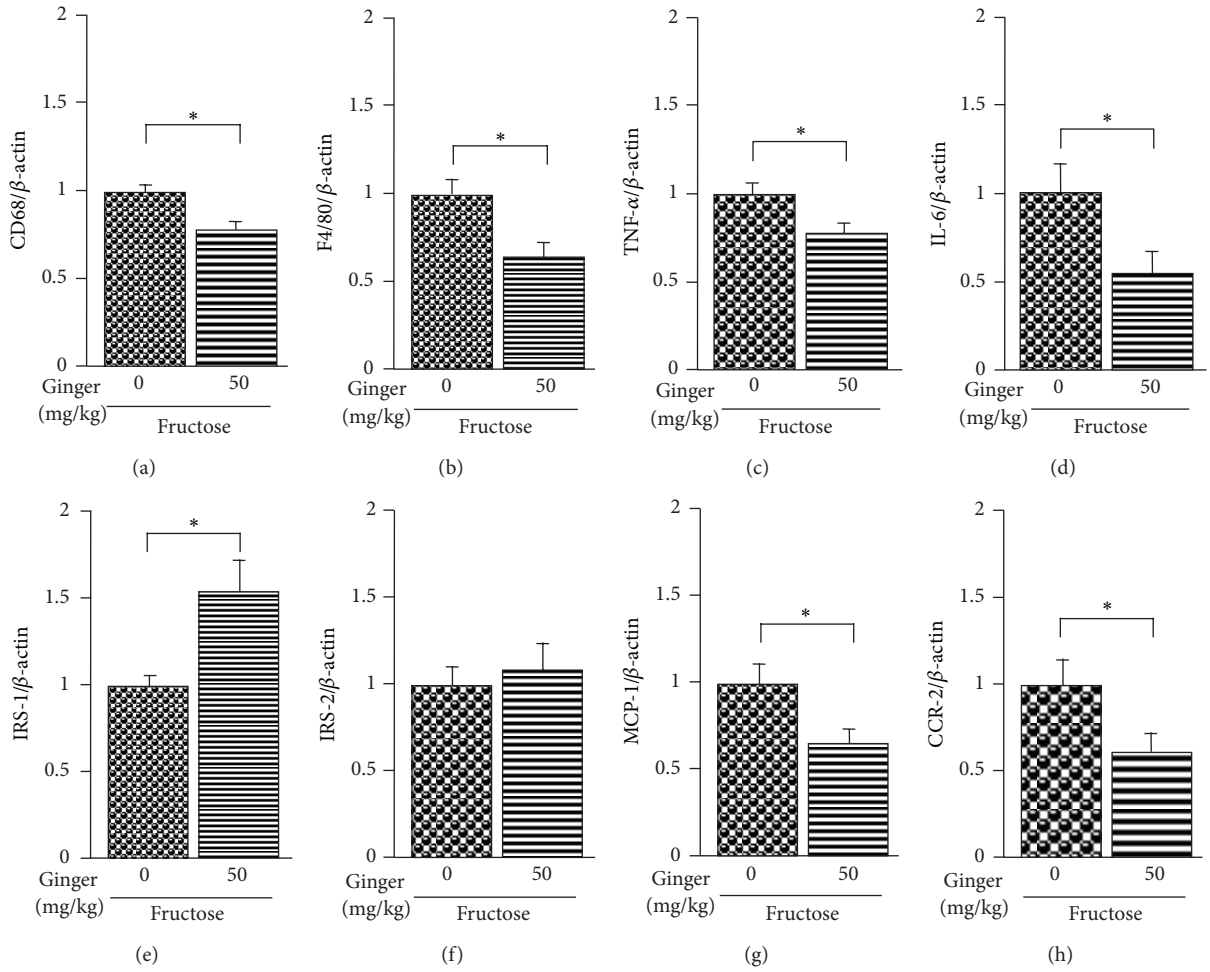

Figure 6: Adipose mRNA expression of CD68 (a), F4/80 (b), tumor necrosis factor (TNF)-α (c), interleukin (IL)-6 (d), insulin receptor substrates (IRS)-1 (e) and IRS-2 (f), monocyte chemotactic protein (MCP)-1 (g), chemokine (C-C motif) receptor (CCR)-2 (h) in fructose-pair-fed rats. The fructose-control rats had free access to 10% fructose in their drinking water, while the consumption of fructose in the ginger-(50 mg/kg) treated (by gavage daily) rats was adjusted to that of the fructose-control rats over 5 weeks. mRNA was determined by real-time PCR and normalized to β-actin. Levels in fructose control rats were arbitrarily assigned a value of 1. Data are means ± SEM ($n = 6$ each group). $^*P < 0.05$.

However, the expression of ChREBP may be tissue dependent. Recent findings from humans have demonstrated that ChREBP mRNA and protein levels are increased in the liver from obese compared to lean subjects, whereas the expression is decreased in adipose tissues [51]. It has been reported that [6]-shogaol acts as a PPAR-γ agonist in 3T3-L1 adipocytes that originally derived from mice [52]. Recently, we have demonstrated that treatment with ginger extract substantially suppressed fructose-stimulated hepatic overexpression of the lipogenic protein/genes ChREBP, ACC-1, FAS, and SCD-1 in rats [16]. In the present study, however, treatment with ginger extract did not alter adipose mRNA levels of PPAR-γ, adiponectin, CD36, ChREBP, SREBP-1c, FAS, and ACC-1 in fructose-fed rats. Thus, our findings in gene expression do not support that the adipose PPAR-γ, ChREBP, and SREBP-1c pathways are involved in the improvement of Adipo-IR by ginger treatment. These results also suggest tissue-specific regulation of the lipogenic protein/genes by ginger treatment. Studies are needed to further clarify whether the difference in animal species (mice versus rats) and/or situation (in vitro versus in vivo) is associated with the discrepancy of the effect of ginger on PPAR-γ.

5. Conclusion

The present results demonstrate that ginger treatment ameliorates fructose-overconsumption-induced adipose tissue insulin resistance in rats, which is associated with suppression of adipose macrophage-related proinflammatory cytokines. Our findings provide new insight into the pharmacological basis of therapeutics, especially for the traditional use of ginger in the prevention and treatment of metabolic derangements.

Conflict of Interests

The authors declare that there is no conflict of interests associated with this paper.

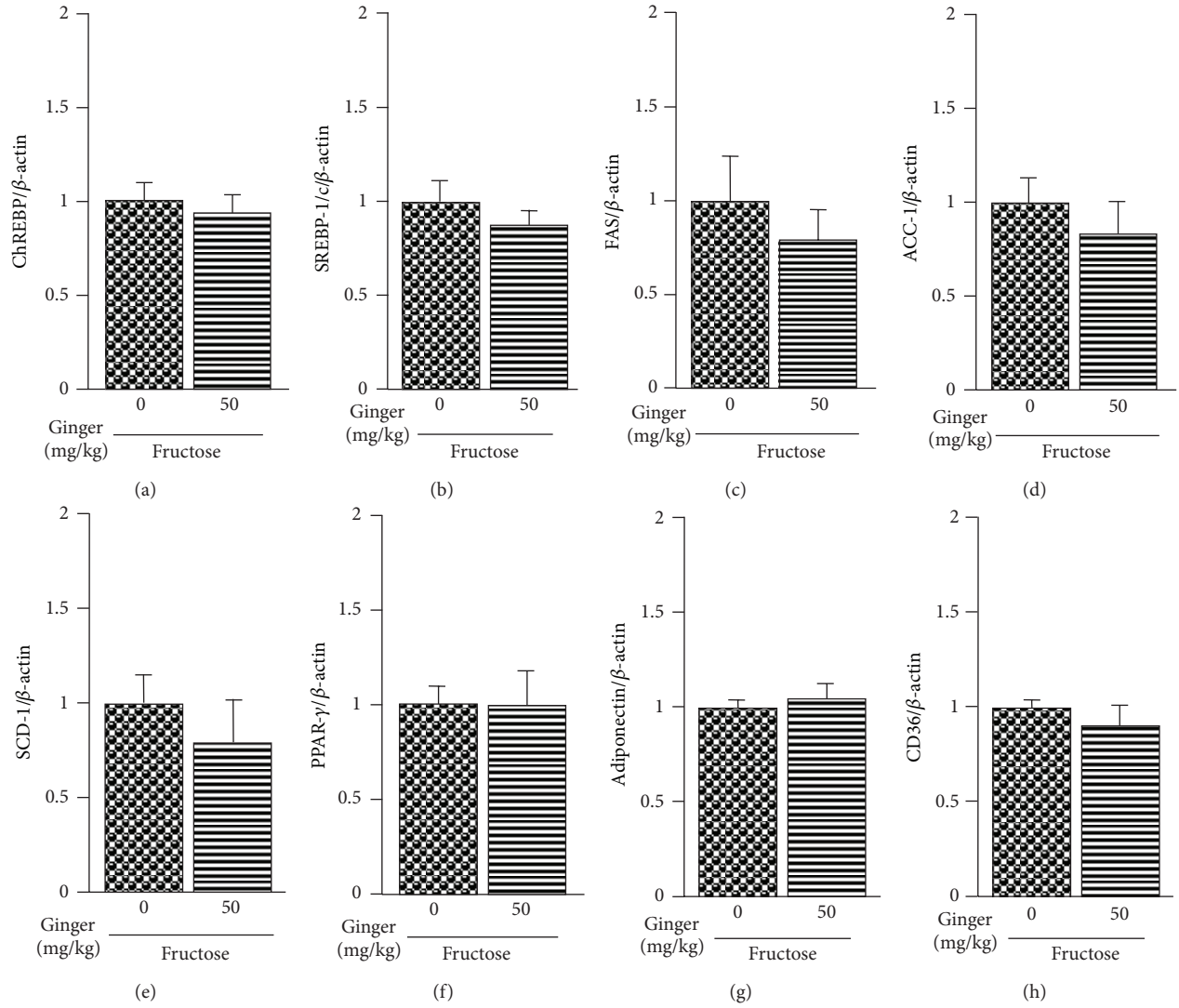

Figure 7: Adipose mRNA expression of carbohydrate-response-element-binding protein (ChREBP) (a), sterol-regulatory-element-binding protein (SREBP)-1c (b), fatty acid synthase (FAS) (c), acetyl-CoA carboxylase (ACC)-1 (d), stearoyl-CoA desaturase (SCD)-1 (e), peroxisome-proliferator-activated receptor (PPAR)-γ (f), adiponectin (g), and CD36 (h) in fructose-pair-fed rats. The fructose-control rats had free access to 10% fructose in their drinking water, while the consumption of fructose in the ginger-(50 mg/kg) treated (by gavage daily) rats was adjusted to that of the fructose-control rats over 5 weeks. mRNA was determined by real-time PCR and normalized to β-actin. Levels in fructose-control rats were arbitrarily assigned a value of 1. Data are means ± SEM ($n = 6$ each group). $^{*}P < 0.05$.

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